Evaluation of a histocompatibility antigen related to hepatitis B virus in patients with hepatocellular carcinoma in the western Brazilian Amazon

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ABSTRACT. Hepatocellular carcinoma is an infection of variable incidence that can be caused by hepatitis B virus (HBV), which is endemic in the Amazon region. The diagnosis of HBV can be performed through the use of serum markers such as the hepatitis B surface antigen. The chronic HBV can cause mutagenesis and carcinogenesis, being the susceptibility of infection due to allele human leukocyte antigen (HLA). Thus, we evaluated the clinical, molecular and laboratory profile (histocompatibility complex) of HBV in 22 patients with hepatocellular

carcinoma in Amazonia, including 18 males and 4 females, using a blood sample for generic HLA class II. The results showed increased frequency of disease evolution in adults between 25 and 64 years old, who comprised 19 of the 22 patients studied. Most patients (16/22) presented high levels of alpha-fetoprotein and transaminases (14/22). The most common HLA alleles were DRB1*04 (8/44), DRB1*08 (9/44), DRB*03 (16/44), and DQB1*04 (9/44). When we compared specific phenotype frequencies of HLA-DRB1* between patients and controls, we found that patients had a significantly higher frequency of allele DRB1*08 and a significantly lower frequency of DRB1*07 and DRB1*12 compared to previous studies on Asian and Amazonian populations suggesting ethnic differences. We suggest that alleles HLA-DRB*08, HLA-DRB*03 and HLA-DQB1*04 may be risk factors for hepatocellular carcinoma in Amazon.

Key words: Hepatocellular carcinoma; Hepatitis B; Histocompatibility antigen

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important human viruses. It affects approximately 300 million people worldwide and is responsible for 1-2 million annual deaths. Although most people recover from HBV infection, approximately 10% of infected individuals develop chronic hepatitis, remaining asymptomatic or developing serious liver disease such as cirrhosis or hepatocellular carcinoma (HCC) (Ferreira et al., 2006). One report has pointed out that HBV transmission occurs through blood or bodily fluids such as semen, vaginal discharge, or saliva from infected individuals, with sexual contact being the most common transmission pathway and has indicated that 15% of the population has had contact with HBV and 1% is chronically sick (Chávez et al., 2003).

Serum markers are critical for HBV diagnosis. The hepatitis B surface antigen (HBsAg) is the first marker to appear. Its persistence in serum for 6 months or more characterizes virus carriers, although it does not show complete virion replication, and an individual carrier may be asymptomatic and free of liver injury. The persistence of HBs and HBe antigens in blood circulation, antibodies against HBV core antigen (anti-HBc), and sometimes antibodies against HBs (anti-HBs) in addition to viral DNA indicates HBV chronic replication, which may be followed by progressive liver injury. A study on the prevalence of serum HBV in Brazil demonstrated that it is endemic in northern regions of the Amazonas as well as in areas of the city of Espírito Santo and the southern States of Paraná and Santa Catarina (Miranda et al., 2004).

In HCCs related to HBV, HBsAg and anti-Hbc are usually found in serum, although HBsAg may be negative at low levels in some patients, maintaining positivity for anti-Hbc. The integration of viral DNA into host DNA may be the initial event affecting cellular and HBV genome alterations causing mutagenesis and carcinogenesis. The HBV DNA sequences may be identified using polymerase chain reaction (PCR) in lump tissues of HBsAg-negative patients and those with anti-Hbc and even positive serum anti-HBs (Lee, 1997). The application of molecular biology techniques has allowed notable achievements in HBV research (Ferreira, 2000).

Acute or chronic illness is conditioned by the mechanism of immune response regulated by the human leukocyte antigen (HLA) molecule. The chronic susceptibility of infection is due to allele HLA-A0206 in the Taiwanese population, B35 in the Chinese, B18, B35, B40, and Cw3 in Russians, and B44-Cw 0501 in white Americans. HLA-B8 in white Europeans is associated with the absence of response to HBV vaccine. In the Chinese population, HLA-DR3 is associated with vertical transmission (Singh et al., 2007).

