Patient HLA-DRB1* and -DQB1* allele and haplotype association with hepatitis C virus persistence and clearance

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INTRODUCTION

Hepatitis C virus (HCV) infection is pandemic worldwide and long-term persistence of this virus is known to cause liver cirrhosis. HCV infects about 3% of the world population. In some individuals, HCV infection is self-resolving but does not provide permanent immunity. Most often, HCV infection becomes chronic, manifesting itself as chronic hepatitis, liver carcinoma and cirrhosis (Sinn et al., 2008). The antiviral treatment choice for such individuals is a combination therapy with standard interferon (IFN) and ribavirin, especially in underdeveloped countries where the patients cannot afford the standard pegylated IFN therapy. It should be noted that the response to IFN therapy depends on viral genotype, as some viral strains are resistant to the treatment (Hnatyszyn, 2005). In addition, even among patients infected with the same genotype, the response to IFN therapy is variable, indicating that some host genetic factors such as interleukin (IL) 28B or human leukocyte antigen (HLA) might be influencing the clinical outcome of IFN therapy. A polymorphism near IL28B is associated with the response to IFN therapy for patients with chronic genotype 1 HCV infection (Ge et al., 2009).

In general, the host innate immune response against HCV infection involves the release of type I IFNs (IFN-α and -β) followed by the activation of many genes including cellular proteinase kinase R, MX proteins, RNA helicases and several other antiviral factors (García-Sastre & Biron, 2006; Sadler & Williams, 2008). The cellular immune response to HCV shows involvement of CD4+ T cells. This has been shown in experiments on chimpanzees where the animals that cleared virus more efficiently showed a strong association with CD4+ T cells (Nascimbeni et al., 2003). Similarly in humans the involvement of CD4+ T cells has been shown in response to IFN-α and ribavirin (Kamal et al.,...
In seropositive individuals exhibiting spontaneous viral clearance, the CD4+ T-cell response is mostly against non-structural proteins (NS3, NS4 and NS5) with the predominant response being against NS3 (Lloyd et al., 2007). An epitope in the NS3 region shows high affinity for HLA-DR, indicating the possible involvement of HLA class II in virus clearance (Fanning et al., 2001).

HLAs are encoded by a complex of genes, which are among the most polymorphic regions of the human genome. Allelic variants of HLA have been reported to be involved in immune responses to infectious agents such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and HCV (Roe et al., 2000; Singh et al., 2007). Previously, several HLA alleles have been found to be associated with susceptibility and resistance to HCV infection, pathogenesis leading to liver damage, cirrhosis and the response to IFN therapy (Fanning et al., 2004; Kikuchi et al., 1998; Kuzushita et al., 1998; López-Vázquez et al., 2004). In addition to variations in the immune response, there also appear to be some geographical differences among various population groups in defining HCV pathogenesis. For example, an interesting study reported that the black population exhibited a relatively greater tendency to develop HCV persistent disease (Thio et al., 2007). Similarly, a number of recent studies have shown an association of HLA class II alleles with persistence or clearance of HCV in patients (Table 1).

In Pakistan, approximately 10 million people are infected with HCV, and the majority of these infections are due to the reuse of syringes and needles (Raja & Janjua, 2008). Although many data have been generated on the mode of spread and disease pathogenesis in Pakistan, our study is the first describing the association of HLA alleles with viral pathogenesis and treatment outcome in this particular geographical location. The data generated from our study may be helpful in future for clinicians to predict the treatment outcome for HCV-seropositive individuals in Pakistan. We emphasize the importance of the use of pharmacogenomic data in the treatment of patients in poor countries, who can ill afford expensive treatment with a negative outcome.

