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Imputation of Class I and II HLA Loci using High-density SNPs from ImmunoChip and Their Associations with Kawasaki Disease in Family-based Study:

HLA imputation in Kawasaki Disease

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Summary

Kawasaki disease (KD) is the leading cause of acquired heart disease in children in most developed countries including the United States. The etiology of KD is not known; however, epidemiological and immunological data suggest infectious or immune-related factors in the manifestation of the disease. Further, KD has several hereditary features that strongly suggest a genetic component to disease pathogenesis. Human leukocyte antigen (HLA) loci have also been reported to be associated with KD but results have been inconsistent, in part, because of small study samples and varying linkage disequilibrium (LD) patterns observed across different ethnic groups. To maximize the informativeness of single nucleotide polymorphism (SNP) genotypes in the major histocompatibility (MHC) region, we imputed classical HLA I (*A, B, C*) and HLA II (*DRB1, DQA1, DQB1*) alleles using SNP2HLA method from genotypes of 6700 SNPs within the extended MHC region contained in the ImmunoChip among 112 white KD patients and their biological parents from North America and tested their association with KD susceptibility using the transmission disequilibrium test. Mendelian consistency in the trios suggested high accuracy and reliability of the imputed alleles (class I=97.5%, class II=96.6%). While several SNPs in the MHC region were individually associated with KD susceptibility, we report over-transmission of HLA-C*15 (z=+2.19, *P*=0.03) and under-transmission of HLA-B*44 (z=−2.49, *P*=0.01) alleles from parents to KD patients. HLA-B*44 has been associated with KD in other smaller studies and

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Ethical Standards Statement

The study conformed to the procedures for informed written consent (parental permission was obtained) approved by institutional review boards (IRB) at all sponsoring organizations and to human-experimentation guidelines set forth by the United States Department of Health and Human Services and finally reviewed and approved by the UAB IRB.

both HLA-C*15 and HLA-B*44 have biological mechanisms that could potentially be involved in KD pathogenesis. Overall, inferring HLA loci within the same ethnic group, using family based information is a powerful approach. However, larger families are warranted to evaluate the correlations of the strength and directions between the SNPs in MHC region and the imputed HLA alleles with KD.

Keywords

HLA imputation; Immunochip; Kawasaki disease; MHC

INTRODUCTION

Kawasaki disease (KD) is the most common cause of acquired heart disease among children in developed countries, including the United States (Newburger *et al.*, 2004). In the U.S. alone, even with only passive surveillance nearly 4248 cases were estimated in 2009 (Holman *et al.*, 2012), and based on the system dynamics modeling simulations, there will be an average of 6200 new patients each year with a total of 161, 776 individuals (half of them adults) with a history of KD by 2030 (Huang *et al.*, 2013). KD is considered an autoimmune disease and includes life-threatening acute vasculitis that diffusely involves multiple organ systems in children and has a predilection for involvement of the coronary arteries (Kawasaki, 1967).

Based on epidemiologic studies and surveys, several features of KD strongly suggest a genetic component to disease pathogenesis: 1) The incidence of KD is 10-fold higher among Asians and Asian Americans than European Americans; 2) Siblings and twins of KD patients have higher risk of KD (relative risk > 10) than the general population (Uehara *et al.*, 2003); and 3) emerging recognition of KD in successive generations. Parents of KD patients in Japan have two-fold higher prevalence than general populations (Uehara *et al.*, 2003). Additionally, multiple genome-wide association (GWA) and candidate gene studies, including our own, have consistently identified the Fc gamma receptors (FcGRs) as major players in the pathogenesis and treatment response for KD (Khor *et al.*, 2011; Makowsky *et al.*, 2013; Onouchi *et al.*, 2012; Shendre *et al.*, 2014; Shrestha *et al.*, 2012; Shrestha *et al.*, 2011). However, there are reports of other genes that have not been adequately studied, including the human leukocyte antigen (HLA) genes in the major histocompatibility complex (MHC) region. As a result of their involvement in immune response, HLA genes have been extensively studied in association with outcomes of autoimmune and infectious diseases. Unlike in other diseases, the role of HLA genes in relation to KD has not been well defined, although a few early reports exist (Fildes *et al.*, 1992; Huang *et al.*, 2007; Kaslow *et al.*, 1985; Kato *et al.*, 1978; Keren *et al.*, 1982; Krensky *et al.*, 1981; Krensky *et al.*, 1983; Matsuda *et al.*, 1977; Oh *et al.*, 2008). Recently, we replicated findings from GWA studies showing an association between a SNP (rs2857151) in HLA Class II region and KD susceptibility (Shendre *et al.*, 2014). In general, the correlations of individual SNPs in the MHC region are not well-defined in relation to being functional or tagging another functional SNP or HLA alleles. Recently, methods have been developed using the haplotype structure of the SNPs surrounding an HLA locus to make prediction of HLA alleles. In this