HBV infection clearance was specifically associated with HLA-DR13 in several ethnic groups. In Gambia, the HLA-DRB1-1302 allele is associated with protection against persistent HBV infection. A European study has confirmed that alleles HLA-DRB1-1302 and HLA-DRB-1301 also show resistance to chronic infection in whites (Carrilho et al., 2010). In a study of 91 American adults and Africans, 60 presented persistent HBV infection related to the alleles HLA-DQA1 0501 and HLA-DQB1 0301. Another study has shown a strong association of HLA-DRB1 0301, HLA-DQA1 0501, and HLA-DQB1 0301 with susceptibility to chronic hepatitis B (Kummee et al., 2007). We evaluated the serum profile and identified the antigen of histocompatibility (HLA class II), aiming to verify risk factors for HCC in a population infected by hepatitis in a health clinic in the western Brazilian Amazonia.

MATERIAL AND METHODS

Study model

This study was approved by the Ethics Council for Research of Human Beings of the Federal University of Amazon (MEMO - No. 0132.0.115.000-10-CAAE). This case-control study included patients of both genders, older than 13 years, who were carriers of HBV and HCC and were seen at the Oncology Control Foundation Center of the Amazonas State (FCECON) between August 2010 and July 2011. The controls were patients of both genders, aged 13 years and older, who were non-carriers of HBV and HCC and registered in the database of the Laboratory of Immunology of the Federal University of Amazonas, and who performed HLA tests.

Study population

Patients were included in this study according to the following criteria: they were hepatitis B patients with HCC, had been seen at the Cancer Foundation during the study period, were older than 13 years, had positive serum for HBV evaluated through specific markers (HBsAg, anti-HBs, total anti-HBc, HBeAg, and anti-HBe), and had confirmed presence of HCC on imaging tests including ultrasound, tomography, or magnetic nuclear resonance and alpha-fetoprotein dosage. All patients signed informed consent forms after the medical procedures were explained.

HBV and HCC patients attended the ambulatory unit of the FCECON on Tuesdays beginning at 10:00 am. The patients completed a questionnaire, and 10 mL venous blood was drawn by a phlebotomist. A total of 5 mL blood was collected in a tube with anticoagulant and 5 mL was collected in a tube without anticoagulant. All samples were kept in a polystyrene container with ice gel at the Immunology Laboratory of the Federal University of Amazonas and analyzed for HLA using the methods described below.

DNA extraction

DNA extraction from venous blood was carried out with a rapid technique using salts of tetraethylammonium bromide [dodecyltrimethylammonium bromide (DTAB)/cetyl-trimethylammonium bromide (CTAB)] described by Gustincich et al. (1991) with the following adaptations. The leukocyte layer, the "buffy coat", was obtained from centrifuged blood at 250 g for 10 min after mixing with an equal volume of lysis buffer [DTAB 12% (12% DTAB, 25 M $NaCl_2$, 150 mM Tris 150, pH 8.6, and 75 mM ethylenediaminetetraacetic acid)] and incubated for 10 min at 68°C. Two volumes of chloroform were added after strong agitation. After centrifugation at 10,000 g for 2 min, the supernatant was recovered and added to two volumes of CTAB 0.5% (5% CTAB, 0.4 M NaCl) and then homogenized until DNA/CTAB precipitated.

After additional centrifugation at 10,000 g for 2 min, the precipitation was suspended in 300 µL 1.2 M NaCl and 750 µL absolute ethanol and centrifuged for 2 min at 16,000 g. Additional washings and centrifugations were carried out with 70% ethanol. The supernatant was discharged, and the precipitation was dissolved in 200 µL H_2O.

