Introduction

Kawasaki disease (KD) is a systemic vasculitis which develops exclusively in young children. Multiple genes are related to the pathogenesis of KD. The high incidence of KD in Asian ethnic groups has stimulated extensive searches for the genetic susceptibility for KD. Most of the susceptible genes are related to inflammation and aberrant immune reactions. Whatever the initiating agent of inflammatory reaction is, the individual differences of a series of immunologic inflammatory cascades due to a given genetic polymorphism are the primary focus of etiologic research on KD. Interleukin-6 (IL-6) gene polymorphisms, tumor necrosis factor-α (TNF-α) gene polymorphisms, CD40 ligand gene polymorphisms, genetic polymorphisms of encoding chemokine receptors, programmed death-1 (PD-1) gene polymorphisms, and other genome wide association studies have been researched.

In addition, the paucity of regulatory T cells (Treg cells) and the plasticity of T cell populations have been investigated in the development of KD. Recently T follicular helper cells (Tfh cells) have been a focus of interest in autoimmune vasculitis. Tfh cells are involved in the germinal center formation for effective B cell differentiation to antibody-producing plasma cells. T cells and B cells have an effect on each other in every step of adaptive immune reaction. Therefore, we concentrated on Tfh cells as an emerging factor for balancing T cell plasticity after infection and affecting B cell development. King et al. reported that homeostatic expansion of T cells during im-
mune insufficiency generates autoimmunity. Therefore, repetitive viral infections or potent live vaccinations in infants may induce relative immune insufficiency and high turnover of T cells may direct to autoimmunity; the state of KD. T cells work effectively via IL-21/IL-21 receptor (IL-21R) pathway. IL-21 promotes B cell differentiation but if there is a defect in IL-21R, IgE is increased and immunoglobulin class switching recombination is decreased. Jin et al. reported that IL-21 directs B cell growth arrest for nonspecifically or inappropriately activated B cells while normally it promotes B cell maturation during T cell dependent B cell activation. Therefore, in KD, we could infer that IL-21 may arrest growth of B cell through lacking of IL-21R expression due to IL-21R gene polymorphism. Ineffective B cell differentiation is prominent in KD compared with other febrile diseases. IL-21 may be increased due to ineffective B cell differentiation but lack of IL-21R may lead to functional defect in immunoglobulin switching and increased IgE level.

Now, we speculated that KD might develop in certain young individuals who have IL-21R gene polymorphism when IL-21R expression down regulated transiently, and immunoglobulin switching may be arrested by ineffective IL-21R expression. In advance we checked the serum level of IL-21 in patients with KD and it was increased. Hence, we investigated the IL-21R gene polymorphisms in the promoter region which could decrease the expression of IL-21R gene in aspect of the genetic susceptibility of KD.

Subjects and Methods

Subjects
A total of 200 samples (100 samples of patients with KD and 100 of healthy controls) were studied from February 2010 to October 2011 in Wonju Christian Hospital, Wonju, South Korea. KD was diagnosed according to KD criteria. The control group was composed of healthy adults who were recruited into a hypertension cohort study and had no medical history of KD or autoimmune disease and have normal blood pressure. Whole blood was collected after receiving informed consent. This study was approved by the Institutional Review Board of Hospital, Yonsei University (CR309043).

Deoxyribonucleic acid extraction, polymerase chain reaction, and sequencing
Genomic deoxyribonucleic acids were extracted from whole blood samples of patients and controls by using LaboPass™ Blood Kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer’s instruction. The nucleotide changes in the promoter region of IL-21R gene (-2500 bp~ +1 bp) according to the position from the transcription starting base of the IL-21R gene (NG_012222.1) (Fig. 1) were identified by PCR using ExTaq polymerase (Takara Bio Inc, Shiga, Japan) with 5 pairs of primers listed in Table 1. PCR conditions were 1 cycle of 3 minutes at 95°C, followed by 35 cycles of 3 steps that were at 95°C for 30 seconds, at 58°C for 30 seconds, at 72°C for 1 minute each, and 1 cycle of 10 minutes at 72°C. The PCR products were separated by gel electrophoresis on 1% agarose gel.