study, we utilized the dense set of 6700 SNPs, across the extended MHC (xMHC) region in the ImmunoChip, among trios of Caucasian KD population, imputed HLA class I and class II alleles using established methodology and tested their associations with KD susceptibility using a trio family based transmission disequilibrium test (TDT) analysis.

MATERIALS AND METHODS

Study population

We included 112 case-parent trios of European-American descent from a retrospective KD cohort, as previously described (Shrestha *et al.*, 2012). The KD patients and their biological parents were enrolled from children's hospitals at three locations: Seattle, WA; Oakland, CA; and Salt Lake City, UT. Informed consent was obtained from the parents and the study was approved by the Institutional Review Boards at each of the participating institution and University of Alabama at Birmingham.

Kawasaki Disease Diagnosis

Kawasaki disease was defined based on the guidelines recommended by the American Heart Association (AHA) and the American Academy of Pediatrics (AAP) (Newburger *et al.*, 2004; Shrestha *et al.*, 2011). The standard epidemiological criteria of presence of fever for ≥5 days and at least 4 of 5 clinical features were used for the diagnosis of KD (Newburger *et al.*, 2004). In addition, we also included patients with at least 2 clinical features accompanied by coronary artery involvement, and or laboratory criteria as recommended by AHA guidelines.

DNA extraction, genotyping and quality control

As previously described, genomic DNA was extracted from blood or saliva samples obtained from the probands and their biological parents using the Versagene DNA purification kit (Gentra Systems, Minneapolis MN) (Shrestha *et al.*, 2012). The PicoGreen assay for double stranded DNA was used for quantification and the final concentration was adjusted to 100ng/μl in Tris-EDTA.

Genotyping of 195, 806 SNPs was performed using the ImmunoChip, an iSelect HD custom genotyping array (Illumina, San Diego CA) developed by the ImmunoChip Consortium (Cortes & Brown, 2011; Shendre *et al.*, 2014). The loci were selected on the basis of their associations with autoimmune or inflammatory diseases and ImmunoChip was designed using early 1000 Genome Pilot data (February 2010 release) (Cortes & Brown, 2011; Parkes *et al.*, 2013). The ImmunoChip also contains a dense representation of 6,700 SNPs at the xMHC (6p21.3), a region carrying disease-susceptibility genes in many infectious diseases, which will enable imputation of the major classical HLA loci and also a dense SNP set across the KIR/LILR complex. Known 591 duplicate xMHC SNPs in the ImmunoChip were removed.

HLA Imputation

Using 6,700 SNPs in the HLA region (chr 6: 26,009,112–33,478,210 bp), we inferred two-digit and four-digit HLA class I (*A, B* and *C*) and class II (*DRB1*, *DQA1*, and *DQB1*) alleles

with an imputation program using the SNP2HLA method (Jia *et al.*, 2013). Amino acid residues were inferred from the corresponding imputed nonsynonymous SNPs (nsSNPs). Briefly, SNP2HLA uses all available SNPs to impute classical HLA alleles and nsSNPS with the Beagle software package (Browning & Browning, 2009; Jia *et al.*, 2013) from a training data set consisting of the Type 1 Diabetes Genetics Consortium (T1DGC) panel that contained 5,868 SNPs (genotyped with Illumina Immunochip) and 4-digit classical HLA types for *HLA-A*, *-B*, *-C*, *-DPA1*, *-DPB1*, *-DQA1*, *-DQB1* and *-DRB1* for 5,225 unrelated individuals (http://www.broadinstitute.org/mpg/snp2hla/). Previous studies using Illumina ImmunoChip have shown 99.3% accuracy with HLA allele calls and near-perfect correlation between the imputed and genotyped amino acid frequencies and 99.3% of polymorphic amino acid positions reached $r^2_{dosage}>0.8$ (Jia *et al.*, 2013). For consistency, the amino acid nomenclature followed the following pattern "AA_[Gene]_[Amino acid position]_[Genetic Position]_[Allele]" as described in the SNP2HLA manual (v1.0) (http://www.broadinstitute.org/mpg/snp2hla/snp2hla_manual.html). For quality control, Mendelian inconsistency checks were performed for all class I and class II alleles for each parent pair in all trios.