Generic HLA class II (*DRB1*, *DRB3*, *DRB4*, *DRB5*, and *DQB1*)

After DNA extraction and normalization, the samples were typed for generic HLA class II using a micro-sequence-specific primer (SSP) plate from One Lambda®. The SSP technique described by Olerup and Zetterquist (1992) was used with modification. Samples from three patients in triplicate were mixed with Taq polymerase and PCR reagents before loading onto a One-Lambda 96-well DMIX plate according to manufacturer instructions.

The amplification of a 150-bp product was checked with 1.5% agarose gel electrophoresis under ultraviolet light. The final HLA allele typing analysis was performed using DNALMT version 3.81 (One Lambda).

Analysis and data interpretation

The variables were tabulated in Excel 2007 and the statistical analyses were carried out with MINITAB version 14.1. The frequencies of the HLA class II antigens were recorded in tables of frequency distributions. The relationships between the histocompatibility HLA class II antigen and HBV disease evolution with respect to anti-HBc and gender were determined with chi-square and Fisher exact tests (when the chi-square test was unavailable) considering a level of 5% of significance in all verifications.

RESULTS

The study population was 81.8% men and 18.2% women. All patients were in an advanced stage of disease, with some displaying cirrhosis. The majority of patients were aged 20-45 years, whereas the minority was older than 65 years. A follow-up of the study population verified that approximately 73% became sick and 14% died, which constituted 87% of the sample. Only 14% were cured (Figure 1). Most (86.4%) presented reactivity to the anti-HBc marker, whereas only 13.6% showed no reactivity.
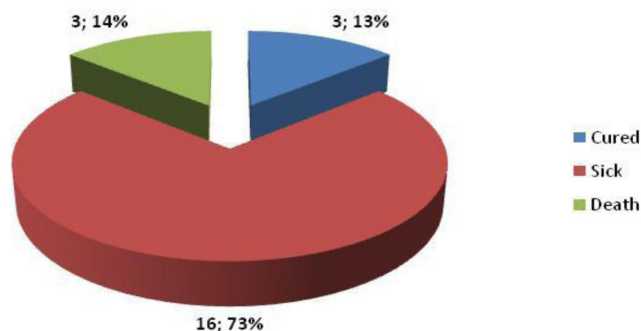
Figure 1. Distribution changes in the population studied.

DNA was extracted from 22 blood samples, quantified, and submitted to generic class II complex of histocompatibility typing (HLA-DRB1*, HLA-DRB3*, HLA-DRB4*, HLA-DRB5*, and HLA-DBQ1*). Because each individual represents two alleles, 44 alleles were analyzed. As shown in Figure 2, HLA-DRB1*08 prevailed, followed by HLA-DRB1*04; both composed approximately 39% of the evaluated samples. No results were found for HLA-DRB1*10 and HLA-DRB1*09.
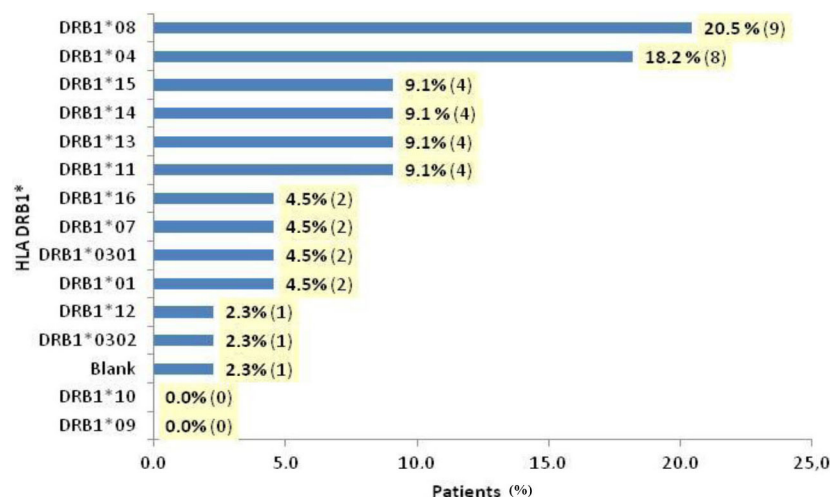

Figure 2. Distribution of human leukocyte antigen (HLA)-DRB1* generic among patients with hepatitis B and hepatocellular carcinoma.