**RESULTS**

In this study, we evaluated the association of HLA alleles with HCV pathogenesis and treatment outcome among 204 HCV-seropositive individuals. For comparison purposes, our study also consisted of 102 uninfected controls (C). Out of the 204 patients, 150 (75 males and 75 females) responded to standard IFN therapy, i.e. had a sustained virological response (SVR), whilst 54 patients (26 males and 28 females) did not respond to the therapy (non-SVR) (Table 2). These patients were tested at two intervals, 6 months and 1 year after completion of the standard IFN

**Table 1. HLA class II association with HCV persistence and clearance across global populations**

<table>
<thead>
<tr>
<th>Associated HLA allele</th>
<th>Treatment outcome</th>
<th>Population/country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1<em>0405 and DQB1</em>0401</td>
<td>Viral persistence</td>
<td>Japan</td>
<td>Aikawa et al. (1996)</td>
</tr>
<tr>
<td>DRB1*0301</td>
<td>Viral persistence</td>
<td>Germany</td>
<td>Hohler et al. (1997)</td>
</tr>
<tr>
<td>DRB1<em>0405 and DQB1</em>0401</td>
<td>Viral persistence</td>
<td>Japan</td>
<td>Kuzushita et al. (1998)</td>
</tr>
<tr>
<td>DRB1*07</td>
<td>Viral persistence</td>
<td>Caucasians</td>
<td>Afric et al. (1999)</td>
</tr>
<tr>
<td>DQB1*06</td>
<td>Viral clearance</td>
<td>Caucasians</td>
<td>Afric et al. (1999)</td>
</tr>
<tr>
<td>DRB1<em>1001 and DRB1</em>1101</td>
<td>Viral persistence</td>
<td>Italian</td>
<td>Asti et al. (1999)</td>
</tr>
<tr>
<td>DQB1*0502</td>
<td>Viral persistence</td>
<td>Italian</td>
<td>Mangia et al. (1999)</td>
</tr>
<tr>
<td>DRB1<em>0701, DRB1</em>15 and DRB4*0101</td>
<td>Viral persistence</td>
<td>UK (European)</td>
<td>Thursz et al. (1999)</td>
</tr>
<tr>
<td>DRB1*01</td>
<td>Spontaneous clearance</td>
<td>Irish</td>
<td>Fanning et al. (2000)</td>
</tr>
<tr>
<td>DRB1<em>0301 and DQB1</em>0201</td>
<td>Viral persistence</td>
<td>Thai</td>
<td>Vejbaeya et al. (2000)</td>
</tr>
<tr>
<td>DRB1<em>11 and DQB1</em>03</td>
<td>Viral clearance</td>
<td>Caucasians</td>
<td>Harcourt et al. (2001)</td>
</tr>
<tr>
<td>DRB1*13 allele</td>
<td>Viral persistence</td>
<td>Poland</td>
<td>Kryczka et al. (2001)</td>
</tr>
<tr>
<td>DQB1*0201 (male gender)</td>
<td>Viral persistence</td>
<td>France</td>
<td>Hue et al. (2002)</td>
</tr>
<tr>
<td>DRB1*11</td>
<td>Protection</td>
<td>Turkey</td>
<td>Yenigün &amp; Durupinar (2002)</td>
</tr>
<tr>
<td>DRB14 and DRB17</td>
<td>Viral persistence</td>
<td>Italian</td>
<td>Scotto et al. (2003)</td>
</tr>
<tr>
<td>DQB1*0503</td>
<td>Viral persistence</td>
<td>Japan</td>
<td>Yoshizawa et al. (2003)</td>
</tr>
<tr>
<td>DQB1*0201</td>
<td>Viral persistence</td>
<td>Irish</td>
<td>McKiernan et al. (2004)</td>
</tr>
<tr>
<td>DRB1*07</td>
<td>Viral clearance</td>
<td>Chinese</td>
<td>Jiao &amp; Wang (2005)</td>
</tr>
<tr>
<td>DRB1<em>0803, DQB1</em>0601 and DQB1*0604</td>
<td>Viral persistence</td>
<td>Korean</td>
<td>Yoon et al. (2005)</td>
</tr>
<tr>
<td>DRB1*0401</td>
<td>High viral load</td>
<td>Taiwan</td>
<td>Wang et al. (2005)</td>
</tr>
<tr>
<td>DRB1*15</td>
<td>Viral persistence</td>
<td>Tunisians</td>
<td>Ksiaa et al. (2007)</td>
</tr>
<tr>
<td>DRB1*08</td>
<td>Spontaneous clearance</td>
<td>Tunisians</td>
<td>Ksiaa et al. (2007)</td>
</tr>
<tr>
<td>DRB1*04 and DQB1 *02</td>
<td>Viral persistence</td>
<td>Egyptians</td>
<td>El-Chennawi et al. (2008)</td>
</tr>
<tr>
<td>DQB1*06</td>
<td>Protection</td>
<td>Egyptians</td>
<td>El-Chennawi et al. (2008)</td>
</tr>
<tr>
<td>DRB1*07</td>
<td>Viral persistence</td>
<td>Brazilian</td>
<td>Corghi et al. (2008)</td>
</tr>
</tbody>
</table>
therapy. Patients who remained negative for HCV during this period were grouped as SVR whilst the others were grouped as non-SVR.

