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Relationship between *HLA-DRB1* allele polymorphisms and familial aggregations of hepatocellular carcinoma

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ABSTRACT

Objective We explored the relationship between *HLA-DRB1* allele polymorphisms and familial aggregation of hepatocellular carcinoma (fHCC).

Methods Polymerase chain reaction sequence-specific primers were used to determine *HLA-DRB1* genotypes for 130 members of families with 2 or more liver cancer patients and for 130 members of families without any diagnosed cancers. The genotype profiles were then compared to explore the relationship between *HLA-DRB1* gene polymorphism and fHCC.

Result Of 11 selected alleles, the frequencies of *DRB1**11 and *DRB1**12 were significantly lower in the fHCC group than in no-cancer group ($p < 0.05$; odds ratio: 0.286; 95% confidence interval: 0.091 to 0.901; and odds ratio: 0.493; 95% confidence interval: 0.292 to 0.893). Differences in the frequencies of the other 9 alleles were not statistically significant in the two groups ($p > 0.05$).

Conclusions Our research suggests that if genetic factors play a role in fHCC, the deficiency in the *DRB1**11 and *DRB1**12 alleles might be the risk factor at work in Guangxi Zhuang Autonomous Region, P.R.C.

Key Words *HLA-DRB1* alleles, polymorphisms, hepatocellular carcinoma, familial aggregation, PCR-SSP

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary tumour of liver. Worldwide, HCC constitutes an important problem for health care systems because of its high morbidity, mortality, and increasing incidence[1]. It is the 5th most common cancer in men, the 7th most common in women, and the 3rd most frequent cause of cancer death. Estimates suggest that the incidence of HCC will continue to rise into the foreseeable future[2]. This aggressive tumour usually develops in a cirrhotic liver with limited functional reserve, and without treatment, survival after diagnosis is short.

Rates of HCC are particularly high in East and Southeast Asia and in Africa, intermediate in southern Europe, and low in most high-income countries. The incidence of HCC and the distribution of HCC risk factors vary widely by geographic region. China and Africa are areas of high HCC incidence, where the primary cause of HCC is chronic infection with the hepatitis B virus (HBV), with dietary exposure to aflatoxin being an important cofactor. In areas of low HCC incidence (including Europe and North America), diverse environmental factors including chronic infection with HBV or hepatitis C virus (HCV), heavy alcohol use, diabetes, obesity, and tobacco use have been shown to contribute to the local burden of HCC[3–5]. However, the facts that only a small proportion of the people with established risk factors eventually develop HCC and that HCC can cluster within families both suggest that genetic factors might play a role in the development of HCC.

To date, many genetic factors have been reported to be related to a susceptibility to HCC: polymorphisms of tumour necrosis factor α[6,7], of epidermal growth factor and epidermal growth factor receptor[8,9], of the transforming growth factor β1 gene[10], and of major histocompatibility complex (MHC) or human leucocyte antigen (HLA)[11–15],

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among others. Of the foregoing genetic factors, MHC plays a key role in antivirus activity and tumour defense. The function of HLA is to regulate the immune response to foreign antigens and to discriminate self from non-self antigens. The HLAs are encoded by a series of closely linked genetic loci found on chromosome 6[16,17]. Polymorphism in HLA is implicated in conferring genetic susceptibility to a large number of immune-mediated diseases, including some cancers.

Statistical data show that half of all new HCC cases and deaths reported worldwide occur in China, where the case distribution has obvious regional differences. An epidemiologic investigation indicated that the incidence of, and mortality from, liver cancer in Guangxi are significantly higher than the national average, and liver cancer in Guangxi showed a tendency toward familial aggregation (FHCC). Most patients with HCC had family history of liver cancer. The risk of developing HCC increased greatly when 1st- and 2nd-degree relatives also had the disease. We therefore designed a project to probe the relationship between *HLA-DRB1* allele polymorphisms and FHCC.

