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Human leukocyte antigen (HLA) class II association in chronic hepatitis B patients with hepatocellular carcinoma in a Malay population: A pilot study

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Abstract. Asian countries account for almost three quarter of hepatocellular carcinoma (HCC) reported globally and chronic hepatitis B infection is one of the main contributors. Clinical observations show that Malay patients with chronic hepatitis B and HCC tend to have a worse outcome, when compared to other two major races in Malaysia. The objectives of this study was to determine the frequency of human leukocyte antigen (HLA) class II alleles in chronic hepatitis B patients with HCC among Malays compared to the general population to identify potential associations of HLA alleles with this disease. HLA class II typing was performed in chronic hepatitis B patients with hepatocellular carcinoma (n=12) by -polymerase chain reaction, sequence specific primer (PCR-SSP) method. There were higher allelic frequencies of certain HLA-DQB1 and HLA-DRB1 alleles; HLA-DQB1*03 (07) (41.7%), and HLA-DRB1*12 (41.7% vs 28.6%) and compared to controls (41.7% vs 29.7%). However, there was no significant statistical correlation found when compared with the normal healthy general population. This study provides an insight into the HLA Class II association with chronic hepatitis B and hepatocellular carcinoma in Malays. However, findings from this study should be validated with a larger number of samples using a high resolution HLA typing.

INTRODUCTION

Hepatitis B virus (HBV) infects more than two billion people of whom 257 million are chronically infected (O'Brien & Lim, 2019). HBV infection accounts for 30.3% of liver cirrhosis deaths and 45.4% of liver cancer deaths (Trépo *et al.*, 2014). Chronic HBV causes 60-80% of the world's primary liver cancer. In Asia where HBV is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC), they constitute 75% global chronic hepatitis B infection (Merican *et al.*, 2000). Western Pacific and Southeast Asia have the highest level of endemicity globally, with seroprevalence ranging from 2% to 31%. However, in Malaysia it is estimated that there are 1 million people chronically infected with hepatitis B virus (Raihan, 2016) with prevalence rate of less than 2% (Hudu *et al.*, 2013). Chronic hepatitis B infection has been attributed with more than 80% of the hepatocellular carcinoma (HCC) cases in Malaysia, with an incidence rate of 36%, 26% and 15% among the Chinese, Malays and Indians respectively (Raihan, 2016).

Genetic factors such as; polymorphisms of tumour necrosis factor α, epidermal growth factor and epidermal growth factor receptor, transforming growth factor β1 gene, and major histocompatibility complex (MHC) or human leukocyte antigen (HLA) have been identified to be related to the susceptibility to hepatocellular carcinoma. However, MHC plays a vital role in antivirus activity and tumour defense, by regulating the immune response to foreign antigens such as the HBV antigen and to discriminate self from non-self antigens (Burroughs *et al.*, 2004). These proteins are encoded by a large genomic region or gene family that is found in most vertebrates on the short arm of chromosome 6, with the prime role to present antigens to T cells (Delves & Roitt, 2000).

The MHC class I (HLA-A,-B and -C) and class II (HLA-DR,-DP and -DQ) antigens are highly polymorphic membrane proteins (Zavaglia *et al.*, 1996), hence, reported to play significant role in the control of disease, resistance and susceptibility. It seems likely that HLA determines development of HCC in chronic Hepatitis B antigen positive patients (Karra *et al.*, 2018) and this might be one of the reasons for racial difference in incidence and prevalence of HBV globally. To date, there is limited data on HLA association in HCC patients with hepatitis B in Malaysia which is a multi-Ethnic country. Prevalence of HCC in the different ethnic groups could be determined by a different reaction of the host to HBV infection which is associated with types of HLA. Based on clinical data, it was observed that the Malays have a poor outcome as compared to the other two major races in Malaysia (unpublished medical record). Similarly, according to Malaysian cancer registry the prevalence of chronic HBV infection and HCC is higher in Chinese than Malay. Therefore in this study it is hypothesized that HLA allele variation may play a significant role in HCC pathogenesis in Malays compared with other race from peninsula Malaysia. Thus, the objectives of this study were (1) to determine the frequency of HLA class II alleles in chronic hepatitis B patients with HCC among Malays and (2) compare with frequencies of HLA alleles in the general Malay population as reported.