We screened the known single nucleotide polymorphism (SNP) sites for IL-21R in patients with KD: rs11074858, rs56189459, and rs2214032 for IL-21R gene, which are located in exons. However there was no significant change of these genetic sites in patients with KD. Therefore we studied the promoter region of IL-21R. Among the 5 experiments for screening SNP of promoter genes, we found significant SNPs in -500 bp~ +1 bp region. We analyzed the sequences using direct sequencing method using ABI 3730 capillary sequencer (Applied Biosystems, CA, USA) and compared sequences with reference sequence through Human BLAT program (developed by Jim Kent in UCSC Genome Browser).

Measurement of interleukin–21 and total IgE in sera
We measured the serum IL-21 level in patients with KD by enzyme linked immunosorbent assay kits (e-Bioscience, San Diego, CA, USA) and total IgE by immunoassay system (Siemens, Munich, Germany).

| Primer | Strand | Sequence (5’–3’)
|--------|--------|-----------------
| N1 primer | Forward | CCT TCT GCC GAT AAT G |
| C1 primer | Reverse | CTG ATG AGT GCC AGA GTT GG |
| N2 primer | Forward | TCT GGC ACT CAT CAG |
| C2 primer | Reverse | AAG CTC ATT GTG TGC CAG |
| N3 primer | Forward | CAC AAT GAG CTC TTA GC |
| C3 primer | Reverse | AGC CAA GAA GAA TTA GAG CTG G |
| N4 primer | Forward | ATT CTT CAT GGC TTA TGC |
| C4 primer | Reverse | CTC CAT TCC TCT TGG ATC |
| N5 primer | Forward | AAT GAG GCA GAG AGC C |
| C5 primer | Reverse | GAG GAT GTG GTG AGC CG |

Table 1. Primers used for polymerase chain reaction on the promoter regions of interleukin-21 receptor gene

Fig. 1. The IL-21R gene site in chromosome 16 (27,413,483…27,463,363). IL-21R: interleukin 21 receptor.
Statistical analysis

Sample size was determined by the PASS program 2008 (NCSS Statistical Software, Kaysville, UT, USA). The difference of allele frequencies between KD patients and controls was compared by Fisher’s exact test using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). The difference of serum level of IL-21 or total IgE according to the presence of IL-21R gene polymorphisms was calculated by Mann-Whitney test. The difference of prevalence of coronary arterial dilatation (CAD) according to IL-21R gene polymorphisms was calculated by Fisher’s exact test. Statistical significance was defined as p<0.05.

Results

Characteristics of patients with Kawasaki disease

A total of 100 patients and 100 healthy controls were enrolled in this study. The median age of patients with KD was 3 years of age (range: 0.3–11.0) and gender ratio (male : female) was 55 : 45 (1.2 : 1). The gender ratio of healthy controls was 1 : 1. The proportion of atypical KD patients was 28% and that of refractory KD patients was 6%. The number of patients with CAD when the echocardiogram was performed in febrile period was 29 (29%).

Single nucleotide polymorphisms in the promoter region of interleukin-21 receptor gene and nomenclature

Among the SNPs in the promoter region of IL-21R gene, SNPs of which minor allele frequency (MAF) >0.01 were depicted in Table 2. Most SNPs were located in -500 bp~ +1 bp from transcription starting site. In this study, we could not find the known SNPs of this region such as rs2189522 or -83 T>C in both groups. Several SNPs which had not been reported in dbSNP site were found. Therefore, we named these SNPs as either the chromosome position in the chromosome 16 region (NC_000016.9) or the position from the transcription starting base of the IL-21R gene (NG_012222.1).