METHODS

Our project was approved by the National Natural Science Foundation of China and the Science Foundation of the Health Bureau of Guangxi Zhuang Autonomous Region. It enrolled 260 healthy individuals with no known disease, among whom many had various degrees of consanguinity. All subjects came from 11 areas of high HCC incidence in Guangxi. Of the 260 enrolled participants, 130 were members of 34 families considered to show FHCC (all included 2 or more HCC patients), and 130 were members of 37 families considered to have no familial cancers (control group). The control participants were matched with the FHCC participants in terms of age (±5 years), presence of the HBV surface antigen (HBsAg), ethnicity, residence, and sex.

When patients were diagnosed with liver cancer at our hospital, we collected their information and subsequently contacted healthy members of their families and other families in their towns of the same ethnicity and residence. Using epidemiology questionnaires, we then obtained basic personal and demographic information and information about risk factors for liver cancer from the healthy individuals. Using the personal information, we created family trees. At the same time, we collected 5-mL samples of non-anticoagulated blood so that serum could be separated for the detection of markers of HBV, anti-HCV, alanine transaminase, aspartate transaminase, albumin, and so on. In the morning, another 5 mL of fasting venous blood was collected in anticoagulant tubes, and DNA for genome study was extracted.

We used polymerase chain reaction (PCR) sequence-specific primers (SSPS) to determine the *HLA-DRB1* genotype for the 130 FHCC participants and the 130 non-cancer participants. The genotypes were then compared to explore the relationships between *HLA-DRB1* gene polymorphisms and FHCC participants. We selected certain *HLA-DRB1* alleles that had been reported to be associated with HCC, and sequence-specific primers were designed based on reference sequences from the GeneBank database and from Olerup and Zetterquist[18]. The primers (Table I) were then synthesized by Shanghai Sangon Biological Engineering Technology and Services (Shanghai, P.R.C.).

The conventional phenol chloroform and proteinase K method using Promega kits (Madison, WI, U.S.A.) was applied to extract genomic DNA from peripheral blood lymphocytes. The DNA concentration was determined by ultraviolet spectrophotometer and subsequently adjusted to 1 μg/μL.

Every PCR reaction tube contained genomic DNA; a PCR mix (including Taq polymerase, deoxyribose nucleoside triphosphate, and Taq buffer); 5′-SSP and 3′-SSP of each *HLA-DRB1* allele, and 5′-SSP and 3′-SSP of the reference sequence; and human growth factor, which was the housekeeping gene in our genomic DNA. Tables II and III present complete information for every reaction tube and the PCR conditions for each *HLA-DRB1* allele. The PCR product was analyzed in 2% agarose gel. The SPSS statistical software package (version 16.0: SPSS, Chicago, IL, U.S.A.) was used for data analysis.

RESULTS

Table IV shows the frequencies of the *HLA-DRB1* alleles. We detected a total of 11 alleles at the *HLA-DRB1* locus in members of the FHCC families and the non-FHCC families. Chi-squares and odds ratios (ORS) were calculated for each allele. The frequency of the *DRB1**11 allele was 3.08% (4 of 130) in the FHCC group and 10% (13 of 130) in the no-FHCC group, a difference between the groups that is statistically significant [$p < 0.05$; OR: 0.286; 95% confidence interval (CI): 0.091 to 0.901]. The frequency of the *DRB1**12 allele in the two groups was 16.92% (22 of 130) and 29.23% (38 of 130) respectively, another statistically significant difference ($p < 0.05$; OR: 0.493; 95% CI: 0.292 to 0.893). The significantly lower frequencies of *DRB1**11 and *DRB1**12 in the FHCC group might indicate that those alleles confer some resilience against HCC.