Statistical methods

TDT was employed to assess susceptibility to KD using family-based association test (FBAT), as described previously (Horvath *et al.*, 2001; Shrestha *et al.*, 2012). Only SNPs that were informative in at least 10 trio families without Mendelian errors were included in the analysis. Briefly, alleles were tested independently of each other, using binomial probability to determine whether alleles were differentially transmitted from heterozygous parents to the affected child. Single marker analysis was conducted to test the association of all the individual SNPs in the xMHC regions, imputed HLA class I and class II alleles under the null hypothesis of no linkage and no association using the dominant genetic model. The direction and frequency of transmission is provided through the Z statistic and p-values. Similar analyses were performed with imputed amino acids.

The tagging SNPs for individual HLA alleles were identified using the SNAP server available at http://www.broad.mit.edu/mgp/snap/ (Johnson *et al.*, 2008). SNPs associated with other diseases, specifically autoimmune diseases and/or gene expression quantitative trait loci (eQTL) in various cell lines were also examined for their involvements in gene regulation using SCAN (http://www.scandb.org/newinterface/about.html), GWASdb (http://jjwanglab.org/gwasdb) and/or RegulomeDB (http://regulome.stanford.edu) databases. For all significantly associated amino acid residues, additional computation was performed to identify if multiple HLA alleles were tagging them.

RESULTS

SNP variants and KD susceptibility

Results from the conventional single SNP (in xMHC region) association analyses are shown in Figure 1, with rs7741091 in Chr6:31384854 being the most significantly associated variant in the additive model ($p< 2.2$ E-04) and rs9269080 in Chr6:32473192 being the most significantly associated variant in the dominant model ($p< 6.5$ E-06).

HLA imputation and association with KD susceptibility

We identified 48 two-digit HLA alleles for class I, 22 two-digit HLA alleles for class II, 64 four-digit HLA alleles for class I and 45 four-digit HLA alleles for class II resulting from the imputation of 6,700 SNPs. However, only 28 class I and 16 class II two-digit alleles and 31 class I and 24 class II 4-digit alleles were informative for TDT analysis. There were only 17 (2.5%) Mendelian errors in total with class I alleles and 23 errors (3.4%) with class II alleles in 112 trios, with none for HLA-C*15 and HLA-B*44 alleles.

HLA-C*15 and HLA-B*44 were the two alleles in class I HLA that were statistically significant in the TDT analysis (Table 1). HLA-C*15 allele was over-transmitted (n=12, z= +2.19, *P*=0.03) from parents to affected offspring whereas HLA-B*44 was under-transmitted (n=33, z=−2.49, *P*=0.01) from parents to KD patients (Table 1). All of HLA-C*15 alleles were typed to four-digit HLA-C*15:02, showing the same association (data not shown). HLA-B*44 were typed to B*44:02 and B*44:03 with only B*44:03 indicating borderline significant under-transmission (n=20, z=−1.9, *P*=0.055) (data not shown). No other four-digit HLA class I alleles were significantly associated. None of the class II HLA alleles were associated with KD susceptibility (Tables 2). Several amino acid residues were statistically significant in the TDT analysis (Supplementary Table), with the most significant one (AA_B_67_31432515_FC) being an under-transmission of phenylalanenine (F) or Cysteine (C) at position 67 in gene B compared to alternate amino acids, serine (S) and tyrosine (Y).

