Clinical Study

CD40 Gene Polymorphisms Associated with Susceptibility and Coronary Artery Lesions of Kawasaki Disease in the Taiwanese Population

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1. Introduction

Kawasaki disease (KD) is characterized by acute, febrile, systemic vasculitis and was first described by Kawasaki et al. in 1974 [1]. The most serious complication of KD is the occurrence of coronary artery lesions (CALs) [2]. In developed countries, KD is the leading cause of acquired heart diseases in children. KD primarily affects children less than 5 years of age [3], and the prevalence is highest in Japan, followed by Korea and Taiwan, and lowest in Europe [4].

The etiology of KD is still under investigation. Esper et al. reported that human coronavirus is associated with KD but cannot be reproduced by other groups [5]. Previous studies have either failed to identify the causative pathogen for KD or reported discrepant results [5–7]. Evidence suggests that immune activation with vascular endothelial inflammation...
may be involved in the pathogenesis of KD and CAL formation. Acute stage of KD is reported to be associated with overactivation of immunologic factors including immune competent cell activation [4], cytokines [8, 9], nitric oxide production [10], autoantibody production [11, 12], and adhesion molecule expression [13, 14].

CD40 ligand (CD40L, CD154, gp 39), a transmembrane protein structurally related to tumor necrosis factor alpha, was originally identified on activated CD4+ T cells. Both membrane-bound and soluble forms of CD40L may interact with CD40, which is mainly expressed on B cells, macrophages, endothelial cells, and vascular smooth muscle cells, resulting in various immune and inflammatory responses [15, 16]. Interaction between CD40L and CD40 plays a central role in the activation of the immune system, such as immunoglobulin G (IgG) switching, autoimmune disease, antiviral effect, allograft rejection, cytokines regulation, atherosclerosis, and endothelial cell interaction [17, 18]. CD40-CD40L system is also associated with both prothrombotic and proinflammatory effects [19, 20]. The soluble form of CD40L (sCD40L) is derived mainly from activated platelets and contributes to the pathophysiology of atherosclerosis and atherothrombosis [20]. Indeed, sCD40L has autocrine, paracrine, and endocrine activities, and it enhances platelet activation, aggregation, and platelet-leucocyte conjugation that may lead to atherothrombosis. It also has been suggested that sCD40L may play a pathogenic role in triggering acute coronary syndromes [19]. By detecting expression level of CD40L from clinical samples, our previous study provides evidence that supports a functional role of CD40L in the susceptibility of KD and CAL formation [21].

Several reports have indicated that genetic polymorphisms may contribute to the susceptibility and disease severity of KD. For example, polymorphisms in the genes including IL-10, CASP3, IL-18, and inositol 1,4,5-triphosphate 3-kinase C (ITPKC) have been reported to be involved in the development of KD [22–26]. In 2004, Onouchi and colleagues were the first group reporting the association between CD40L gene polymorphisms and KD [27]. Our previous studies have shown that expression of CD40L on CD4+ T cells was correlated to the coronary artery lesion (CAL) and disease progress in KD [21]. However, another group from Taiwan reported the lack of an association between CD40L polymorphisms and susceptibility KD in a Taiwanese population [28]. Therefore, we strived to investigate the potential genetic role of CD40 in the susceptibility of KD and CAL formation in the Taiwanese population.

2. Material and Methods

2.1. Patients Studied. All subjects studied were children who filled the diagnostic criteria for KD according to 2004 AHA criteria [29] and were admitted to the Kaohsiung Chang Gung Memorial Hospital between 2000 and 2010. All patients were treated with a single infusion of IVIG (2 g/kg) administered over a 12-hour period. Aspirin was administered until all signs of inflammation were resolved or regression of CAL was detected under two-dimensional (2D) echocardiography as in our previous reports [30–32]. This study was approved by the Institutional Review Board of the Chang Gung Memorial Hospital. CAL was defined by the internal diameter of the coronary artery being at least 3 mm (or 4 mm if the subject was over the age of 5 years) or the internal diameter of a segment being at least 1.5 times of an adjacent segment in the echocardiogram [22, 33]. IVIG responsiveness was defined as defervescence within 48 hours after the completion of IVIG treatment and being without fever (temperature, >38°C) [2, 34].

2.2. DNA Extraction. Blood cells were subjected to DNA extraction by treating them first with 0.5% SDS lysis buffer and then protease K (1 mg/mL) for digestion of nuclear protein for 4 h at 60°C. Using the Gentra extraction kit followed by 70% alcohol precipitation, total DNA was harvested.