With respect to the other 9 alleles, the difference in frequency between the two groups was statistically non-significant ($p > 0.05$): *DRB1**03 (16.15%, 18.46%), *DRB1**04 (11.53%, 9.23%), *DRB1**07 (0.76%, 2.36%), *DRB1**08 (6.92%, 4.62%), *DRB1**09 (18.46%, 16.92%), *DRB1**13 (2.31%, 3.08%), *DRB1**14 (32.31%, 22.31%), *DRB1**15 (43.08%, 36.15%), and *DRB1**16 (18.46%,17.69%).

We also determined the frequencies of the *DRB1**11 and *DRB1**12 alleles in the study participants who had been infected with HBV, dividing them into groups depending on whether they were HBsAg-positive or -negative (Table V). The frequencies of the *DRB1**11 and *DRB1**12 alleles in those groups were 4.17% and 7.08%, and 14.58% and 25% respectively, differences that were nonsignificant ($p > 0.05$).

DISCUSSION AND CONCLUSIONS

Human leucocyte antigen, the gene product of MHC, is the first genetic system to have been discovered to be related to disease. The HLA complex, a closely linked gene cluster, is found in the short arm of the human 6th chromosome. The antigens encoded by the HLAs determine an organism's rejection reaction and are related to immune response and

TABLE I Sequence-specific primers for *HLA-DRB1* alleles

Allele	Primer sequences		PCR product size (bp)
	5′-Sequence 5′→3′	3′-Sequence 5′→3′	
*DRB1**03	TACTTCCATAACCAGGAGGAGA	TGCAGTAGTTGTCCACCCG	151
*DRB1**04	GTTTCTTGGAGCAGGTTAAACA	CTGCACTGTGAAGCTCTCAC	260
*DRB1**07	CCTGTGGCAGGGTAAGTATA	CCCGTAGTTGTGTCTGCACAC	232
*DRB1**08	AGTACTCTACGGGTGAGTGTT	CTGCAGTAGGTGTCCACCAG	214
*DRB1**09	GTTTCTTGAAGCAGGATAAGTT	CCCGTAGTTGTGTCTGCACAC	236
*DRB1**11	GTTTCTTGGAGTACTCTACGTC	CTGGCTGTTCCAGTACTCCT	176
*DRB1**12	ACTCTACGGGTGAGTGTT	ACTGTGAAGCTCTCCACAG	244
*DRB1**13	TACTTCCATAACCAGGAGAGA	CCCGCTCGTCTTCCAGGAT	130
*DRB1**14	GTTTCTTGCAGTACTCTACGTC	TCTGCAATAGGTGTCCACCT	224
*DRB1**15	TCCTGTGGCAGCCTAAGAG	CCGCGCCTGCTCCAGGAT	197
*DRB1**16	TCCTGTGGCAGCCTAAGAG	CTCCGTCACCGCCCGGT	137
HGF	CAGTGCCTTCCCAACCATTCCCTTA	ATCCACTCACGGATTTCTGTTGTGTTTC	432

PCR = polymerase chain reaction.

TABLE II Specific polymerase chain reaction system for *HLA-DRB1* alleles

Allele	Premix Taq (μL)	Reference (μL)		Primer (μL)		ddH_2O (μL)	DNA (μL)
		5′-SSP	3 ′-SSP	5 ′-SSP	3 ′-SSP		
*DRB1**03	12.5	0.5	0.5	0.3	0.3	8.9	2
*DRB1**04	12.5	0.5	0.5	0.3	0.3	8.9	2
*DRB1**07	12.5	0.4	0.4	0.5	0.5	8.2	2
*DRB1**08	12.5	0.6	0.6	0.2	0.2	8.9	2
*DRB1**09	12.5	0.4	0.4	0.5	0.5	8.2	2
*DRB1**11	12.5	0.3	0.3	0.4	0.4	9.1	2
*DRB1**12	12.5	0.3	0.3	0.5	0.5	8.9	2
*DRB1**13	12.5	0.2	0.2	0.7	0.7	9.7	1
*DRB1**14	12.5	0.3	0.3	0.4	0.4	9.1	2
*DRB1**15	12.5	0.5	0.5	0.5	0.5	8.5	2
*DRB1**16	12.5	0.6	0.6	0.2	0.2	8.9	2

