Human Immunology 75 (2014) 867–872

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

The polymorphisms of human leukocyte antigen loci may contribute to the susceptibility and severity of severe aplastic anemia in Chinese patients

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ARTICLE INFO

Article history:
Received 8 August 2013
Accepted 19 June 2014
Available online 27 June 2014

Keywords:
Aplastic anemia
HLA
Susceptibility
Severity
Chinese

ABSTRACT

The human leukocyte antigen (HLA) system has been reported to be involved in the development of aplastic anemia (AA). We compared and analyzed HLA-A, B, C, DRB1 and DQB1 alleles in 96 Chinese severe AA (SAA) patients to those in 600 healthy people chosen randomly from the China Marrow Donor Program to investigate the association of HLA class I and II allele polymorphisms with disposition of SAA and its severity degree in Chinese population. The DNA of patients was extracted and HLA high-resolution genotyping was conducted using polymerase chain reaction-sequence based typing technique. The gene frequencies of A*02:01, A*02:06, B*13:01, DRB1*07:01, DRB1*09:01, DRB1*15:01 and DQB1*06:02 in SAA patients were significantly higher than in controls (all $P < 0.05$), while the allelic frequencies of A*02:07, A*11:01 and B*40:01 were notably lower in SAA patients than those in the controls ($P = 0.001$, 0.002, 0.005, respectively). Comparison among different severity of SAA groups exhibited significant increases of DRB1*15:01 ($P = 0.027$) and DQB1*06:02 ($P = 0.013$), but obviously lower frequencies of B*46:01 ($P = 0.023$) and DRB1*09:01 ($P = 0.020$) in non-VSAA patients than in VSAA patients. Thus, our results identified several risk and protective HLA alleles for Chinese SAA patients. Moreover, DRB1*15:01, DQB1*06:02, B*46:01 and DRB1*09:01 may be associated with severity of SAA.

1. Introduction

Aplastic anemia (AA) is a life-threatening hematopoietic stem cell (HSC) disorder characterized by pancytopenia in peripheral blood and hypoplasia in bone marrow (BM) [1]. AA can be classified as chronic AA (CAA) and severe AA (SAA, including non-VSAA and VSAA) according to disease severity. Though immune-mediated mechanism plays an important role in the pathogenesis of acquired AA, the pathogenesis might differ between the types of AA and little is known about the factors associated with the severity of AA [2,3]. Genetic risk factors and environment were considered to be crucial determinants of susceptibility to AA [4–9]. The human leukocyte antigen (HLA) located at chromosome 6p21.3 served as an important gene responsible for immune regulation. So far, a number of studies have reported strong associations between HLA and several autoimmune diseases, such as ankylosing spondylitis with HLA-B27, psoriasis with HLA-B13 and HLA-B17-Cw6, rheumatoid arthritis with HLA-DR1 and HLA-DR4, juvenile diabetes mellitus with HLA-B8, HLA-B15 and HLA-B54, and multiple sclerosis with MS, HLA-B7 and HLA-DR2 [4]. Meanwhile, AA has also been reported to have positive correlation with HLA alleles in different regions worldwide [5–9].

The HLA genes are the most polymorphic human genes with more than 8000 alleles [10], and the polymorphisms themselves show differences according to varying ethnicities and regions. Most of the polymorphic sequence motifs of the HLA class II loci are localized to the second exon. The amino acid residues encoded by the exon form the peptide binding cleft and interact with the peptide and the T cell receptor [11]. So HLA-II has always been the research priority for predisposition to AA. However, most of such studies conducted the HLA typing using low-resolution genotyping, which can only reach antigen-level resolution in verification of alleles due to the technical limitations. Because most

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http://dx.doi.org/10.1016/j.humimm.2014.06.011
0198-8859/

antigen-level allele types contain diversified subtypes, which might perform different roles, when it comes to the high-resolution genotyping, which reaches the level of the nucleotide sequences, the results might be more precise, significant and convincing, even if maybe widely different.

Studies have suggested a marked geographic variation in AA rates, with comparatively large numbers of patients in Asia relative to Europe and the United States [12,13]. This suggested that it was the genetic component and/or combined with numerous environmental factors that provided the key background for susceptibility to AA and its variable incidence worldwide [14]. China was burdened with a huge amount of AA patients with an enormous base population [15]. The current study aimed to explore the association of the gene frequencies of HLA-A, B, C, DRB1 and DQB1 alleles with susceptibility to severe AA (SAA) together with the varying degrees of SAA severity in Chinese patients.

2. Materials and methods

2.1. Patients and controls

A total of 96 SAA patients were investigated, all of whom were diagnosed and underwent HLA detections at Blood Diseases Hospital Chinese Academy of Medical Sciences & Peking Union Medical College from January 2000 to May 2013. Among the 96 patients, 56 were males and 40 females, with an median age of 19 years old (ranging from 6 to 53 years old). 48 cases were very severe AA (VSAA), and the remaining 48 cases were non-VSAA (non-VSAA).