The relative allele frequencies of the HLA-DRB1 locus in the C, SVR and non-SVR groups are given in Table 3. In the non-SVR group, HLA-DRB1*07 was found as the most common allele (27.8 %), whereas it was found at lower levels in the C group (16.7 %) and also in the SVR group (13.0 %). HLA-DRB1*11 was found in the C and SVR groups at 15.2 and 14 %, respectively and was found at lower levels in the non-SVR group (5.6 %). HLA-DRB1*04 was found at a relatively higher level in the C group (11.8 %) compared with the other groups. In addition to the HLA-DRB1 locus, the HLA-DQB1 allele frequencies were also obtained in all the groups studied (Table 3). The DQB1*0301 allele was found at similar frequencies in the C (16.7 %) and SVR (17.7 %) groups, whereas in the non-SVR group it was found at only 6.5 %. Multivariate analysis of variance (MANOVA) did not show a significant association of HLA-DRB1 and DQBI alleles with age or gender (Wilks’ λ, P value = 0.531).

In addition to the allele frequencies, the two-locus haplotype frequencies for HLA-DRB1 and -DQB1 were also calculated in the C, SVR and non-SVR groups and the values compared statistically (Tables 4 and 6). The total number of haplotypes found in the C, SVR and non-SVR groups were 49, 56 and 35, respectively (data not shown). A comparison of the most common haplotypes is shown in Table 4. In the controls, the most common haplotype was 07–02 (20.4 %), a frequency much higher than found in either of the other two groups. In this group, the other common alleles 03–02 (16.6 %) and 15–0601 (13.0 %) were found at almost the same levels as in the C and SVR groups.

**Association of genotype with disease outcome**

To study the association between HCV patients’ HLA genotypes and standard IFN therapy, several comparative analyses were performed among the groups, i.e. HLA allele frequency comparison of all the patients (P = SVR + non-SVR patients) with C, comparison of SVR and non-SVR with C, and also SVR compared with non-SVR (Table 5). When the allele frequency for the total 204 (2n = 408) patients at each locus was compared with that obtained for the 102 (2n = 204) controls (P vs C), a significant difference was found only for allele HLA-DRB1*04 [P = 0.004, Bonferroni-corrected P value (pc) = 0.047] with an odds ratio (OR) of 0.41 [95 % confidence interval (CI) = 0.22–0.75]. This showed an association of the HLA-DRB1*04 allele with the controls (Table 5), where it was found at much higher frequency (11.8 %) than in the patients (5 %) (Table 3). Another association was found for HLA-DQB1*0603 (P = 0.03), which became non-significant after applying the Bonferroni correction.

