Original article

HMGB1 gene polymorphism is associated with coronary artery lesions and intravenous immunoglobulin resistance in Kawasaki disease

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Abstract

Objectives. Kawasaki disease (KD) is an acute systemic vasculitis of unknown aetiology that affects infants and young children. Recent reports of elevated serum high mobility group box 1 (HMGB1) level during the acute phase of KD and its relationship to poor response to IVIG treatment suggest a possible association of HMGB1 polymorphisms with KD. We investigated the association between the polymorphisms of the HMGB1 gene, KD susceptibility, coronary artery lesions, and KD response to IVIG treatment.

Methods. Whole genome sequencing of the HMGB1 gene was performed to identify causative variants. Two tagging single nucleotide polymorphisms of the HMGB1 gene were selected using linkage disequilibrium analysis. The tagging single nucleotide polymorphisms were genotyped using the TaqMan Allelic Discrimination assay in a total of 468 subjects (265 KD patients and 203 controls).

Results. The HMGB1 single nucleotide polymorphisms were not associated with KD susceptibility. However, in KD patients, there was a significant association of rs1412125 with coronary artery lesions formation in the recessive model (GG vs AA + GA: odds ratio = 4.98, 95% CI = 1.69–14.66, P = 0.005). In addition, rs1412125 was associated with IVIG resistance in the recessive (GG vs AA + GA: odds ratio = 4.11, 95% CI = 1.38–12.23, P = 0.017) and allelic models (G vs A: odds ratio = 1.80, 95% CI = 1.06–3.06, P = 0.027).

Conclusion. The rs1412125 in HMGB1 might be a risk factor for the development of coronary artery lesions and IVIG resistance in KD patients.

Key words: Kawasaki disease, HMGB1, coronary artery lesion, IVIG resistance

Introduction

Kawasaki disease (KD) is an acute febrile vasculitis of childhood with the classic complication of coronary artery lesions (CAL). The aetiology of KD remains unknown, although it is thought that symptoms of KD are related to hyperactivation of the immune system triggered by infection in patients with genetic susceptibility [1]. High mobility group box 1 (HMGB1) is a highly conserved, non-histone chromosomal protein that plays a role as a DNA chaperone regulating DNA replication, V(D)J recombination, transcription, DNA repair and the stabilization of nucleosome formation by DNA binding and bending activities [2]. In response to injurious or infectious stimuli, HMGB1 also acts as a damage-associated molecular pattern (DAMP) to induce inflammation, proliferation and migration of immune cells [2, 3]. With the recent numerous studies on HMGB1 and its role as a DAMP molecule in conjunction with other inflammatory mediators, HMGB1 has been implicated as a critical molecular target in inflammatory mechanisms in the pathogenesis of many autoimmune and inflammatory diseases.

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Rheumatology key messages

- SNP rs1412125 is associated with coronary artery lesions formation in Kawasaki disease.
- SNP rs1412125 is associated with IVIG resistance in Kawasaki disease.
- The HMGB1 polymorphism may be a predictor of coronary artery lesions development and IVIG resistance.
Two recent studies have reported an association between serum HMGB1 levels and KD. Hoshina et al. reported that children in the early acute phase of KD showed the highest levels of HMGB1, and levels decreased significantly during the late acute phase and convalescent phase of the disease. In the study of Eguchi et al. serum HMGB1 levels were significantly higher in poor-responders to IVIG than good-responders to IVIG. The evidence suggests that HMGB1 may play an important role in the pathogenesis of KD. So far, there are no data on the association between single nucleotide polymorphisms (SNPs) of HMGB1 and clinical outcomes such as development of CAL in KD patients. We hypothesized that SNPs of HMGB1 could influence clinical outcomes, including CAL formation and response to IVIG treatment, in patients with KD.

Methods

Study subjects

KD patients were recruited from the Department of Pediatrics at Severance Children’s Hospital, Seoul, Korea from 2012 to 2015. Diagnosis of KD was made by pediatricians according to the criteria of the Japanese Kawasaki Disease Research Committee. Incomplete or atypical KD cases were excluded. All patients were enrolled at the time of initial diagnosis. Control group consisted of healthy children without history of KD, who had visited the outpatient clinic of the hospital for their symptoms other than KD (Fig. 1). CAL was defined by echocardiography as aneurysm proposed by the Japanese Ministry of Health Criteria or dilation (Z-score > 2.5), according to American Heart Association criteria. IVIG resistance was defined as the presence of persistent or recurrent fever (≥ 38°C) at ≥ 36 h after IVIG treatment completion.