2.3. Genotyping. Two tagging SNPs (rs4810485 and rs1535045) in the CD40 gene with a minor allele frequency of >10% in the Han Chinese population were selected from the HapMap database (http://www.hapmap.org/). Genotyping was performed by using the TaqMan allelic discrimination assay (Applied Biosystems, Foster city, CA, USA) was noted in our previous report [22, 24, 35]. Briefly, PCR primers and TaqMan minor groove binding probes were designed by applied Biosystems. The polymerase chain reaction (PCR) was carried out in a 96-well microplate with the ABI 9700 Thermal Cycler. After PCR, fluorescence was detected by ABI 7500 Real-Time PCR system and data was analyzed using the System SDS software (version 1.2.3).

2.4. Statistical Analysis. JMP 9.0 for Windows was used for analysis. The statistical differences between case and control in genotype and allele frequency were assessed by the χ²-test. Statistical differences in genotype and allele frequency of KD patients with/without CAL formation were assessed using the χ² test. Linkage disequilibrium (LD) was assessed for one pair of SNPs, and haplotype blocks were defined using the default setting of the Haplovievew software 4.1 (Broad Institute, Cambridge, MA, USA).

3. Results

3.1. A Significant Association between Genetic Polymorphisms in CD40 and Susceptibility to Kawasaki Disease. A total of 950 subjects including 381 KD patients and 569 controls were recruited in this study. Table 1 shows the characteristics of the subjects. The average age of cases was 1.7 years and that in the controls is 5.7 years. CAL was observed in 9.7% (37/381) of KD patients 8 weeks after disease onset, and 12.9% (49/381) of the KD patients were resistant to initial IVIG treatment. The genotype frequencies of the controls and patients were in the Hardy-Weinberg equilibrium (Table 2). Polymorphism rs1535045 in the CD40 was found to have significant influence with regards to the susceptibility of KD (P = 0.0405).
Table 1: Basal characteristics of patients with Kawasaki disease and normal controls.

| Characteristics          | Patients with KD | Normal control |
|--------------------------|------------------|----------------|
| N                        | 381              | 569            |
| Mean (SD) age (years)    | 1.7 ± 1.6        | 5.7 ± 4.9      |
| Age range (years)        | 0–11             | 0–51           |
| CAL formation            | 37 (9.7%)        |                |
| IVIG resistance          | 49 (12.9%)       |                |

CAL: coronary artery lesions; IVIG: intravenous immunoglobulin; SD: standard deviation.

Table 2: Genotype and allele frequencies of the CD40 gene in controls and patients with Kawasaki disease.

| Genotype   | Case (%) (n = 381) | Control (%) (n = 569) | Allele | Case (%) (n = 381) | Control (%) (n = 569) | Genotype P value | Dominant P value | Recessive P value | Allelic P value |
|------------|--------------------|-----------------------|--------|--------------------|-----------------------|------------------|------------------|------------------|-----------------|
| rs4810485  |                    |                       |        |                    |                       |                  |                  |                  |                 |
| TT         | 50 (13.2)          | 99 (17.7)             | T      | 284 (37.4)         | 460 (41.1)            | 0.1632           | 0.3489           | 0.0609           | 0.1005          |
| GT         | 184 (48.4)         | 262 (46.9)            | G      | 476 (62.6)         | 658 (58.9)            |                  |                  |                  |                 |
| GG         | 146 (38.4)         | 198 (35.4)            |        |                    |                       |                  |                  |                  |                 |
| rs1535045  |                    |                       |        |                    |                       |                  |                  |                  |                 |
| TT         | 44 (11.5)          | 61 (10.8)             | T      | 262 (34.4)         | 346 (30.6)            | 0.1160           |                  | 0.0405*          | 0.7180          |
| CT         | 174 (45.7)         | 224 (39.6)            | C      | 500 (65.6)         | 784 (69.4)            |                  |                  |                  |                 |
| CC         | 163 (42.8)         | 280 (49.6)            |        |                    |                       |                  |                  |                  |                 |

*Significant (P < 0.05) values are in bold.