In other comparisons where the patient samples were divided into two groups, i.e. SVR and non-SVR, and when each group was compared separately with C, significant associations were observed at various loci, which became non-significant after applying the Bonferroni correction.
Finally when the non-SVR group was compared with the SVR group, a highly significant association was found for HLA-DRB1*07 (P=0.0008; OR=2.60, 95 % CI=1.50–4.40; pc=0.008), where the non-SVR group had a significantly higher frequency of this allele (27.8 %) compared with the SVR group (13.0 %) (Table 3). In this comparison, the HLA-DQB1*0301 allele also showed a significant association (P=0.004; OR=0.32, 95 % CI=0.14–0.73; pc=0.044), this allele being found at a frequency of 17.7 % in the SVR group and at a frequency of only 6.5 % in the non-SVR group. Another significant association was also found at HLA-DQB1*02 (P=0.02), this allele being found at a frequency of 39.0 % in the non-SVR group and at only 6.5 % in the SVR group. However, this association became non-significant after applying the Bonferroni correction.

Similarly, group comparisons were performed for the two-locus haplotypes, where only two haplotypes, HLA-DRB1*07–DQB1*02 and HLA-DRB1*11–DQB1*0301, showed significant associations when the non-SVR group was compared with the SVR group (Table 6). Haplotypes HLA-DRB1*11–DQB1*0301 became non-significant after applying the Bonferroni correction. Linkage disequilibrium between the two loci HLA-DRB1*11 and HLA-DQB1*0301 was also calculated to determine the association between the two loci (Δ=0.071, P<0.001). However, the haplotype HLA-DRB1*07–DQB1*02 showed a highly significant difference even after applying the Bonferroni correction (P=0.0005; OR=3.1, 95 % CI=1.64–5.80; pc=0.028). The linkage disequilibrium value between these two loci was Δ=0.067 (P<0.001).

**DISCUSSION**

In the current study, we have reported the association of viral persistence or clearance with HLA class II antigens of patients on standard IFN therapy infected with HCV genotype 3a. The HLA-DRB1*07 allele was found to be significantly associated with virus persistence, whilst HLA-DQB1*0301 was found to be associated with virus clearance.
In addition, haplotype HLA-DRB1*07–DQB1*02 was found to be associated with disease persistence, i.e. patients with this haplotype did not have a sustained virological response to IFN therapy (non-SVR group).

In a comparison of the allelic distribution of the HLA-DRB1 and -DQB1 alleles in all patients (P=SVR+non-SVR) with the C group, a statistically significant association of HLA-DRB1*04 with the C group was observed, indicating a protective advantage towards HCV infection of this allele. As this allele was found at a comparatively lower frequency in the patients, we postulate that this allele is probably responsible for spontaneous clearance of the virus, in agreement with the previous study of Cramp et al. (1998).

When the HLA allele differences were compared between the SVR and non-SVR groups with the C group, no significant association was observed at any locus. However, when the non-SVR group was compared with the SVR group, the HLA-DQB1*0301 allele was found to be associated with virus clearance and HLA-DRB1*07 with virus persistence following IFN therapy, the former being consistent with previous findings (Cursino-Santos et al., 2007; Hong et al., 2005; Thio et al., 2001; Yee, 2004). In addition, Cramp et al. (1998) observed a significantly higher frequency of HLA-DQB1*0301 in patients who spontaneously cleared the virus as opposed to chronically infected patients, although we did not see any statistically significant difference in the frequency of this allele in patients and normal controls. However, when the effect of IFN therapy in patients was analysed, we observed a statistically higher frequency of this allele in SVR patients compared with non-SVR patients. This association is consistent with the observations of Harcourt et al. (2001); although they did not report the HCV genotype, it was most probably genotype 1, which is the most prevalent in UK patients in contrast to our study population, which consisted of genotype 3a only, the most common genotype in Pakistan (Idrees & Riazuddin, 2008). These observations are interesting as they indicate a possible mechanism in which the HLA-DQB1*0301 allele is somehow involved in clearance of virus from patients spontaneously by natural immunity or by an IFN-supplemented immune system. However, it must be pointed out that this virus clearance following IFN treatment might be dependent on viral genotype (Jiao & Wang, 2005). We believe that our HLA-DQB1*0301 patients infected with HCV genotype 3a may not have spontaneously cleared the virus, but they did clear the virus following IFN therapy.