SSP = single specific primer; ddH_2O = double-distilled water.

immunologic regulation. The HLA gene family is classified into 3 groups—HLA-I, HLA-II, and HLA-III—by their polymorphisms, the distribution of their coding regions, and their function. The classical HLA-I group has 3 functional sites, HLA-A, HLA-B, and HLA-C, which were the first members of the HLA family to be discovered[19,20]. Genes in the HLA-I group have a very high rate of polymorphism; as of October 2014, 2964 alleles of HLA-A, 3693 alleles of HLA-B, and 2466 alleles of HLA-C had been identified according to the IMGT/HLA database of the ImMunoGeneTics project (http://www.ebi.ac.uk/imgt/hla/stats.html).

The HLA-I gene products are located mainly on the surface of all nucleated cells, where they present foreign antigens to CD8+ T cells, enabling recognition and lysis of virus-infected cells[21]. Class II HLAs—named HLA-D and subclassified into HLA-DR, HLA-DQ, HLA-DP, and so on—are expressed mainly in immunocells. They function as labelled molecules to activate the immune response and regulate the interaction of immunocells[22–24].

Given the high polymorphism of the HLA complex, finding the same phenotype in multiple individuals is very rare, and thus HLA has become an important target in the

TABLE III Specific polymerase chain reaction amplification conditions for *HLA-DRB1* alleles

Allele	Initial denaturation (°C, min.)	Circulation (°C, s)			Cycles (*n*)	Last elongation (°C, min.)
		Denaturation	Renaturation	Elongation		
*DRB1**03	94, 3	94, 30	59, 40	72, 60	35	72, 5
*DRB1**04	94, 3	94, 30	58, 40	72, 60	35	72, 5
*DRB1**07	94, 5	94, 35	57.5, 35	72, 60	35	72, 10
*DRB1**08	94, 3	94, 30	61, 40	72, 60	35	72, 5
*DRB1**09	94, 3	94, 30	58, 30	72, 60	35	72, 5
*DRB1**11	94, 3	94, 30	60, 30	72, 60	35	72, 5
*DRB1**12	94, 3	94, 35	59, 35	72, 60	35	72, 10
*DRB1**13	94, 3	94, 35	56, 35	72, 60	38	72, 5
*DRB1**14	94, 5	94, 50	58, 40	72, 60	36	72, 10
*DRB1**15	94, 5	94, 50	58, 40	72, 60	35	72, 10
*DRB1**16	94, 3	94, 30	56, 40	72, 60	35	72, 5

TABLE IV Distribution of *HLA-DRB1* alleles in the case and control groups

Allele	Polymerase chain reaction results for ...				*p* Value	OR	95% CI
	Cases (fHCC)		Controls (no fHCC)				
	Positive	Negative	Positive	Negative			
*DRB1**03	21	109	24	106	0.358	0.739	0.386 to 1.412
*DRB1**04	15	115	12	118	0.542	1.283	0.576 to 2.858
*DRB1**07	1	129	3	127	0.622	0.328	0.034 to 3.197
*DRB1**08	9	121	6	124	0.425	1.537	0.531 to 4.450
*DRB1**09	24	106	22	108	0.745	1.111	0.588 to 2.103
*DRB1**11	4	126	13	117	0.024#	0.286	0.091 to 0.901
*DRB1**12	22	108	38	92	0.019&	0.493	0.272 to 0.893
*DRB1**13	3	127	4	126	0.702	0.744	0.163 to 3.392
*DRB1**14	42	88	29	101	0.70	1.662	0.956 to 2.889
*DRB1**15	56	74	47	83	0.254	1.336	0.812 to 2.200
*DRB1**16	24	106	23	107	0.872	1.053	0.560 to 1.981

fHCC = familial hepatocellular carcinoma; OR = odds ratio; CI = confidence interval.

investigation of many diseases, including autoimmune, infectious, and malignant diseases, among others[25–28]. High expression of HLA-DR is a marker of T-cell activation. According to statistics from the International Histocompatibility Working Group, the number of alleles in the noetic DRB1 sites of HLA-II antigens is 494, with 1582 alleles having been identified for the *HLA-DRB1* gene. That polymorphism is the main genetic factor[29,30] involved in the variety of immune responses and the varying susceptibility to disease presented by different individuals in a group. Both of the foregoing HLA groups play very important roles in antigen recognition and immune response and regulation[31,32].