DISCUSSION

Kawasaki disease is considered an immune-mediated disease and HLA loci in the MHC region that are involved in antigen processing and presentation could have potential role in the disease pathogenesis. In this study, we focused on the association of 6700 SNPs in the MHC region and imputed HLA class I and class II alleles. Evaluation of Mendelian consistency in the trios showed high accuracy (97.5% in class I and 96.6% in class II) of the inferred HLA alleles. We also report HLA-C*15 and HLA-B*44 alleles in HLA class I to be associated with Kawasaki disease.

Of the previous HLA association studies, two have reported the association of HLA-B*44, alone (Krensky *et al.*, 1983) or in combination with other HLA alleles with KD among European Americans (Kaslow *et al.*, 1985; Krensky *et al.*, 1983). Both of these associations were observed among two smaller sets (23 cases in Boston area and 16 cases in Maryland) of epidemic cases of KD which indicated potential involvement of infectious trigger in the etiology of KD. Of note, while we observed an under-transmission of HLA-B*44 from parents to KD patients, the previous two studies showed increased frequency of B*44 among incidence KD cases. B*44 has two major alleles, B*44:02 and B*44:03 and in our study, B*44:03, which was less frequent (4.8%), was borderline significant (n = 20, z = −1.913, p = 0.055) in relation to under-transmission, but B44*02 did not show any significant association. It is possible that there is allelic heterogeneity at 4-digit level between our participants and those from previous two epidemic KD case studies. For instance, based on Kaslow et al report, combination of B*44-C*05 was over-represented in KD patients; however, it is known that HLA-B*44:02 is in LD with C*05 and not B*44:03

(Cao *et al.*, 2001). Since both epidemic studies had similar results, it is possible that B*44:02 (or another allele in LD) is a risk allele and B*44:03 (or another allele in LD) observed in our study is a protective allele. HLA B*44 has also been shown to be associated with other autoimmune diseases such as Grave's disease (Gough & Simmonds, 2007) and with Crohn's disease associated ankylosing spondylitis in combination with HLA-B*27 (Purrmann *et al.*, 1988). In Africans, HLA-B*44:03 was highly favorable during acute and early chronic HIV-1 infection (Tang *et al.*, 2011). HLA-B*44:03 has a frequency of 2.2% and is tagged by two SNPs, rs12526186, and rs7747738 in Caucasians. It is interesting that some adult HIV patients have been reported to have presented KD-like symptoms (Blanchard *et al.*, 2003; Johnson *et al.*, 2003; Johnson *et al.*, 2001; Stankovic *et al.*, 2007; Yoganathan *et al.*, 1995); thus, it is possible that this subset population could share HLA alleles that could have triggered in response to an etiological antigen. Additionally, other genetic variants in *CCR2*, *CCR5* and *CCL3L1* genes associated with HIV susceptibility have also been shown to be important in KD pathogenesis (Burns *et al.*, 2005; Mamtani *et al.*, 2010).

Overall, the frequencies of most of the HLA alleles were similar those reported in Caucasian population (http://www.allelefrequencies.net/hla6006a.asp). The frequency of HLA-C*15 in our study is 4.3%, which is higher than the known frequency of 1.9 in other white populations (3.6% in Hispanics and 2.6% in Asians). In Caucasians, HLA-C*15:02 has a frequency of 2.8% and is tagged by two SNPs, rs3764808 and rs4379333, both of which are known eQTLs (but not included in the ImmunoChip). Further, amino acid residues, Leucine in position 116 in HLA-C corresponding to variant in genomic position hg18 31347029 (AA_C_116_31347029_L) and histidine in position 113 in HLA-C corresponding to variant in genomic position hg18 31347038 (AA_C_113_31347038_H), both of which were significantly associated in TDT analysis ($z = +2.188$, $p < 0.03$, supplementary table) were perfectly tagged by HLA_C*15. Thus there is potential functional relevance, which warrants further research. SNP rs4379333 along with variant rs887466 also predicts HLA-C*06:02 ($r^2 = 1.0$) which has been associated with psoriasis (de Bakker *et al.*, 2006). HLA-C*15 has also been associated with multiple sclerosis (Gough & Simmonds, 2007).