In the present study, the HLA-DRB1*07 allele was found to be associated with disease persistence even after IFN therapy, which is consistent with the results of Alric et al. (1999) who also observed a higher prevalence of HLA-DRB1*07 in non-SVR compared with SVR patients. However, upon Bonferroni correction, this difference became non-significant in their study population; in

Table 6. Statistical analysis of HLA haplotype association with response to IFN therapy among the different groups

<table>
<thead>
<tr>
<th>HLA haplotype</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
<th>P-value</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1*–DQB1*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03–02</td>
<td>1.10 (0.67–1.75)</td>
<td>0.87</td>
<td>1.09 (0.43–2.50)</td>
<td>1.00</td>
<td>1.09 (0.73–1.64)</td>
<td>1.02</td>
<td>1.09 (0.73–1.64)</td>
<td>1.02</td>
</tr>
<tr>
<td>07–02</td>
<td>0.85 (0.51–1.42)</td>
<td>0.97</td>
<td>0.75 (0.39–1.47)</td>
<td>0.78</td>
<td>0.75 (0.39–1.47)</td>
<td>0.78</td>
<td>0.75 (0.39–1.47)</td>
<td>0.78</td>
</tr>
<tr>
<td>11–0301</td>
<td>0.85 (0.51–1.42)</td>
<td>0.97</td>
<td>0.75 (0.39–1.47)</td>
<td>0.78</td>
<td>0.75 (0.39–1.47)</td>
<td>0.78</td>
<td>0.75 (0.39–1.47)</td>
<td>0.78</td>
</tr>
<tr>
<td>15–0601</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
</tr>
<tr>
<td>13–0603</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
</tr>
<tr>
<td>15–05</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
</tr>
<tr>
<td>10–05</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
<td>0.89 (0.59–1.37)</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Maximum number of haplotypes found that were used for Bonferroni correction=56.
contrast, our observations remained highly significant even after Bonferroni correction. This could be due to the fact that their patients were mostly infected with genotype 2. For HLA-DRB1*11, we also observed a qualitatively similar result to that for the HLA-DQB1*0301 allele, but this became statistically non-significant once the Bonferroni correction was applied. Linkage disequilibrium results showed that HLA-DRB1*11 is appearing as a significant allele because of its linkage to the HLA-DQB1*0301 allele. Minton et al. (1998) observed that patients with the HLA-DRB1*11 allele cleared the virus more effectively, and this remained statistically significant even after correction for multiple testing. Similar results have been reported in various other studies (Tillmann et al., 2001; Yenigun & Durupinar, 2002). It is worth mentioning that the HENCORE group did not see any significant association (after Bonferroni correction) between any MHC class II allele and the response of patients to IFN therapy (Thursz et al., 1999). This again could be due to population differences as the patients in that study were from all over Europe. It must be pointed out that the current study focused on patients from the Pothohar region, which is where all invaders passed through to reach the Indian subcontinent. This population is thus similar to that of the Ancestral North Indians, which Reich et al. (2009) showed to be derived from Middle Eastern, Central Asian and European populations. Our previous work also substantiates this conjecture (Qamar et al., 2002).

Two-locus haplotype analysis revealed a statistically significant association of the haplotype HLA-DRB1*07–DQB1*02 with persistence of disease. The non-responders (non-SVR) had this haplotype at almost three times the frequency of responders (SVR) and at twice the frequency of the normal control population. This is an important finding as, to date, we have not come across any report that has demonstrated the association of an HLA-DRB1–DQB1 haplotype with persistence of HCV in Pakistani patients. However, Fanning et al. (2001) reported a positive response to IFN therapy in patients from Ireland with the haplotype HLA-DRB1*0701–DQB1*02, again indicating the need to conduct such studies in ethnically different patient populations. In agreement with the data of Correa et al. (2002), we also found this haplotype to be the most common in non-responders and in linkage disequilibrium in our population.