The study protocol was reviewed and approved by the Yonsei University Health System Institutional Review Board, Seoul, Korea (4-2008-0055). The study was conducted in accordance with good clinical practices (national regulations and ICH E6) and the principles of the Helsinki Declaration. Written informed consent was obtained from the parents or legal guardians of the patients prior to sample collection following a detailed explanation of schedules and contents of the study.

Discovery of variations and tag SNP selection

Genomic DNA was extracted from blood cells using a QiAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). The quantity of DNA was measured using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA).

For causative SNP discovery, we sequenced the HMGB1 gene in 24 subjects (12 KD and 12 controls). Genomic sequencing data for the HMGB1 gene were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/). PCR primers that amplified the exon and promoter regions of the gene were designed using Primer3.
software (http://carbon.bineer.co.kr/primer3plus). PCR reaction mixtures consisted of 2.5 mM MgCl₂, 10× reaction buffer, 2.5 mM of each dNTP, 0.5 p.m. of each primer, 0.25 U of Taq DNA polymerase (SolvTM) and 10 ng of genomic DNA in a 30-µl reaction volume. Amplification conditions for all reactions were as follows: initial denaturation at 96°C for 5 min; 35 cycles at 96°C for 30 s, 62°C for 30 s and 72°C for 60 s; and a final extension cycle of 72°C for 10 min. PCR products were purified by treatment with MEGA quick-spin total Fragment DNA Purification kit (iNTRON Biotechnology, Korea). Purified PCR products were sequenced using BigDye Terminator chemistry on an ABI Prism 3730xl DNA analyser (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The PolyPhred program (http://droog.gs.washington.edu/mpg/haploview/) was used to identify DNA polymorphisms [10]. The tagging SNPs were selected using Haplovie 4.2 software (http://www.broad.mit.edu/mpg/haplovie/).

Genotyping

Genotyping was carried out using the TaqMan fluorogenic 5'-nuclease assay (Applied Biosystems, Foster City, CA, USA). Briefly, PCR was performed using a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, USA). Fifteen nanograms of DNA were amplified in a total volume of 10µl containing TaqMan Universal PCR Master Mix (Applied Biosystems) including 40× TaqMan SNP Genotyping Assay. The thermal cycle conditions were as follows: denaturing at 95°C for 10 min, followed by 45 cycles of denaturing at 95°C for 15 s and annealing and extension at 60°C for 1 min. After PCR, fluorescence was measured and analysed using QuantStudio 6 FlexReal-Time PCR software v1.2 (Applied Biosystems).

Statistical analysis

All statistical analyses were performed using R software v3.4.0, on a Windows 10 platform. The statistical differences between KD patients and controls in genotype and allele frequency were assessed using the χ² test or Fisher's exact test. Statistical differences in genotype and allele frequency of KD children with/without CAL formation and patients with IVIG resistance/responsiveness were assessed using the χ² test. The Bonferroni test was used to correct for multiple tests. Binary multiple logistic regression was performed for the odds ratio (OR), 95% CI, and corresponding P values, with adjustment for age and sex.

Results

No association between HMGB1 polymorphisms and susceptibility to KD

Study subjects included 265 KD patients and 203 controls (Supplementary Table S1, available at Rheumatology online). Average ages were 35.31 months in cases and 116.96 months in controls. Of the 265 KD patients, 45 (17.0%) developed CAL, and 38 (14.3%) were resistant to initial IVIG treatment. Fourteen (5.3%) had both CAL formation and IVIG resistance.

A total of two SNPs were identified by the entire HMGB1 gene sequencing for causative SNP discovery. These SNPs were identical to those in the dbSNP database and no novel SNPs were identified (https://www.ncbi.nlm.nih.gov/snp). Based on the results of linkage disequilibrium analyses, two tagging SNPs (rs1412125 and rs117077167) were selected for genotyping analysis. However, none of the tagging SNPs were significantly associated with the genotype or allele frequency of KD children and controls under five genetic models (codominant, dominant, recessive, overdominant or log-additive models) (Table 1).

Association of HMGB1 polymorphisms with CAL formation in KD patients

The SNP rs1412125 was found to be associated with CAL formation in the recessive model. The frequency of individuals carrying the GG genotype of HMGB1 (rs1412125) was 15.6% for KD with CAL and 4.1% for KD without CAL. KD patients with the GG genotype showed a higher rate of CAL development (OR = 4.98, 95% CI = 1.69–14.66, P = 0.005) (Table 2). The SNP rs117077167 was not statistically associated with the development of CAL in KD patients.