Associations with other HLA alleles and in different race/ethnicities have also been reported. One small, previous study suggested that some HLA-B (B*35 and B*75) and HLA-C (Cw09) alleles were associated with KD in Korean children (Oh *et al.*, 2008). However, of interest, amino acid valine in position 194 of HLA-B corresponding to variant in genomic position hg18 31431315 (AA_B_194_31431315_V), which was significantly associated in the TDT analysis ($z = +2.59$, $p<0.0095$, supplementary table) was perfectly tagged by a combination of HLA_B*35 and B*51, although they were not significantly associated when examined individually. In two other early studies, HLA-Bw51 was associated with KD in Israeli patients (Keren *et al.*, 1982) and HLA-Bw22 (Bw54) was associated with KD in a Japanese population (Kato *et al.*, 1978). However, we did not observe associations with any of these alleles possibly because the ethnicity researched in our study was different compared to most studies. Moreover, we did not see any effect with the imputed HLA class II alleles in this study, similar to what has been previously reported (Barron *et al.*, 1992; Fildes *et al.*, 1992; Huang *et al.*, 2007). Overall, the correlations of

HLA genes with KD either in the Class I or the Class II region have been inconsistent. This may, in part, be a function of varying race/ethnicity of the study participants, smaller sample sizes of the earlier studies, or complexity of the HLA region as a result of the increased linkage disequilibrium observed within and between the two HLA class regions.

In the secondary analysis, the most significantly associated amino acid residue (AA_B_67_31432515_FC), was located in position 67 of HLA-B where an under-transmission of phenylalanenine (F) or Cysteine (C) was observed from parents to KD child. The amino acid change corresponds to a quadri-allelic variant rs1071816 at genomic position 31432515 (HG18) in chromosome 6, which codes for four different amino acids (F (0.35), C (0.14), Y (0.15) and S (0.36)). Upon replication of these associations in larger cohorts, functional work of these amino acids and others (supplementary table) could help understand the biological mechanism of KD.

We have also replicated the associations of the previous GWAS SNP rs2857151 ($P<0.04$), in the intergenic region of the HLA Class II histocompatibility antigen, DQ beta 2 chain and DO beta (*HLA-DQB2* and *HLA-DOB*), in relation to susceptibility to KD. However, another SNP rs9269080 in this region was observed to be the most significant SNP in the dominant TDT model and rs7741091 was most significant in the additive TDT model (Fig 1a & 1b). SNP rs2857151 was shown to be an eQTL for several of the HLA genes, namely, *HLA-DOB*, *-DRB1*, *-DRB5*, *-DQA1*, *-DQA2*, as well as the transporter 2, ATP-binding cassette, sub-family B (*TAP2*) gene. SNP rs9269080 is in LD with several other SNPs in the vicinity of the SNP rs9268877 and include SNPs that are known eQTLs for *HLA-DQA1* or *-DQA2*. The potential involvement of immune-related genes, specifically in the MHC region further implies that KD is an infectious or autoimmune disease.

The strengths of our study include the availability of a homogenous population of European Americans for whom the ethnicity was genetically determined using Ancestry Informative Markers. The trio family-based association test using TDT is usually robust to confounding effects and variance inflation therefore allowing us to replicate previous findings even with a small sample size. Our limited sample size restricted our ability to perform multiple testing and therefore there is a possibility that some of the observed associations are false positives. However, most methods for multiple testing corrections assume an independent test, which is not the case with ImmunoChip in the MHC region, where there is strong LD between SNPs and therefore they would be highly conservative in our study.

The use of the ImmunoChip provided us with a higher density of markers in the xMHC region and allowed inclusion of more than 6500 SNPs for HLA imputation, and the use of SNP2HLA ensured high call rates of 96.7% (Jia *et al.*, 2013). Although SNPs in the xMHC region are not comprehensive and may not include the whole spectrum of SNPs and genes related to immune response, we report associations of HLA-B*44 and HLA-C*15 alleles with KD. We also acknowledge that most imputation methods, including the one that we used, have been developed for specific class I and II HLA genes, and cannot perform analyses with other HLA class I and II genes (e.g. HLA-G and HLA-E) or class III genes (Kim *et al.*, 2008; Lin *et al.*, 2009; Maggioli *et al.*, 2014), where associations have been previously reported; hence, there is need for future studies with larger samples and typed