Considering the phenotype frequencies for specificities of HLA-DRB1* (Table 1) between patients and controls, patients presented significantly lower HLA-DRB1*07 frequency and significantly higher HLA-DRB1*08 frequency compared with those in controls (P < 0.05). No evidence of a significant association was found between HLA-DRB1* specificities and anti-HBc serum markers in the sample studied (Table 2; P > 0.05). No evidence of significance was found between the HLA-DRB1* specificities and disease evolution in the sample studied (P > 0.05).

Table 1. Comparison between the phenotypic frequencies of human leukocyte antigen (HLA)-DRB1* alleles among patients infected with the hepatitis B virus and the group of uninfected patients (control).

HLA-DRB1*	Pacients [(N = 44) %]	Controls [(N = 44) %]	P value
Blank	2.3	13.6	0.05
DRB1*01	4.5	4.5	0.62
DRB1*04	18.2	13.6	0.77
DRB1*07	4.5	20.5	0.02
DRB1*08	20.5	13.6	0.02
DRB1*09	0.0	4.5	0.25
DRB1*10	0.0	0.0	-
DRB1*11	9.1	0.0	0.06
DRB1*12	2.3	0.0	0.50
DRB1*13	9.1	13.6	0.73
DRB1*14	9.1	2.3	0.35
DRB1*15	9.1	2.3	0.61
DRB1*16	4.5	6.8	0.50
DRB1*0301	4.5	4.5	0.69
DRB1*0302	2.3	0.0	0.50

Table 2. Distribution of human leukocyte antigen (HLA)-DRB1* generic towards the scoring results for anti-HBc in patients with hepatocellular carcinoma.

Results of HLA-DRB1*	Anti-HBc				Total	P value
	Positive	%	Negative	%		
Blank	1	100.0	0	0.0	1	0.86
DRB1*01	2	100.0	0	0.0	2	0.74
DRB1*0301	2	100.0	0	0.0	2	0.74
DRB1*0302	1	100.0	0	0.0	1	0.86
DRB1*04	7	87.5	1	12.5	8	0.70
DRB1*05	1	100.0	0	0.0	1	0.86
DRB1*07	2	100.0	0	0.0	2	0.74
DRB1*08	7	77.8	2	22.2	9	0.35
DRB1*11	3	75.0	1	25.0	4	0.45
DRB1*12	0	0.0	1	100.0	1	0.13
DRB1*13	4	100.0	0	0.0	4	0.54
DRB1*14	3	75.0	1	25.0	4	0.45
DRB1*15	3	100.0	0	0.0	3	0.63
DRB1*16	2	100.0	0	0.0	2	0.74
Total	38	86.4	6	13.6	44	

We noticed that the phenotype frequency was significantly lower in male patients compared to that in females for the HLA-DRB1*14 allele (P < 0.05), as presented in Table 3.

Table 3. Distribution of human leukocyte antigen (HLA)-DRB1* generic in relation to gender of patients with hepatocellular carcinoma.