Differences in allele frequencies of single nucleotide polymorphisms between patients with Kawasaki disease and controls

Table 3 shows the allele frequencies of SNPs in patients and control groups. Among the SNPs, -237 A>T polymorphism was found in 15 patients with KD only and significant difference in allele frequency between the patients and the controls was found (OR: 36, 95% confidence interval (CI): 2.1–618, p<0.0001). Because there were more SNPs of which MAF <0.01 and one individual had more than one SNP, we divided the study population into individuals with any SNPs and those with no SNP (Table 4). There was a significant difference in terms of number of individuals who have any SNPs of IL-21R gene between the patients group and controls (OR: 3.0, 95% CI: 1.6–5.6, p=0.0005).

Relationships between interleukin–21 receptor gene polymorphisms and the serum levels of interleukin–21

When we searched for differences in the serum level of IL-21 by the presence of IL-21R gene polymorphisms in 25 patients with KD, no significant difference was shown (Fig. 2). The mean serum level of IL-21 in patients who have polymorphisms was 612±480 pg/mL and that of patients who have no polymorphism was 414±290 pg/mL.

Table 2. Identified SNPs in promoter region of interleukin-21 receptor gene

| Chromosome site | Position | SNPs | MAF in subjects |
|-----------------|----------|------|-----------------|
| 2741802         | -1681    | G→T  | 0.15            |
| 27413104        | -379     | G→A  | 0.06            |
| 27413151        | -332     | G→C  | 0.145           |
| 27413246        | -237     | A→T  | 0.075           |
| 27413430        | -53      | G→A  | 0.04            |

*Reference sequence: NC_000016.9, †From the starting transcription site (+1); Chromosome site: 27413483, ‡Minor allele frequency in controls and patients. SNPs: single nucleotide polymorphisms, MAF: minor allele frequency

Table 3. Comparison in terms of allele frequencies of the SNPs in promoter region of interleukin–21 receptor gene between patients with KD and healthy controls

| SNPs | KD (%) | Control (%) | OR (95% CI) | p   |
|------|--------|-------------|-------------|-----|
| -1681 G>T | 11/51 (21.6) | 12/100 (12.0) | 2.0 (0.8–5.0) | 0.15 |
| -379 G>A | 9/100 (9.0) | 3/100 (3.0) | 3.2 (0.8–12.1) | 0.13 |
| -332 G>C | 16/100 (16.0) | 13/100 (13.0) | 1.3 (0.6–2.8) | 0.68 |
| -237 A>T | 15/100 (15.0) | 0 | 36 (2.1–618) | <0.0001 |
| -53 G>A | 5/100 (5.0) | 3/100 (3.0) | 1.7 (0.4–7.3) | 0.72 |

SNPs: single nucleotide polymorphisms, KD: Kawasaki disease, OR: odds ratio, CI: confidence interval
there was no significant difference between two groups (p=0.18).

Association of interleukin-21 receptor gene polymorphisms and total IgE

Serum levels of total IgE were also checked in the same group above. There was no significant difference in terms of total IgE level between patients with polymorphisms and those with no polymorphism (Fig. 3). Our data couldn’t show the significant difference of the median values of serum total IgE between the patients who have IL-21R gene polymorphisms and those who have not (30.1 pg/mL vs. 58.6 pg/mL, p=0.17).

