이학석사 학위논문

가와사끼병에서 면역글로불린 관련 유전자의 질병 연관성 연구

Association study of immunoglobulin-related genes in Kawasaki disease

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이 논문을 이학석사 학위 논문으로 제출함

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감사의 글

먼저 2017 년 1 월에 입사하여 유전학 분야에 대해 아무것도 모르던 저에게 오늘까지 가르침을 주신 이종극 교수님께 진심으로 깊은 감사를 드립니다. 많이 부족한 저에게 하나의 작은 일에서도 완벽하게 마무리하고 성과를 이루는 가르침을 주시어 3 년이라는 시간을 통해 석사학위를 마무리하며 작은 결실을 맺도록 지도해 주셔서 감사합니다. 늘 자신이 모범을 보이며 교수님의 연구에 대한 열정에 존경심을 표합니다. 또한 저의 석사학위 논문 심사 심사위원을 맡아주신 송규영 교수님과 유정진 교수님, 바쁘신 가운데도 논문이 완성될 수 있도록 가르침을 주셔서 감사의 말씀을 전합니다.

유전체 연구단에 일하며 얻은 것들 중 가장 큰 하나는 연구실 사람들과의 소중한 인연입니다. 먼저 업무에 있어서 가장 많이 의지하고 가르침을 받았던 김재정 박사님, 저의 우문에 항상 현답을 해주시고, 심적으로 힘들때 인생의 선배로서 조언에 큰 힘이 되었습니다. 제가 또 많이 의지한 허선희 선생님. 항상 옆에서 실험적인 지식뿐 아니라 개인적인 이야기와 상담이 연구실 생활에서 정말 큰 도움이 되었습니다. 그리고 언제나 활기찬 기운을 주시는 진옥 선생님, 항상 친절한 민지 선생님, 그리고 개인적인 일에 격려와 기도해주신 혜림 선생님, 연구실에 일하는 동안 함께 생활하며 희로애락을 함께하며 응원해주셔서 감사합니다. 작은 질문에도 꼼꼼하게 설명해주는 태호 선생님과 묵묵히 맡은 일을 열심히 하는 정민 선생님, 저의 부탁에 항상 친절하게 들어주는 준영 선생님에게 고마운 마음을 전합니다.

마지막으로 무엇보다 소중한 나의 가족들. 항상 든든하게 제가 하는 일에 응원과 후원을 해주신 부모님, 감사합니다. 그리고 언제나 저에게 객관적인 충고와 석사학위 마무리할 수 있도록 지원해준 남편, 그리고 석사과정 중 연구로 태교하여 탄생한 소중한 우리 아들 시현이, 아직까지도 철없는 동생을 걱정하는 두 아이의 아버지가 된 친오빠와 친언니처럼 이야기 들어주고 조언을 준 새언니, 모두에게 사랑하는 마음과 함께 이 논문을 바칩니다.

2020 년 8 월

김혜지

ABSTRACT

Kawasaki disease (KD), the leading cause of pediatric acquired heart disease in the developed countries, is an acute, systemic vasculitis. The etiology of KD remains unknown. However, the current consensus is that KD can result from environmental or infectious trigger occurring in genetically susceptible children. Treatment for KD is a single dose of intravenous immunoglobulin (IVIG) and high-dose aspirin and it reduces the fever in most patients and the rate of coronary artery aneurysms (CAA). In spite of IVIG treatment, more than 10% of KD patients do not respond to the therapy and show persistent fever. So, the identification of the genetic variants associated with susceptibility and IVIG resistance in KD would help the KD patients for the understanding of pathogenesis and the additional treatments to prevent increased risk of developing coronary artery abnormalities. The susceptibility of KD and the mechanism of IVIG therapy are little known. We hypothesized that immunoglobulin-related genes, such as immunoglobulin Fc receptor genes and genes involved in glycosylation of immunoglobulin G, play a crucial role for the susceptibility and the mechanisms of IVIG therapy in KD. The objective of this thesis research is to identify the genetic risk factors associated with susceptibility of KD and IVIG-resistance in the immunoglobulin-related genes. This study consists of three chapters.

In the first chapter, we initially sought to identify nonsynonymous SNPs (nsSNPs) in the coding region of a total of 15 immunoglobulin Fc receptor genes by capillary sequencing using pre-made ABI primer sets (Waltham, Massachusetts, USA) with DNA samples of 98 KD cases and 96 controls. Seven candidate nsSNPs were selected from capillary sequencing data for large scale case-control test. The genotyping for association study was performed using TaqMan^{TM} SNP genotyping system with 569 KD cases and 570 controls. The result of the association study for 7 candidate nsSNPs in Fc receptor genes did not show any significant association with KD.