For ethnically matched controls, 600 healthy BM donors were selected randomly from the China Marrow Donor Program (CMDP). They were all genotyped at the HLA-A, -B, -C, -DRB1 and DQB1 loci. All the SAA patients and controls belonged to the Chinese Han population and were from various regions of China. Besides, all SAA patients were unrelated individuals. The study was approved by the Institutional Committee for Medical Care and Safety, and written informed consents were obtained from all SAA patients or their guardians before inclusion in the study.

2.2. Diagnosis of AA

Diagnosis of AA was based on those of Camitta [16] and on the International Agranulocytosis and Aplastic Anaemia Study Group (1987) [17] criteria. Patients had to have hypocellularity in BM biopsy and at least 2 of the 3 following full blood count criteria: (1) a hemoglobin concentration of less than 10 g/dL; (2) a white blood cell count of $\leqslant 3.5 \times 10^9$/L or a neutrophil count $\leqslant 1.5 \times 10^9$/L; and (3) a platelet count of $\leqslant 50 \times 10^9$/L. Also, BM should not exhibit fibrosis or infiltration by leukemia, lymphoma, or carcinoma. SAA was diagnosed if BM cellularity was <25% or between 25% and 30% but the proportion of remnant hematopoietic cells <30%; Moreover, SAA diagnosis had to satisfy at least two of the following three criteria: (1) neutrophil count $< 0.5 \times 10^9$/L; (2) platelet count $< 20 \times 10^9$/L; and (3) reticulocyte count $< 20 \times 10^9$/L. If the criteria for severe disease were fulfilled and the neutrophil count was $<0.2 \times 10^9$/L, VSAA could be diagnosed. Non-VSAA were the SAA patients who didn't meet the VSAA criterion. Patients who received chemotherapy, immunotherapy, or radiaotherapy and had hypoplastic anemia secondary to a known drug were excluded. Congenital forms of AA, such as Fanconi anemia or dyskeratosis congenita, were excluded by a set of "sandwich" tests, namely cytogenetic diagnosis, mitomycin C-induced chromosomal fragility test, and mononuclear cell gel electrophoresis [18].

Table 1
Genotyping distribution in patients and controls.

Loci genotyped	Non-VSAA *n*	VSAA *n*	All patients *n*	Control *n*
HLA-A, B, C, DRB1 and DQB1	32	32	64	600
HLA-A, B and DRB1	16	16	32	0
Total	48	48	96	600

2.3. HLA typing

Two milliliters of venous blood were drawn from SAA patients, anticoagulated with ethylene diamine tetraacetic acid (EDTA) and then kept at 4 °C. DNA was extracted using a QIAamp DNA mini kit (Qiagen, Germany). The purity and concentration of DNA were detected using a Pharmacia Gene Quant RNA/DNA Calculator. The concentration of DNA was 175–200 ng/L, and A260/A280 as 1.8–2.0. All DNA was stored at −20 °C until tested. Genomic DNA was amplified by the HLA locus-specific primers that we designed based on the standard ones. Then, HLA typing was performed by polymerase chain reaction-sequence based typing (PCR-SBT) high-resolution genotyping, using a BigDye TM Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequencing was performed using an ABI Prism® 3730 Genetic Analyzer (Applied Biosystems). For HLA-A, -B, and -C, exons 2 and 3 were sequenced, and for DRB1 and DQB1, only exon 2 was sequenced. The HLA-SBT typing results were analyzed by means of SBT engine software 3.0 (Genome Diagnostics B.V., Utrecht, Netherlands), according to the current IMGT/HLA Database release 3.15.0 [10]. The ambiguous genotyping samples were identified by using high resolution polymerase chain reaction-sequence specific primer (PCR-SSP) genotyping to confirm the results [19]. Of all 96 patients, 64 had been typed for HLA-A, B, C, DRB1 and DQB1 alleles (32 were non-VSAA, and 32 VSAA) and remaining 32 were only typed for HLA-A, B and DRB1 alleles (16 were non-VSAA, and 16 VSAA) (Table 1).

2.4. Statistical analysis

For comparison of the frequencies of HLA alleles between patients and control groups, we used Pearson's χ^2 analysis in a 2×2 contingency tables and two-sided Fisher's exact test when appropriate; P values that were $\leqslant 0.05$ were considered statistically significant, and odds ratios (OR) with 95% confidence intervals (CI) were calculated for those comparisons demonstrating significant P values. Alleles with very low frequencies that would cause obvious deviations or meaningless results would be excluded from analysis. Each typed locus in the controls was tested for Hardy–Weinberg equilibrium (HWE) by ARLEQUIN software based on Guo and Thomson exact test [19,20]. The statistical analyses were performed by using the SPSS 17.0 statistical software.

3. Results

3.1. Number and frequency of HLA polymorphisms

A total of 1392 alleles were detected in this study, with all the patient and control samples were genotyped for HLA-A, -B, and -DRB1 ($696 * 2 = 1392$), but that only 64 patient and 600 control samples (totally 664/696 samples) were typed for HLA-C and -DQB1 ($664 * 2 = 1328$). The genotyping results showed 31 kinds of polymorphisms in HLA-A alleles, 71 in HLA-B, 34 in HLA-C, 43 in HLA-DRB1, and 20 in HLA-DQB1 for all our patients and controls. No deviations from HWE were observed in the control individuals at each locus. Among these polymorphisms, A*11:01 (25.6%),