HLA data to replicate as well as to determine novel or causal associations (both with SNPs and HLA alleles) in relation to KD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The parent study and this genetic sub-study conformed to the procedures for informed consent (parental permission) approved by institutional review boards at all sponsoring organizations and to human-experimentation guidelines set forth by the United States Department of Health and Human Services. We thank the participating patients and their parents. We also thank investigators (Dr. Neil E Bowles at Department of Pediatrics, University of Utah, Salt Lake City, UT; Dr. Hitendra Patel at Children's Hospital of Oakland, Oakland, CA), pediatricians and staffs of the participating clinics. This study was supported by National Institutes of Health grant NHLBI-R21-HL90558 & The American Heart Association.

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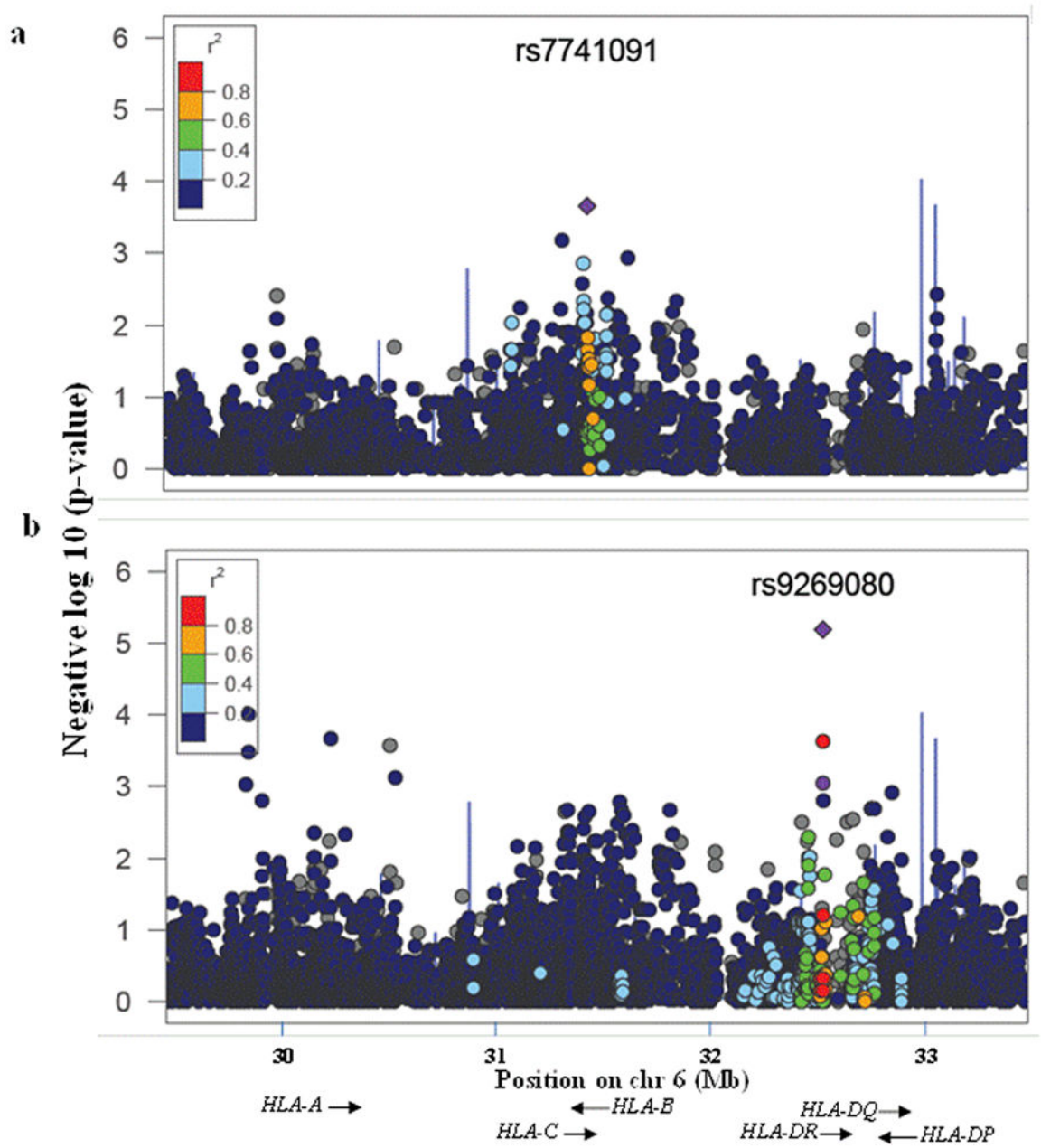

Figure 1.