Association of HMGB1 polymorphisms with IVIG resistance in KD patients

The SNP rs1412125 was associated with IVIG resistance in the recessive (OR = 4.11, 95% CI = 1.38–12.23, P = 0.017) and allelic models (OR = 1.80, 95% CI = 1.06–3.06, P = 0.027) (Table 3). The frequency of patients carrying the GG genotype of HMGB1 (rs1412125) was 15.8% for IVIG resistant KD and 4.4% for IVIG responsive KD. KD children with the GG genotype showed a higher rate of IVIG unresponsiveness. The prevalence of the rs1412125 G allele was significantly higher in IVIG non-responders (32.9%) than in IVIG responders (21.4%). The SNP rs117077167 had no significant association with IVIG resistance in KD patients.

Discussion

In this study, we investigated the association of HMGB1 SNPs with KD in the Korean population. There were no differences in the distribution of HMGB1 genotypes between KD patients and controls, indicating that HMGB1 polymorphisms do not play a role in the susceptibility to KD. However, the HMGB1 SNP rs1412125 was associated with IVIG resistance as well as development of CAL in KD patients. KD patients with the GG genotype for HMGB1 (rs1412125) showed higher rates of CAL development and IVIG unresponsiveness. In addition, the rs1412125 G allele was significantly higher in the IVIG resistant group than in the IVIG responsive group. Our results suggest that the polymorphism of the HMGB1 gene may play an important pathogenic role in CAL and IVIG resistance in KD.
HMGB1 is a ubiquitous nuclear DNA binding protein located on chromosome 13 [11]. It acts in the nucleus as a DNA chaperone under physiologic conditions [2, 12]. However, HMGB1 can be actively secreted from immune cells like macrophages, monocytes and dendritic cells or passively released from necrotic, damaged cells or from apoptotic cells when exposed to infection and injury [11, 13, 14]. Extracellular HMGB1 functions through specific receptors to activate the NF-κB signalling pathway inducing the production of cytokines and chemokines [15, 16]. These findings indicate that HMGB1 plays an important role in regulating inflammatory, injurious and infectious responses.

The role of HMGB1 in KD has been investigated in recent studies. Hoshina et al. [5] showed that serum HMGB1 concentrations are higher in the early acute phase of KD compared with the late acute phase and the convalescent phase. Even in the latter phase, HMGB1 concentrations were higher than in normal controls and were comparable to levels in sepsis patients. It is known that HBGB1 is elevated not only in KD, but also in other systemic diseases, such as infectious diseases, ischaemia, immune disorders, neurodegenerative diseases, metabolic disorders and cancer [17]. Therefore, HMGB1 may be related to severity or complications rather than to the diagnostic specificity of KD. No association between HMGB1 SNPs and susceptibility to KD in this study supports this hypothesis.

KD is characterized by multisystem involvement and inflammation of all the medium-sized arteries including the coronary artery. Various proinflammatory cytokines and chemokines are increased through the prominent immunologic cascade during the acute febrile phase of KD, when inflammatory cell infiltration into KD vascular tissue leads to vascular damage [1, 18, 19]. Although the specific immunologic pathways that develop CAL in KD remain unclear, ongoing coronary artery inflammation may be a risk of CAL complication [20]. Active immune cells and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Genotype and allele frequencies of the HMGB1 gene in patients with Kawasaki disease and normal controls</th>
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<tbody>
<tr>
<td>SNP</td>
<td>Genotype (KD (%) (n = 265))</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>rs1412125</td>
<td>AA (159 (60))</td>
</tr>
<tr>
<td></td>
<td>GA (90 (34))</td>
</tr>
<tr>
<td></td>
<td>GG (16 (6))</td>
</tr>
<tr>
<td></td>
<td>rs117077167</td>
</tr>
<tr>
<td></td>
<td>CC (250 (94.3))</td>
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<td></td>
<td>CA (15 (5.7))</td>
</tr>
</tbody>
</table>

| KD: Kawasaki disease; SNP: single nucleotide polymorphisms; HMGB1: high mobility group box 1. |

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<tr>
<th>Table 2</th>
<th>Genotype and allele frequencies of rs1412125 in patients with or without CAL formation</th>
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<tbody>
<tr>
<td>SNP</td>
<td>Genotype (CAL (% (n = 45)) Without (% (n = 220))</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>rs1412125</td>
<td>AA (26 (57.8))</td>
</tr>
<tr>
<td></td>
<td>GA (12 (26.7))</td>
</tr>
<tr>
<td></td>
<td>GG (7 (15.6))</td>
</tr>
</tbody>
</table>

*Significant (P < 0.05) values are in bold. CAL: coronary artery lesions; SNP: single nucleotide polymorphisms. |