Results of HLA-DRB1*	Male	%	Female	%	Total	P value
Blank	1	100.0	0	0.0	1	0.82
DRB1*01	2	100.0	0	0.0	2	0.67
DRB1*0301	2	100.0	0	0.0	2	0.67
DRB1*0302	1	100.0	0	0.0	1	0.82
DRB1*04	6	75.0	2	25.0	8	0.45
DRB1*05	1	100.0	0	0.0	1	0.82
DRB1*07	2	100.0	0	0.0	2	0.67
DRB1*08	7	77.8	2	22.2	9	0.53
DRB1*11	4	100.0	0	0.0	4	0.43
DRB1*12	1	100.0	0	0.0	1	0.82
DRB1*13	4	100.0	0	0.0	4	0.43
DRB1*14	1	25.0	3	75.0	4	0.02
DRB1*15	2	66.7	1	33.3	3	0.46
DRB1*16	2	100.0	0	0.0	2	0.67
Total	36	81.8	8	18.2	44	

HLA-DRB3* was predominant for these specificities, whereas HLA-DRB5* was less frequent. Considering phenotype frequencies for the HLA-DRB3*, HLA-DRB4*, and HLA-DRB5* specificities, no evidence of significant differences between allele frequencies were found between patients and controls (P > 0.05). In addition, we found no evidence of an association between HLA-DRB3*, HLA-DRB4*, and HLA-DRB5* specificities and the anti-HBc serum marker in the sample studied (P > 0.05). As shown in Table 4, no significant association was found between HLA-DRB3*, HLA-DRB4*, and HLA-DRB5* specificities and the disease evolution in the sample studied (P > 0.05).

Table 4. Distribution of generic human leukocyte antigen (HLA)-DRB3*, -DRB4* and -DRB5* in relation to the evolution of patients with hepatocellular carcinoma.

Results of HLA-DRB3*, -DRB4*, -DRB5*	Healthy	%	Sick	%	Total	P value
Blank	1	8.3	11	91.7	12	0.47
DRB3*	3	18.8	13	81.3	16	0.38
DRB4*	2	20.0	8	80.0	10	0.41
DRB5*	0	0.0	6	100.0	6	0.39
Total	6	13.6	38	86.4	44	

In relation to gender, no significant difference was found between the HLA-DRB*3, HLA-DRB4*, and HLA-DRB5* alleles (P > 0.05), as shown in Table 5.

Table 5. Distribution of generic human leukocyte antigen (HLA)-DRB3*, -DRB4* and -DRB5* in relation to gender of patients with hepatocellular carcinoma.

Results of HLA-DRB3*, -DRB4*, -DRB5*	Male	%	Female	%	Total	P value
Blank	10	83.3	2	16.7	12	0.62
DRB3*	13	81.3	3	18.8	16	0.62
DRB4*	8	80.0	2	20.0	10	0.59
DRB5*	5	0.0	1	16.7	6	0.70
Total	36	81.8	8	18.2	44	

Figure 3 shows that HLA-DBQ1*04 (20.5%) prevailed, followed by HLA-DRB1*0301 (18.2%), and HLA-DBQ1*06 (18.2%), comprising approximately 57% of the study.
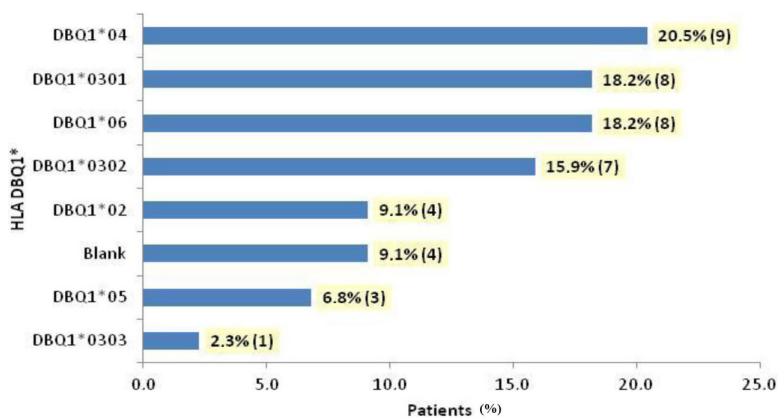

Figure 3. Distribution of human leukocyte antigen (HLA)-DBQ1* generic among patients with hepatitis B and hepatocellular carcinoma.