MATERIALS AND METHODS

This is a descriptive pilot study targeting Malaysian population with Malay ethnicity, above 18 years of age and diagnosed with chronic hepatitis B infection as defined by the presence of HBsAg or HBV DNA persisting for more than 6 months, liver Fibroscan that show stiffness of the liver with more than 11 KPa score, and elevation of serum ALT two times or more than the upper normal limit. Similarly, HCC patients were selected based on medical ultrasound or computed tomography (CT) scan showing space occupying lesions such as mass or nodules, while inconclusive HBV markers or HCC status as well as other hepatic virus co-infection and autoimmune diseases were excluded. Written informed consent was obtained from patients and peripheral blood samples of approximately 3mls were taken. Ethical approval was obtained from the Medical Research Ethics Committee (MREC), Universiti Putra Malaysia with reference number: UPM/TNCPI/RMC/1.4.18.1 (JKEUPM)/F1. Judgmental sampling was used for this study in which subjects were picked on the basis inclusion criteria. Blood samples were collected at the Hepatology Clinic of Hospital Selayang from chronic hepatitis B patients seen or admitted in the hepatology unit of Hospital Selayang from January to December, 2014.

The genomic DNA from the peripheral blood was extracted by using the QIAmp DNA Blood Mini Kit according to manufacturer's instructions (QIAGEN, Germany). The Nano Drop spectrophotometer (ThermoScientific, USA) was used to determine the DNA concentration and purity. The HLA class II typing was performed using polymerase chain reaction- Sequence specific primer (low resolution *Olerup* SSP® DQ-DR SSP Combi Tray Kit, Sweden). The Bio-Rad thermocycler was used to run the PCR for cycles using the following PCR conditions: 2 minutes initial denaturation at 94°C for 1 cycle, then 10 seconds at 94°C and 60 seconds at 65°C for 10 cycles followed by 10 seconds at 94°C, 50 seconds at 61°C and 30 seconds at 72°C. The PCR products were then aliquoted into individual prepared gel and

electrophoresed in TAE buffer. The gel images were then captured in UV trans-illuminator and photographed for analysis.

Data was analyzed using Microsoft Excel software for analysis of allele frequency. The odds ratio (OR) and 95% confidence interval (CI) were analyzed using the online statistical software, http://www.vassarstats.net/ and http://web1.sph.emory.edu/cdckms/ctab-logbased-exact.html. The association between HLA alleles from this study was compared with the published data from the normal healthy Malay population as shown by Tan *et al*. (2016) by using the Pearson Chi-square or Fisher's exact test where appropriate. A *p*-value of less than 0.05 was considered significant. The 4-digit high resolution HLA typing from other studies were compared with this low resolution HLA typing by looking at the serological equivalence at http://www.ebi.ac.uk/ipd/imgt/hla. Besides that, the serological equivalence was also cross checked using 'HLA Dictionary' (Schreuder *et al*., 2005).

RESULTS

A total of twelve patients with HCC were analyzed in this study. The demographic data of the patients are tabulated in Table 1. The frequency of HLA-DQB1 and HLA-DRB1 was plotted on a worksheet based on the interpretation table given by the manufacturer (Olerup SSP). Table 2 and 3 shows the frequencies of HLA-DQB1 and HLA-DRB1 alleles respectively in chronic hepatitis B with hepatocellular carcinoma among Malay patients (n=12) including heterozygous or homozygous alleles for each individual (2n=24). HLA-DQB1*03 (07) was the most frequent allele among the HLA-DQB1 alleles with about 41.7%, followed by HLA-DQB1*05 with 25%. HLA-DQB1*02 and HLA-DQB1*06 both had a frequency of 12.5%. Both HLA-DQB1*03 (08) and HLA-DQB1*04 was not detected in these patients.