Classical HLAs act as genetic markers of tumour susceptibility, and many tumour-related studies are currently being performed. Allele polymorphism in *HLA-DRB1* has been reported to be associated with certain cancers and autoimmune diseases, including cervical squamous cell carcinoma, rheumatoid arthritis, systemic lupus erythematosus, autoimmune hepatitis, inflammatory bowel disease, multiple sclerosis, and type 1 diabetes, among others.

Many studies have already reported the relationship between *HLA-DRB1* and HCC. El-Chennawi *et al.*[12] studied the association of HLA class II *DRB1* and *DQB1* polymorphisms with HCC in Egyptian patients and investigated

TABLE V Distribution of HLA-*DRB1**11 and HLA-*DRB1**12 in study participants with and without hepatitis B viral infection

Variable	HLA-DRB1 allele			
	*11		*12	
	Positive	Negative	Positive	Negative
HBsAg				
Positive	2	46	7	41
Negative	15	197	53	159
Statistic				
Chi-square	0.542		2.392	
p Value	0.462		0.122	
Odds ratio	0.571		0.512	
95% CI	0.126 to 2.585		0.217 to 1.210	

HBsAg = hepatitis B surface antigen; CI = confidence interval.

the role of those polymorphisms as risk factors for the development of HCC. Those authors found a significantly increased frequency of *DRB1**04 and *DQB1**02 ($p = 0.016$ and 0.032 respectively) and a significantly decreased frequency of *DQB1**06 ($p = 0.032$) in their HCC patients compared with a control group. They concluded that the *DRB1**04 and *DQB1**02 alleles might be risk factors for the occurrence of HCC (OR: 4.373 and 3.807 respectively) and that *DQB1**06 might be a protective allele (OR: 0.259).

Even earlier, Donaldson *et al.*[11] had also investigated HLA class II as a risk factor for the development of HCC in Hong Kong Chinese. Their study reported that the alleles *DRB1**1501 (36% of HCC patients vs. 19% of controls; OR: 2.44), *DQA1**0102 (42% vs. 26%; OR: 2.07), and *DPB1**0501 (80% vs. 63%; OR: 2.35) were significantly more common in patients with HCC, and that the alleles *DQA1**03 (36% vs. 56%; OR: 0.53), *DQB1**0302 (4.% vs. 13%; OR: 0.25), and *DPB1**0201 (14% vs. 29%; OR: 0.4) were found at significantly lower frequencies.

Controversially, in 1987 in Taiwan, Lin *et al.*[33] studied the distribution of HLA-A, -B, -C, and -DR antigens in Chinese patients with HCC. Their results suggested that no specific patterns or frequencies of those antigens were associated with the development of HCC.