<table>
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<th>Table 3</th>
<th>Genotype and allele frequencies of rs1412125 in patients resistant and responsive to IVIG</th>
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<tbody>
<tr>
<td>SNP</td>
<td>Resistant (% (n = 38))</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------</td>
</tr>
<tr>
<td>rs1412125</td>
<td>AA (19 (50.0))</td>
</tr>
<tr>
<td></td>
<td>GA (13 (34.2))</td>
</tr>
<tr>
<td></td>
<td>GG (6 (15.8))</td>
</tr>
</tbody>
</table>

*Significant (P < 0.05) values are in bold. SNP: single nucleotide polymorphisms. |
damaged cells are the sources of increased HMGB1 release in serum during the acute febrile phase. We speculate that increased levels of HMGB1 in the serum of acute phase KD function as DAMP in conjunction with other inflammatory factors and then activate the ongoing coronary artery inflammatory response, leading to CAL formation. In this study, HMGB1 SNP rs1412125 was associated with CAL complication. This result suggests that the polymorphism of the HMGB1 gene may play a crucial role in pathogenesis of CAL in KD.

High-dose IVIG is the primary treatment for KD. However, ~10–20% of patients are resistant to initial IVIG and have an increased risk of developing CAL [21–23]. Like the mechanism of action of IVIG in KD treatment, the immunological mechanism for IVIG resistance has not yet been clarified. The possible mechanisms of the beneficial effect of IVIG include modulating cytokine production, augmenting regulatory T cell activity, neutralizing toxins or other pathogens, downregulating antibody synthesis and providing anti-idiotypic antibodies [24]. Extracellular HMGB1 not only acts as a DAMP with cytokine and chemokine activities but also has several effects on T lymphocytes [4, 25]. Regarding regulatory T cells, HMGB1 has been shown to reduce the expression of CTLA4 (cytotoxic T-lymphocyte antigen 4) and Foxp3 (forkhead box P3), and to suppress the release of IL-10, inducing decreased regulatory T cell activity [26, 27], which is in contrast to the anticipated mechanism of action of IVIG in treatment of KD. Eguchi et al. [6] showed that mean HMGB1 levels were higher in IVIG poor responders than in IVIG good responders and were correlated with leucocyte counts, a known predictor of unresponsiveness to IVIG. These findings suggest that HMGB1 may be a promising candidate as a prognostic marker for IVIG unresponsiveness in KD. Recent studies have shown that genetic factors in the host, such as polymorphisms in the Fc gamma receptors [28, 29], may play a role in the mechanisms for both IVIG response and resistance. In this study, we demonstrated that HMGB1 SNP rs1412125 was associated with IVIG unresponsiveness. The current findings suggest that the variant in the HMGB1 gene plays a crucial role in IVIG resistance.

Interestingly, in this study, rs1412125 was associated with both CAL formation and IVIG resistance in the recessive model. The GG genotype of HMGB1 (rs1412125) increased the risk for CAL development and IVIG resistance. Many studies have shown that poor responders to initial IVIG are at increased risk of CAL development [30–32]. The consistent association of HMGB1 SNP rs1412125 with the two clinical outcomes in this study suggests that the polymorphism of HMGB1 gene may be a potential and independent predictor of those in KD patients. In particular, a sex-stratified analysis suggested that rs1412125 was associated with both CAL formation and IVIG resistance in males but not in females (Supplementary Table S2, available at Rheumatology online). The sex-determining region Y gene on the Y chromosome, which is responsible for male sexual differentiation, includes an HMGB domain [17]. Therefore, such findings demonstrate that HMGB1 variant might be a male-specific risk factor for the development of CAL and IVIG resistance.

Our study had several limitations. First, the modest sample size may not have had sufficient power to detect the small genetic effect of HMGB1 in KD. Future analysis in other populations, with a larger sample size, is required to verify the result of the present study. Second, the control group was not age-matched. However, as future development of KD in young, healthy children cannot be ruled out, older subjects without a history of KD may be a better choice as the control group. Third, control population was different from KD cohort in sex ratio. KD affects boys more commonly than girls [33]. In our study, 64.7% of KD patients were male. We conducted further analysis by sex, but found that HMGB1 polymorphisms were not significantly associated with genotype or allele frequency of KD children and controls by sex. In order to overcome this drawback, we performed statistical adjustments for age and sex.

In conclusion, we identified a significant association between the recessive model of HMGB1 SNP rs1412125 and risk of developing KD with CAL and IVIG resistance in Korean children. To our knowledge, this study is the first to investigate the potential clinical relevance of all known SNPs within the entire HMGB1 gene in relation to the development of CAL and IVIG unresponsiveness in KD patients. Although verification by larger-scale studies is needed, our results suggest that variants in the HMGB1 gene may be useful as potential markers for the prediction of CAL formation and poor response to IVIG.

Acknowledgements

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Supplementary data

Supplementary data are available at Rheumatology online.

References