As for HLA-DRB1 alleles, HLA-DRB1*12 recorded the highest frequency with 41.7% followed by HLA-DRB1*15 with 20.8%. HLA-DRB1*03 (17), HLA-DRB1*09 and HLA-DRB1*14 have only a frequency of 8.3%. Table 2 and 3 depicts the frequency comparison of HLA-DQB1 and HLA-DRB1 respectively between this study and previous reported data from normal populations. The results were considered not significant based on the *p*-value obtained.

DISCUSSION

The study of the association of HLA genes in chronic hepatitis B with hepatocellular carcinoma among Malay patients encompassed the identification of HLA genotypes,

Table 1. Demographic data of the study subjects

Variables	Frequency (n=12)
Gender	
a) Male	8 (66.7%)
b) Female	4 (33.3%)
Male: Female ratio	2:1
Mean age (+/- SD)	55.1

Table 2. HLA DQB1 allele frequencies in chronic hepatitis B with HCC patients and healthy controls

Allele	Patients 2n = 24 (%)	Controls** 2n = 1902 (%)	OR (95% CI)	*P-value*
DQB1*02	3 (12.5)	259 (13.6)	0.91 (0.27-3.06)	ns
DQB1*03(07)	10 (41.7)	564 (29.7)	1.69 (0.75-3.84)	ns
DQB1*03(08)	0 (0)	72 (3.8)	0.52 (0.03-8.56)	ns
DQB1*03(09)	2 (8.3)	74 (3.9)	2.25 (0.52-9.73)	ns
DQB1*04	0 (0)	52 (2.7)	0.72 (0.04-11.9)	ns
DQB1*05	6 (25)	601 (31.6)	0.72 (0.29-1.83)	ns
DQB1*06	3 (12.5)	280 (14.7)	0.83 (0.25-2.79)	ns

HLA human leukocyte antigen, *HCC* hepatocellular carcinoma, *OR* odds ratio, 95% *CI* 95% confidence interval, *ns* not significant.

Table 3. HLA DRB1 allele frequencies in chronic hepatitis B with HCC patients and healthy controls

Allele	Patients 2n = 24	Controls 2n = 1902 (%)	OR (95% CI)	*P-value*
DRB1*01	0 (0)	14(0.7)	2.66 (0.15-45.8)	ns
DRB1*03 (17)	2 (8.3)	94(4.9)	1.75 (0.41-7.55)	ns
DRB1*03 (18)	0 (0)	2(0.1)	15.5 (0.73-331.6)	ns
DRB1*04	0 (0)	122(6.4)	0.29 (0.02-4.90)	ns
DRB1*07	1 (4.2)	192(10.1)	0.39 (0.05-2.88)	ns
DRB1*08	0 (0)	47(2.5)	0.80 (0.05-13.3)	ns
DRB1*09	2 (8.3)	64(3.4)	2.61 (0.60-11.3)	ns
DRB1*10	0 (0)	37(2.0)	1.02 (0.06-17.0)	ns
DRB1*11	1 (4.2)	59(3.1)	1.36 (0.18-10.2)	ns
DRB1*12	10 (41.7)	543(28.6)	1.79 (0.79-4.05)	ns (0.158)
DRB1*13	1 (4.2)	65(3.4)	1.23 (0.16-9.24)	ns
DRB1*14	2 (8.3)	85(4.5)	1.94 (0.45-8.40)	ns
DRB1*15	5 (20.8)	503(26.4)	0.73 (0.27-1.97)	ns
DRB1*16	0 (0)	75(3.9)	0.49 (0.03-8.20)	ns

HLA human leukocyte antigen, *HCC* hepatocellular carcinoma, *OR* odds ratio, 95% *CI* 95% confidence interval, *ns* not significant.