Our findings may have important implications for the disease management of patients suffering from HCV infection in Pakistan, as in the case of other studies (Jiao & Wang, 2005). We also observed that the host immune system plays an important role in virus clearance, which is probably viral genotype dependent. However, in contrast to the work of Jiao & Wang (2005), who found a higher response rate to IFN in patients with the HLA-DRB1*07 allele, we found that in our population these patients were unable to clear the virus. This is important for disease management, as the normal clinical practice in Pakistan is that patients are treated with standard IFN therapy (due to cost issues), so if HLA typing of these patients was performed before the start of therapy and an allele or haplotype associated with persistence was found, these patients could be given a more aggressive therapy, such as pegylated IFN, instead of treating them with the standard IFN therapy. It is important to note that the implications of the current study are relevant only for patients undergoing standard IFN therapy and might not hold true for patients on pegylated IFN; however, our study is valid for poor countries where patients cannot afford the latter and where the standard of care is mostly the former.

**METHODS**

**Subjects.** Blood samples were collected from 204 HCV patients and 102 healthy volunteers (Table 2). The samples were collected only after the subjects gave their explicit written informed consent. This study was approved by an Institutional Review Board and is in compliance with the prevalent laws in the country and also in accordance with the Helsinki Declaration. The HCV patients included in the current study were recruited from the outpatient department of KRL General Hospital, Islamabad, over the period 2000–2007. The age of the study participants ranged from 19 to 65 years. At the time of recruitment, all patients were HCV-positive by PCR with genotype 3a and for at least 6 months (72 injections) had been on standard IFN therapy, i.e. 3 × 10^6 U IFN three times a week and 600 mg ribavirin twice a day. These patients were non-cirrhotic, which was confirmed by liver biopsy. Most of the patients acquired HCV infection during dental or surgical procedures. The exclusion criteria included HBV infection and HIV positivity. In this cohort, some patients showed a rapid virological response with HCV titres becoming undetectable after 4 weeks of treatment. They were tested for an SVR at regular intervals and remained HCV-negative by PCR for at least 1 year. In addition to these, some patients did not clear the virus upon standard IFN therapy and these were grouped into the non-responder (non-SVR) category. The control samples consisted of age-, sex- and ethnicity-matched, unrelated, HCV-seronegative individuals who had volunteered to be a part of this study.

**HCV detection and genotyping.** Plasma/serum from the patient and control samples were separated within 1 h of blood collection and stored at −20 °C. HCV in the plasma/serum was detected using a commercially available RT-PCR kit (AMPLICOR Hepatitis C Virus test, v2.0; Roche Molecular Systems). HCV genotyping was performed as described previously (Ohno et al., 1997). This method detects genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6.

**HLA typing.** For HLA typing, DNA was isolated from blood samples of control and seropositive individuals by a standard organic extraction method (Sambrook et al., 1989). Briefly, the protocol consisted of treating the blood samples with detergent and proteolytic enzymes, followed by phenol/chloroform/isoamylalcohol extraction of the DNA and precipitation with 2-propanol. HLA class II (DRB1* and DQB1*) typing was performed by the phototyping method of Bunce et al. (1995), which uses sequence-specific primers for PCR amplification.

**Statistical analysis.** The distribution of HLA-DRB1* and HLA-DQB1* alleles in HCV patients and controls was analysed by a χ² or Fisher’s exact test. ORs and CIs were calculated using VassarStats (http://faculty.vassar.edu/lowry/tabs.html). P < 0.05 was considered statistically significant. In order to avoid a type I error in the analysis (a false-positive finding), Bonferroni correction was applied to the P-values (http://www.quantitativeskills.com/sisa/calculations/bonfer.htm). For HLA-DRB1
and HLA-DQB1, the number of alleles used for Bonferroni correction was 13 and 11, respectively. Two-locus haplotypes were constructed using the data for HLA-DRB1 and HLA-DQB1 by Arlequin (v3.11) software. Multivariate analysis was carried out by MANOVA using SPSS v10. Pairwise linkage disequilibrium and the statistical significance of linkage disequilibrium using a $2 \times 2$ contingency $\chi^2$ test were calculated as described previously (Iminishi et al., 1992).

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