The phenotype frequencies for this specificity, shown in Table 6, demonstrate that patients had a significantly lower HLA-DBQ1*02 frequency compared with that in controls (P < 0.05). No evidence of a significant difference between patients and controls was found for the other allele frequencies.

Table 6. Comparison between the phenotypic frequencies of human leukocyte antigen (HLA)-DBQ1* among patients infected with hepatitis B and the group of patients not infected (control).

HLA-DRB1*	Patients [(N = 44) %]	Controls [(N = 44) %]	P value
Blank	9.1	11.4	0.01
DBQ1*02	9.1	27.3	0.02
DBQ1*04	20.5	6.8	0.06
DBQ1*05	6.8	4.6	0.50
DBQ1*06	18.2	20.5	0.79
DBQ1*0301	18.2	11.4	0.54
DBQ1*0302	15.9	15.9	0.78
DBQ1*0303	2.3	2.3	0.75

DISCUSSION

Of the 22 HCC patients studied, 82% were male and 18% were female. As mentioned earlier, this tumor is predominant in males, a finding supported by our results. Patients aged 20-45 years were the largest group of individuals and had an earlier age of onset compared to that of a study population in a European study (Romão, 2009), in which 57% of the patients were older than 70 years, and 43% were 50-70 years old.

Owing to the high level of polymorphism of molecules and genes of the main complex and histocompatibility as well as the magnitude of recent advances in typing methods for these markers, the terminology of the system can be highly confusing for non-specialists. In addition, increased understanding of the role of histocompatibility molecules in immune function have added to the complexity of the mechanisms of association between HLA molecules and diseases.

Few studies of the association between histocompatibility antigens and disease in Brazilian populations have been carried out. Available research in Latin American and international databases includes studies of rheumatic fever, pemphigus or endemic pemphigus, chromoblastomycosis, cutaneous leishmaniasis, paracoccidioidomycosis, schistosomiasis, diabetes mellitus, malaria, myasthenia gravis, 21-hydroxylase deficiency, chronic hepatitis, glaucoma, retinal detachment, hereditary angioedema, rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus, connective tissue disorders, cerebellar ataxia, Chagas disease, Graves' disease, Alport syndrome, acute leukemia, bone marrow failure, and Sydenham's chorea (Donadi, 2000). Our study aimed to explore an association between HCC and HBV carriers and HLA class II.

Given that tumor development is preceded by chronic infection, the immune responses to an infective agent or against tumor antigens may be critical for its development. Various types of HLA molecules have different abilities to connect with and present tumor antigens. We have demonstrated the association of specific HLA alleles susceptible to HBV-related HCC, whereas most previous studies have focused on susceptibility and resistance to chronic hepatitis B (Han et al., 2005). Ramezani et al. (2008) showed that HLA-A*33 is strongly related to susceptibility to hepatitis B persistence and that HLA-DRB*13 is strongly related to protection against hepatitis B persistence in a cross-section of the Iranian population.

Kummee et al. (2007) studied the association of HLA-DRB1*13 and polymorphism of the tumor necrosis factor (TNF)-alpha gene with clearance of chronic hepatitis B infection and HCC risk in a cross-section of a Thai population. When stratifying chronic HBV patients with and without HCC, allele 863A was significantly more frequent in the HCC group than it was in controls. Haplotype analysis (-863/-308/-238) showed that CGG/CGG homozygosity was a protective marker for HCC. Carriers of genotype 863A have increasing levels of TNF-alpha in liver tissue in response to HBV infection, which can damage liver cells and cause HCC.