Table 3: Genotype and allele frequencies of the CD40 gene in patients with or without coronary artery lesion (CAL) formation.

| Genotype   | CAL (%) (n = 37) | Without (%) (n = 336) | Allele | CAL (%) (n = 37) | Without (%) (n = 336) | Genotype P value | Dominant P value | Recessive P value | Allelic P value |
|------------|-----------------|-----------------------|--------|-----------------|-----------------------|------------------|------------------|------------------|-----------------|
| rs4810485  |                 |                       |        |                 |                       |                  |                  |                  |                 |
| TT         | 1 (2.7)         | 49 (14.6)             | T      | 25 (33.8)       | 253 (37.8)            | 0.0666           | 0.6381           | 0.0436*          | 0.5021          |
| GT         | 23 (62.2)       | 155 (46.3)            | G      | 49 (66.2)       | 417 (62.2)            |                  |                  |                  |                 |
| GG         | 13 (35.1)       | 131 (39.1)            |        |                 |                       |                  |                  |                  |                 |
| rs1535045  |                 |                       |        |                 |                       |                  |                  |                  |                 |
| TT         | 2 (5.4)         | 41 (12.2)             | T      | 23 (31.1)       | 233 (34.7)            | 0.4410           | 0.9641           | 0.2192           | 0.5368          |
| CT         | 19 (51.4)       | 151 (44.9)            | C      | 51 (68.9)       | 439 (65.3)            |                  |                  |                  |                 |
| CC         | 16 (43.2)       | 144 (42.9)            |        |                 |                       |                  |                  |                  |                 |

*Significant (P < 0.05) values are in bold.

Table 4: Haplotype frequencies of the CD40 gene in controls and patients with Kawasaki disease.

| rs4810485/rs1535045 | Case (%) (n = 381) | Control (%) (n = 569) | OR (95% CI) | P Value |
|---------------------|--------------------|-----------------------|-------------|---------|
| G/T                 | 258 (34.0)         | 343 (30.7)            | 1.22 (0.98–1.52) | 0.0767  |
| G/C                 | 216 (28.5)         | 317 (28.3)            | 1.10 (0.88–1.39) | 0.3937  |
| T/C                 | 282 (37.2)         | 457 (40.8)            | Reference   |         |

Haplotype frequency less than 1% was excluded.

3.2. CD40 Genetic Polymorphisms Associated with CAL Formation in the KD Patients. The association between CD40 genotypes and CAL formation, the major complication of KD, was evaluated. 37 patients among the 381 KD patients developed CAL. Our results indicated that the TT genotype of rs4810485 has protective effects for CAL formation in KD patients (Table 3).

3.3. Haplotype Analysis of CD40 in the KD Patients. We further calculated pairwise linkage disequilibrium (LD) and analyzed haplotypes of CD40. However, no significant association was found between CD40 haplotype analysis and the susceptibility of KD (Table 4), as well as CAL formation (Table 5).

4. Discussion

Kawasaki disease is caused by activation of the immune system targeting on vascular endothelium, resulting in systemic vasculitis or even coronary artery lesions formation. Interaction of CD40L with its receptor CD40 has been implicated in the modulation of immune and inflammatory responses, which are critical for the activation of tissue structure cells, such as endothelial cells, smooth muscle
cells, epithelial cells, and fibroblast and induce production of a cascade of proinflammatory cytokines [18, 19]. The CD40-CD40L signaling pathway has been associated with the pathogenic processes of chronic inflammatory diseases, including autoimmune diseases, neurodegenerative disorders, graft-versus-host disease, cancer, vasculitis, and atherosclerosis [17, 20]. In the presented study, our results implied that genetic polymorphisms in CD40 may result in the overactivation of proinflammatory signaling pathways that lead to the development of KD.

Our previous report indicated that expression of CD40L on CD4+ T-cells correlated to the CAL formation and disease progress in KD patients [21]. Burns and Glodé also suggested that CD40-CD40L interaction impacts on the vasculitis pathogenesis of KD [4]. Therefore, the CD40-CD40L signaling pathway may play an important pathogenic role in acute coronary syndromes such as the CAL of patients with KD. In this study, we found that CD40 genetic polymorphism (rs1535045) is associated with susceptibility of KD. Interestingly, rs1535045 in CD40 gene has been confirmed to be associated with the coronary artery calcification in diabetic families [36]. These observations in combination with those of the present study support genetic polymorphism effects of CD40 in the vascular diseases. Further study into the mechanisms between CD40-CD40L interaction and long-term coronary arterial vasculitis may provide a better understanding for the pathogenesis of KD.

Although our studies indicated the significant association between genetic polymorphisms of CD40 and the risk of KD, haplotypes of KD did not yield significant results. We acknowledged that the modest sample size in the study was underpowered to detect the small genetic effect of CD40 in the disease severity such as CAL formation. These findings need to be replicated in a second population with a larger sample size.

Taken together, our results indicated that the genetic polymorphisms of CD40 are very likely to be involved in the susceptibility and disease severity of KD in a Taiwanese population.