Discussion

The IL-21/IL-21R pathway is known as an executor of B cell differentiation. IL-21 is produced when the immune reaction reaches its peak by CD4+ T cells and IL-21R is expressed in mature B cells. IL-21 induces PD-1 expression on T cells and plays a role in making a knot of a certain immunologic cascade. To our knowledge, this is the first study to investigate IL-21R gene polymorphisms in KD. We found significant gene polymorphisms in patients with KD compared with controls. Because IL-21 is increased by autocrine manners, we assumed that the structure of IL-21R would be still intact even in the presence of polymorphism. In the preliminary study, we couldn’t find any significant polymorphisms belonging to 9 exons of IL-21R gene in patients with KD. Therefore we searched the promoter region of IL-21R gene. Pène et al. studied -83 T>C in atopic patients and controls and they found more frequent variants in atopic patients compared with controls. Their functional study showed that IL-21R gene polymorphism may increase the serum level of total IgE against the IFN-γ mediated inhibition of IgE synthesis. In our study, 30% of patients with KD had any allergic history and family history of any allergic diseases (data not shown). Our data showed no correlation between IL-21R gene polymorphism and the serum level of total IgE or serum level of IL-21. This result may be due to many other molecules which could affect the serum level of IL-21 such as PD-1, SOCS-1, SAP, Blimp-1, Bcl-6 and WSB-2. Therefore, elevated serum level of IL-21 may not be described only by IL-21R gene polymorphisms. However, our data contribute to the understanding of one of the aberrant immune reactions in KD. No allergy, cytotoxic T cell mediated inflammation or inappropriately activated macrophages can entirely explain the inflammatory reactions of KD. We hypothesized that the polymorphisms of IL-21R gene may be responsible for decreased expression of IL-21R. In promoter region of IL-21R gene, 27413104 G→A change makes the sequence as GAAC/CAAG a symmetric mirror sequences. We don’t know what this change means, but this change may be responsible for the altered the transcription of IL-21R gene. Further functional study should be required to prove the function of SNPs that we found.

There are patients who had more than 2 SNPs. Compared with healthy controls, patients with KD have more combinations of SNPs in IL-21R gene. Although there is no significant difference of MAF of each SNPs between patients group and healthy controls, the fre-
frequency of combination of SNPs was higher in patients group than in controls. We could not find a significant increase of known SNPs of IL-21R gene in this study. Those SNPs had been studied in Caucasian ethnic groups, therefore our results may be explained by ethnic differences.

We have no preventive strategy for KD yet. However, we might speculate that repeated infections or vaccinations may down-regulate the IL-21R and this effect would be aggravated in hosts who have IL-21R gene polymorphisms, which may lead to arrest of B cell differentiation and elevated serum levels of total IgE and IL-21. Therefore, avoiding frequent infections in a short term period may be a feasible strategy for preventing KD.

In addition to this, we cautiously suggest that anti-IL-21 antibody could be a new therapeutic agent for IVIG-resistant KD in patients who have IL-21R gene polymorphisms. Anti-IL-21 antibody is being studied in autoimmune diseases as a new therapeutic agent.

In conclusion, our data suggest that the genetic susceptibility profile for KD may include IL-21R gene.

Acknowledgments

This research was supported partly by Leading Foreign Research Institute Recruitment Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) (2011-00263). We deeply appreciate the valuable comments of Prof. Dong Soo Kim about the B cell differentiation and Kawasaki disease.