Infection with HBV or HCV (or both) is the most important risk factor for development of HCC, and many studies have reported a relationship between *HLA-DRB1* and HBV or HCV. Ali *et al.*[34] evaluated the distribution of HLA alleles and haplotypes in 204 HCV-seropositive individuals from Islamabad, Pakistan, who were receiving standard interferon therapy. The authors concluded that *DRB1**04 imparts a significant protective advantage against HCV infection (Bonferroni-corrected $p = 0.047$). In patients on interferon therapy, *DRB1**11 and *DQB1**0301 were found to be associated with viral clearance (Bonferroni-corrected $p = 0.044$). In contrast, *DRB1**07 individually (Bonferroni-corrected $p = 0.008$), or in combination with *DQB1**02, was found to be associated with viral persistence. A meta-analysis[35] demonstrated a statistically significant correlation between the *DRB1**03 allele and the occurrence of chronic hepatitis B in the Han Chinese population; the *DRB1**03 allele might therefore be a susceptibility allele for the disease. Another meta-analysis showed that the *HLA-DR**04 and *HLA-DR**13 alleles (OR: 0.72; 95% CI: 0.60 to 0.85; and OR: 0.27; 95% CI: 0.19 to 0.37 respectively) were significantly associated with HBV clearance. In contrast, patients carrying the *HLA-DR**03 or *HLA-DR**07 alleles (OR: 1.47; 95% CI: 1.16 to 1.87; and OR: 1.59; 95% CI: 1.24 to 2.03 respectively) had a significantly increased risk of chronic HBV persistence. A significant association of the *HLA-DR**01 polymorphism with HBV clearance was found in the Han Chinese group (OR: 0.48; 95% CI: 0.26 to 0.86), but not in other ethnic groups ($p = 0.191$).

The Guangxi Zhuang Autonomous Region is known as a region of high HBV prevalence in China; it is also a region with a high HCC prevalence. As is well known, HBV infection is the most important risk factor in HCC, but not every HBV infection develops into liver cirrhosis or liver cancer. However, Jin *et al.*[36] analyzed the relationship between specific *HLA-DRB1* alleles and the development of HCC in patients with chronic HBV who had taken antiviral drugs for more than 12 months, finding that the *HLA-DRB1**140101 allele could potentially be associated with an increased risk of HCC development in such patients regardless of HBV replicative activity and responsiveness to antivirals.

In view of all the foregoing evidence, we designed a study to explore the relationship of *HLA-DRB1* allele polymorphisms with FHCC in Guangxi Zhuang Autonomous Region. We also attempted to provide insights into the underlying genetic background of the members of families with FHCC and to discover whether the FHCC aggregation in Guangxi is related mostly to genetic factors or to a combination of genes and HBV infection. After enrolling healthy individuals both from FHCC families ("cases") and from families with no history of cancer ("controls") who lived in 11 areas of high HCC incidence in Guangxi (matched for age, HBsAg, nationality, residence, and sex), we selected certain *HLA-DRB1* alleles that had been reported to be related to liver cancer or HBV infection and used PCR SSPS to determine allele frequencies. We found that the frequencies of *DRB1**11 and *DRB1**12 were significantly lower in the FHCC group than in the control group and that the frequencies of *DRB1**03, *04, *07, *08, *09, *13, *14, *15, and *16 were statistically similar. We speculate that the low frequency of the *DRB1**11 and *12 alleles are the risk factor for the FHCC aggregation in Guangxi Zhuang Autonomous Region.

We subsequently explored the relationships of the *DRB1**11 and *DRB1**12 alleles with HBV infection, finding little relation between them. We therefore indirectly infer that those alleles are connected to FHCC mainly because of their own action and not because they affect HBV susceptibility. With respect to pathogenesis, we hypothesize that deficiency in the *DRB1**11 and *DRB1**12 alleles might result in immune evasion by the tumour because of a connection to the regulation of cytokines; more research to prove this hypothesis will be needed.

Our data suggest that genetic factors play a role in FHCC, with a deficiency of the *DRB1**11 and *DRB1**12 alleles potentially being the risk factor for the aggregation seen in Guangxi Zhuang Autonomous Region. However, our study has limitations, and large case–control studies focused on

the potential genetic components of the local fHCC aggregation and on determining immune cytokines in the patients are required to verify our hypotheses. The potential early diagnostic value of *HLA-DRB1* alleles remains a field to be explored in future.

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CONFLICT OF INTEREST DISCLOSURES

We have read and understood *Current Oncology*'s policy on disclosing conflicts of interest, and we declare that we have none.

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