**Abbreviations**

KD: Kawasaki disease  
IVIG: Intravenous immunoglobulin  
CAL: Coronary artery lesions.

**Conflict of Interests**

The authors declare that no Conflict of interests exists.

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**Table 5: Haplotype frequencies of the CD40 gene in patients with or without coronary artery lesion formation.**

| rs4810485/rs1535045 | CAL (%) | Without (%) | OR (95% CI) | P value |
|-------------------|--------|-------------|-------------|---------|
| (n = 37) | (n = 336) |
| G/T | 23 (31.1) | 231 (34.5) | 1.00 (0.55–1.81) | 0.9991 |
| G/C | 26 (35.1) | 186 (27.8) | 1.40 (0.79–2.51) | 0.2511 |
| T/C | 25 (33.8) | 251 (37.5) | Reference |

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**References**

[1] T. Kawasaki, F. Kosaki, S. Okawa, I. Shigematsu, and H. Yamasawa, “A new infantile acute febrile mucocutaneous lymph node syndrome (MLNS) prevailing in Japan,” *Pediatrics*, vol. 54, no. 3, pp. 271–276, 1974.

[2] H. C. Kuo, C. D. Liang, C. L. Wang, H. R. Yu, K. P. Hwang, and K. D. Yang, “Serum albumin level predicts initial intravenous immunoglobulin treatment failure in Kawasaki disease,” *Acta Paediatrica, International Journal of Pediatrics*, vol. 99, no. 10, pp. 1578–1583, 2010.

[3] Y. W. Park, J. W. Han, I. S. Park et al., “Kawasaki disease in Korea, 2003-2005,” *Pediatric Infectious Disease Journal*, vol. 26, no. 9, pp. 821–823, 2007.

[4] J. C. Burns and M. P. Glodé, “Kawasaki syndrome,” *The Lancet*, vol. 364, no. 9433, pp. 533–544, 2004.

[5] F. Esper, E. D. Shapiro, C. Weibel, D. Ferguson, M. L. Landry, and J. S. Kahn, “Association between a novel human coronavirus and Kawasaki disease,” *The Journal of Infectious Diseases*, vol. 191, no. 4, pp. 499–502, 2005.

[6] L. Y. Chang, B. L. Chiang, C. L. Kao et al., “Lack of association between infection with a novel human coronavirus (HCoV), HCoV-NH, and Kawasaki disease in Taiwan,” *The Journal of Infectious Diseases*, vol. 193, no. 2, pp. 283–286, 2006.

[7] T. Ebihara, R. Endo, X. Ma, N. Ishiguro, and H. Kikutani, “Lack of association between New Haven coronavirus and Kawasaki disease,” *The Journal of Infectious Diseases*, vol. 192, no. 2, pp. 351–352, 2005.

[8] J. Kimura, H. Takada, A. Nomura et al., “Th1 and Th2 cytokine production is suppressed at the level of transcriptional regulation in Kawasaki disease,” *Clinical and Experimental Immunology*, vol. 137, no. 2, pp. 449–449, 2004.

[9] H. C. Kuo, C. L. Wang, C. D. Liang et al., “Association of lower eosinophil-related T helper 2 (Th2) cytokines with coronary artery lesions in Kawasaki disease,” *Pediatric Allergy and Immunology*, vol. 20, no. 3, pp. 266–272, 2009.

[10] C. L. Wang, Y. T. Wu, C. J. Lee, H. C. Liu, L. T. Huang, and K. D. Yang, “Decreased nitric oxide production after intravenous immunoglobulin treatment in patients with Kawasaki disease,” *Clinical and Experimental Immunology*, vol. 20, no. 3, pp. 266–272, 2009.
K.-S. Hsieh, T.-J. Lai, Y.-T. Hwang et al., “IL-10 promoter near TGFβ1 promoter polymorphisms and risk of Kawasaki disease in Taiwanese children,” Journal of Human Genetics, vol. 56, no. 12, pp. 840–845, 2011.

H.-C. Kuo, Y.-J. Lin, S.-H. H. Jue et al., “Lack of association between ORAI1/CRACM1 Gene Polymorphisms and Kawasaki disease in the Taiwanese children,” Journal of Clinical Immunology, vol. 31, no. 4, pp. 650–655, 2011.

K. P. Burdon, C. D. Langfeld, S. R. Beck et al., “Variants of the CD40 gene but not of the CD40L gene are associated with coronary artery calcification in the Diabetes Heart Study (DHS),” American Heart Journal, vol. 151, no. 3, pp. 706–711, 2006.