References

1. Kawasaki T. [Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children]. Aereugi 1967;16:178-222.
2. Onouchi Y. Molecular genetics of Kawasaki disease. Pediatr Res 2009; 65:46R-54R.
3. Burns JC, Gloyd MP. Kawasaki syndrome. Lancet 2004;364:533-44.
4. Cheung YF, Huang GY, Chen SB, et al. Inflammatory gene polymorphisms and susceptibility to Kawasaki disease and its arterial sequelae. Pediatrics 2008;122:608-14.
5. Yeung RS. Kawasaki disease: update on pathogenesis. Curr Opin Rheumatol 2010;22:551-60.
6. Sohn MH, Hur MW, Kim DS. Interleukin 6 gene promoter polymorphism is not associated with Kawasaki disease. Genes Immun 2001;2: 357-62.
7. Ahn HM, Park HS, Hong SJ, Hong YM. Interleukin-6 (-636 c/g) gene polymorphism in Korean children with Kawasaki disease. Korean Circ J 2011;41:321-6.
8. Quasney MW, Bronstein DE, Cantor RM, et al. Increased frequency of alleles associated with elevated tumor necrosis factor-alpha levels in children with Kawasaki disease. Pediatr Res 2001;49:686-90.
9. Huang FY, Chang TY, Chen MR, et al. Genetic polymorphisms in the CD40 ligand gene and Kawasaki disease. J Clin Immunol 2008;28: 405-10.
10. Breunis WB, Biezeveld MH, Geissler J, et al. Polymorphisms in chemokine receptor genes and susceptibility to Kawasaki disease. Clin Exp Immunol 2007;150:83-90.
11. Chun JK, Kang DW, Yoo BW, Shin JS, Kim DS. Programmed death-1 (PD-1) gene polymorphisms lodged in the genetic predispositions of Kawasaki Disease. Eur J Pediatr 2010;169:181-5.
12. Furuno K, Yuge T, Kusuhiro K, et al. CD25+CD4+ regulatory T cells in patients with Kawasaki disease. J Pediatr 2004;145:385-90.
13. Sohn SY, Song YW, Yeo YK, et al. Alteration of CD4CD25Foxp3 T cell level in Kawasaki disease. Korean J Pediatr 2011;54:157-62.
14. Franco A, Shimizu C, Tremoulet AH, Burns JC. Memory T-cells and characterization of peripheral T-cell clones in acute Kawasaki disease. Autoimmunity 2010;43:317-24.
15. Gómez-Martín D, Díaz-Zamudio M, Romo-Tena J, Ibarra-Sánchez MJ, Alcocer-Varela J. Follicular helper T cells poise immune responses to the development of autoimmune pathology. Autoimmun Rev 2011;10:325-30.
16. King C, Illc A, Koeisch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004;117:265-77.
17. Jin H, Carrio R, Yu A, Malek TR. Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis. J Immunol 2004;173:657-65.
18. Lee HK, Kim DS, Noh GW, Lee KY. Effects of intravenous immune globulin on the peripheral lymphocyte phenotypes in Kawasaki disease. Yonsei Med J 1996;37:357-63.
19. Ozaki K, Kiky K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. Proc Natl Acad Sci U A 2000;97:11439-44.
20. Bae YJ, Kim MH, Lee HY, et al. Elevated Serum Levels of IL-21 in Kawasaki Disease. Allergy Asthma Immunol Res 2012;4:351-6.
21. Newburger JW, Takahashi M, Gerber MA, et al. Diagnosis, treatment, and long-term management of Kawasaki disease: a statement for health professionals from the Committee on Rheumatic Fever, Endocarditis and Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. Circulation 2004;110:2747-71.
22. Pène J, Guglielmi L, Gauchat JF, et al. IFN-gamma-mediated inhibition of human IgE synthesis by IL-21 is associated with a polymorphism in the IL-21R gene. J Immunol 2006;177:5006-13.
23. Caprioli F, Sarra M, Caruso R, et al. Autocrine regulation of IL-21 production in human T lymphocytes. J Immunol 2008;180:1800-7.
24. Cannons JL, Qi H, Lu KT, et al. Optimal germinal center responses require a multistage T B cell adhesion process involving integrins, SLAM-associated protein, and CD84. Immunity 2010;32:253-65.
25. Bennett F, Luxenberg D, Ling V, et al. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. J Immunol 2003;170:711-8.
26. Cui W, Liu Y, Weinstein JS, Craft J, Kaech SM. An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. *Immunity* 2011;35:792-805.

27. Linterman MA, Vinuesa CG. Signals that influence T follicular helper cell differentiation and function. *Semin Immunopathol* 2010;32:183-96.

28. Nara H, Onoda T, Rahman M, et al. Regulation of interleukin-21 receptor expression and its signal transduction by WSB-2. *Biochem Biophys Res Commun* 2010;392:171-7.

29. Hecker M, Bohnert A, König IR, Bein G, Hackstein H. Novel genetic variation of human interleukin-21 receptor is associated with elevated IgE levels in females. *Genes Immun* 2003;4:228-33.

30. Bucher C, Koch L, Vogtenhuber C, et al. IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. *Blood* 2009;114:5375-84.