specifically of HLA-DQB1 and HLA-DRB1. Several studies provided evidence to suggest that cellular immune surveillance is important in the control of hepatitis B virus infection and the development of HCC. The frequency of HLA-DR12 (DRB1*12) was significantly higher in the hepatitis B virus persistent group as compared to the recovered group found among Chinese (Zhang *et al.*, 2006). In another study, a positive co-relation between persistence of hepatitis B virus and HLA-DR15 (DRB1*15) among Indians in 2003 was found (Amarapurpar *et al.*, 2003). Similarly, in this study, it was noted that the serological split alleles for HLA-DQB1*03(07) to be the most prominent allele, accounting for 41.7% among the HCC patients as compared to the control from the general population at 29.7%. As for HLA-DRB1 alleles, we observed a trend of association between HLA-DRB1*12 and HCC as compared with the general population, however, the association was not statistically significant.

In a study performed on six Malay sub-ethnic groups consisting of 176 unrelated individuals found HLA-DRB1 alleles as the most frequent in the total Malay population (TMP) and studied population group (SPG) were –DRB1*12 (15-36%) and –DRB1*15 (15-37%), whereas the most frequent HLA-DQB1 alleles were –DQB1*03 (25-51%) and –DQB1*05 (26-35%), although the specific –DQB1*03 alleles were not elaborated (Edinur *et al.*, 2009). In another study which involved 40 normal healthy Malay individuals from Kelantan, Malaysia, also found –DR12 (DRB1*12) as the most frequent allele (40%) followed by –DR15 (DRB1*15) (37.5%). Among the DQB1 alleles, DQB1*03 (08) which was depicted as DQB1*08 in that study was the most frequent allele (57.5%) followed by HLA-DQB1*03 (07) which was 52.5% (Azira *et al.*, 2013). Similarly, in a study of HLA class II among 1445 Malays registered with the Malaysian Marrow Donor Registry shows, HLA-DRB1*12 (0.36) was the most frequent allele, followed by HLA-DRB1*15 (0.23) and HLA-DRB1*07 (0.07) (Dhaliwal *et al.*, 2007).

A study conducted by Ma *et al.* (2016) which explored the relationship between *HLA-DRB1* allele polymorphisms and familial aggregation of hepatocellular carcinoma (fhcc) found that, of the 11 selected alleles, the frequencies of HLA-*DRB1**11 and HLA-*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). This is in contrast with our study

which found a higher proportion of HLA-DRB1*12 alleles among the hepatocellular carcinoma patients. This may suggest a different causative mechanism.

To the best of our knowledge, this is the first study conducted in Malaysia for chronic hepatitis B with hepatocellular carcinoma among Malay population. Several studies, however, have been carried out in other countries such as the study in Egyptian patients with hepatocellular carcinoma that were found to have higher frequency of HLA-DR4 (DRB1*04), -DQ2 (DQB1*02) and -DR7 (DRB1*07). On the other hand, HLA-DQ6 (DQB1*06) was significantly lower, leading to the conclusion that this allele may be protective against the development of hepatocellular carcinoma (OR= 0.259) (El-Chennawi *et al*., 2008). It was hypothesized that HLA-DR7 (DRB1*07), HLA-DR12 (DRB1*12) and HLA-DR15 (DRB1*15) alleles may be the key host factors to determine the development of diseases from hepatitis B virus infection to HCC in Asians (Zhong *et al*., 2010). This shows some similarities with our study in terms of increased association of HLA-DRB1*12 and HLA-DRB1*15.

Analytical comparison of the HLA-DRB1 and HLA-DQB1 data obtained in this study and that of Tan *et al*. (2016) did not show statistical significance. This may be largely due to the small number of sample size and low resolution typing method used, which is the limitation of this study. The results of this study need to be validated using larger sample size and high resolution HLA typing for both class I and class II involving multiple ethnics group. This is important in quest for personalized and individual targeted therapy is to be achieved in the near future.

CONCLUSION

In conclusion, the frequency of HLA-DQB1*03 (07) and HLA-DRB1*12 were found to be higher in this study population compared to the general Malay population. Therefore, we postulate HLA-DQB1*03 (07) and HLA-DRB1*12 as potential risk factor for hepatocellular carcinoma among Malays.

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