Fine-mapping of SNPs in the extended MHC (xMHC) regions and their associations with Kawasaki Disease Association in family based Transmission Disequilibrium test (TDT) analysis. Negative log (p-values) are shown in the y-axis for each SNP in the xMHC region by the genomic position in chromosome 6 in the x-axis for a) additive and b) dominant genomic models. Linkage disequilibrium measures (r^2) are shown in the range of color for each SNP with respect to the most significantly associated SNP in each model.

Table 1

Associations of two-digit class I alleles with Kawasaki Disease susceptibility in family-based trio study

No.	Alleles 2-digit	frequency	Informative family	Z-statistic	P Value
1	HLA-A*01	0.17	38	−0.33	0.74
2	HLA-A*02	0.28	47	+0.76	0.45
3	HLA-A*03	0.15	44	−1.71	0.09
4	HLA-A*11	0.06	19	+1.04	0.29
5	HLA-A*23	0.06	24	−0.82	0.41
6	HLA-A*24	0.15	35	−0.51	0.61
7	HLA-A*25	0.05	14	−0.27	0.79
8	HLA-A*68	0.05	15	+0.78	0.44
9	HLA-C*01	0.05	14	+1.60	0.11
10	HLA-C*03	0.14	30	+0.74	0.46
11	HLA-C*04	0.10	24	+0.21	0.84
12	HLA-C*05	0.11	26	−0.30	0.77
13	HLA-C*06	0.07	21	−1.00	0.32
14	HLA-C*07	0.35	41	−0.57	0.57
15	HLA-C*12	0.07	26	−0.69	0.49
16	**HLA-C*15**	**0.04**	**12**	**+2.19**	**0.03**
17	HLA-C*16	0.03	15	−1.29	0.19
18	HLA-B*07	0.17	38	−1.07	0.28
19	HLA-B*08	0.13	30	−0.47	0.64
20	HLA-B*15	0.07	17	−0.12	0.90
21	HLA-B*18	0.04	16	−1.13	0.26
22	HLA-B*27	0.07	18	+0.47	0.64
23	HLA-B*35	0.10	23	+0.94	0.35
24	HLA-B*39	0.07	22	+0.54	0.59
25	HLA-B*40	0.05	12	+1.02	0.31
26	**HLA-B*44**	**0.12**	**33**	**−2.49**	**0.01**
27	HLA-B*51	0.11	33	+1.57	0.12
28	HLA-B*57	0.04	13	−0.42	0.67

Table 2

Associations of two-digit class II alleles with Kawasaki Disease susceptibility in family-based trio study

No.	Alleles 2-digit	frequency	Informative family	Z-statistic	P Value
1	HLA-DRB1*01	0.12	31	+0.91	0.36
2	HLA-DRB1*03	0.15	33	−0.36	0.72
3	HLA-DRB1*04	0.20	38	+0.08	0.93
4	HLA-DRB1*07	0.15	42	−0.95	0.34
5	HLA-DRB1*11	0.15	35	+0.43	0.67
6	HLA-DRB1*13	0.14	36	−0.59	0.55
7	HLA-DRB1*14	0.05	14	+0.69	0.49
8	HLA-DRB1*15	0.21	44	−0.86	0.39
9	HLA-DQA1*01	0.42	43	−0.24	0.81
10	HLA-DQA1*02	0.11	35	−1.64	0.10
11	HLA-DQA1*03	0.17	33	−0.53	0.60
12	HLA-DQA1*05	0.27	45	−0.76	0.45
13	HLA-DQB1*02	0.17	41	−0.96	0.34
14	HLA-DQB1*03	0.40	41	−0.33	0.74
15	HLA-DQB1*05	0.11	32	+1.44	0.15
16	HLA-DQB1*06	0.29	46	−0.15	0.88