Lin et al. (2010) reported 8 control-case studies in an Asian population in a meta-analysis studying HLA-DRB1 alleles and showed that HLA-DRB1*07 and HLA-DRB1*12 are significantly associated with HCC risk in the entire population (P = 0.02 and P = 0.02, respectively). No significant association was shown between the HLA-DRB1*15 allele and HCC. Subgroup analysis by ethnicity showed that HLA-DRB1*07, HLA-DRB1*12, and HLA-DRB1*15 alleles significantly increased the risk of HCC in Asians (P = 0.03; P = 0.006, and P < 0.001, respectively). Our results showed a significant increase in the frequency of the HLA-DRB1*08 (P = 0.02) allele and significantly lower rates for HLA-DRB1*07 (P = 0.02) compared to those in controls, and low levels of HLA-DRB1* 12 in both groups, suggesting ethnic differences between Asians and Amazonian populations.

Eight control-case studies were included in the final analysis. Of the three HLA-DRB1 alleles studied, HLA-DRB1*07 and HLA-DRB1*12 were significantly associated with HCC risk in the entire population [odds ratio (OR) = 1.65, 95% confidence interval (95%CI) = 1.08-2.51, P = 0.02 and OR = 1.59, 95%CI = 1.09-2.32, P = 0.02, respectively]. No significant association was established for the HLA-DRB1*15 allele and HCC in the entire population. Subgroup analysis by ethnicity demonstrated that HLA-DRB1*07, HLA-DRB1*12, and HLA-DRB1*15 alleles significantly increased the risk of HCC in Asians (OR = 2.10, 95%CI = 1.06-4.14, P = 0.03; OR = 1.73, 95%CI = 1.17-2.57, P = 0.006; and OR = 2.88, 95%CI = 1.77-4.69, P < 0.001, respectively).

In 2007, Yang et al. found a high frequency of HLA-DRB1*07 in HBV patients in northwestern China (17.6% in HBV patients *vs* 9.3% in controls, P < 0.05). Discordance rates in our study showed a low rate of HLA-DRB1*07 in HBV patients compared with that in the control group (4.5 *vs* 20.5%, P = 0.02).

Zhang et al. (2006) reported that the frequency of HLA-DRB1*12 in patients with persistent HBV was significantly higher than that in a cured Chinese group (0.230 *vs* 0.063, P = 0.004). In 2003, Amarapurpar et al. pointed out a positive association between HLA-DRB1*15 and persistent HBV between Indian subjects having HBsAg positive and healthy controls (57.6 *vs* 25%). Godkin et al. (2005) showed that HBV clearance in acute infection is associated with vigorous CD4+ T-cell response on the core protein. HLA class II glycoproteins present viral peptides for CD4+ T cells and influence immune responses. The affinity bond of the overlay peptide chain, comprising the nucleus protein and HBV envelope, has been measured for HLA glycoproteins encoded by some HLA-DRB1 molecules and compared to published CD4+ peptide-specific cell responses.

Many studies have been conducted using relatively small samples. An insufficient number of individuals may decrease the detection of differences in the distribution of HLA-DRB1*07, HLA-DRB1*12, and HLA-DRB1*15 alleles between HCC patients and controls, even when a significant difference is present. Studies reporting few associations do not confirm the absence of an association. Many studies have not analyzed a control to the correspondent variables, and conflicting factors are a potential cause of variation in estimates in these studies. Various types of control

groups could also potentially cause variation in the results of the studies. The effects of interactions of other environmental, behavioral, or viral factors may be unavoidable. The complex interaction between various genes, instead of a single allele, is likely to modulate HCC development. HLA genotype techniques must be considered, because these methodologies differ in important ways that can hamper effective comparisons between studies and influence combined results.

In conclusion, HLA-DRB*08, HLA-DRB3*, and HLA-DQB1*04 are more frequent in HBV and HCC carriers. High alpha-fetoprotein tends to increase this frequency and the frequency of HLA-DRB1*15. No evidence of important associations between HLA-DBQ1* specificities and disease evolution was found in the population studied. Because serology is a reagent to HBsAg and total anti-HBc, a higher frequency of HLA-DRB*08 allele was observed.

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