In the second chapter, we performed the association study for IVIG-resistance in KD. We initially selected seven candidate nsSNPs in the genes involved in glycosylation of immunoglobulin G from our unpublished whole exome sequencing (WES) data with 94 IVIG non-responders and 106 IVIG responders. An association study for validation was performed

using DNA samples of a total 191 IVIG non-responders and 374 IVIG responders. However, there was no significant association of the candidate nsSNPs selected in the immunoglobulin G glycosylation genes with IVIG resistance.

Lastly, in the third chapter, to identify coding variants associated with IVIG resistance in KD, we re-analyzed our previous genome-wide association study (GWAS) data made with DNA samples of 296 patients with KD, including 101 IVIG non-responders and 195 IVIG responders. Five nsSNPs in five immune-related genes, including a previously reported *SAMD9L* nsSNP (rs10488532; p.Val266Ile), were significantly associated with IVIG non-response (OR = 1.89–3.46, P = 0.0109–0.0035) (Table 13). In a replication study, only one in the interleukin 16 (*IL16*) gene (rs11556218, p.Asn1147Lys) was significantly associated with IVIG non-response (OR = 1.54, P = 0.0078). The same *IL16* nsSNP was more significantly associated with IVIG non-response in combined analysis of all data (OR = 1.64, $P = 1.25 \times 10^{-4}$). These results implicate *IL16* as involved in the mechanism of IVIG resistance in KD.

Key words: Kawasaki disease (KD), susceptibility, intravenous immunoglobulin (IVIG), IVIG resistance, association study, immunoglobulin Fc receptor, glycosylation, interleukin 16 (IL16), nonsynonymous single nucleotide polymorphism (nsSNP)

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ABBREVIATIONS

AIS	adolescent idiopathic scoliosis
ALL	Acute lymphoblastic leukemia
ALT	Alanine aminotransferase
AR	Allergic rhinitis
AS	ankylosing spondylitis
AST	Aspartate aminotransferase
BCG	Bacillus Calmette–Guérin
CAA	Coronary artery aneurysm
CALs	Coronary artery lesions
CD	Crohn's disease
CI	Confidence interval
cKD	Complete Kawasaki disease
CRP	C-reactive protein
ESR	Erythrocyte sedimentation rate
GD	Grave's disease
GWAS	Genome-wide association study
Hb	Hemoglobulin
HWE	Hardy-Weinberg Equilibrium
IBD	Inflammatory bowel disease
IgG	Immunoglobulin gamma
iKD	Incomplete Kawasaki disease
IL16	Interleukin 16
IVIG	Intravenous immunoglobulin
KD	Kawasaki disease
MAF	Minor allele frequency
MI	Myocardial infarction
MS	Multiple sclerosis
nsSNP	nonsynonymous single nucleotide polymorphism

OR	Odds ratio
PCR	Polymerase chain reaction
P	P-value
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SS	Systemic sclerosis
UC	Ulcerative colitis
WBC	White blood cell
WES	Whole exome sequencing

CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

Kawasaki disease (KD) is an acute vasculitis that affects mostly in young children, younger than 5 years old. It is diagnosed by clinical symptoms that over 5 days of prolonged fever with at least 4 of 5 symptoms, erythema of the oral mucosa, lips and strawberry tongue; polymorphous rash; erythema and edema of the hands and feet; and cervical lymphadenopathy (Burns JC, 2004). Approxiamtely 15 – 20 % of patients of delayed diagnosis and treatment have higher risk of complications of cardiovascular aneurysms include myocardial infarction, ischemic heart disease, or sudden death (Kato H, 1996; Burns JC, 1996).

Intravenous immunoglobulin (IVIG) is the standard therapy for KD. It effectively reduces the duration of fever, systemic inflammation and the development of coronary artery lesions (CALs) (Kato H, 1996). However, 10 ~ 20 % of the KD patients did not respond to the IVIG and had persistent or recurrent fever and a high risk of coronary complications, including myocardial infarction, ischemic heart disease, or sudden death (Durongpisitkul K, 2003; Kato H, 1996; Burns JC, 1996). IVIG treatment is also widely used as anti-inflammatory agent for treatment of autoimmune disease, such as, immune thrombocytopenia, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS) (Nimmerjahn F, 2007). Although the cause of KD is unclear, several evidences support that infections of pathogens or immune triggering factor, or antigens can cause a severe immune response to the individuals who are genetically vulnerable. Many studies reported several clinical risk factors of IVIG resistance in KD, such as, age, duration of fever, white blood cell (WBC) count, hemoglobulin (Hb) and albumin concentration, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) concentration, ratio of neutrophil, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (Kobayashi T, 2006; Egami K, 2006; Sano T, 2007). However, no consistent criteria have been established that can identify KD patients with IVIG treatment resistance.

Immunoglobulin Fc receptor, including Fc receptor-like molecules, play important roles by interacting with immunoglobulins which are recognizing the antigens that are

attached to the surface of microbes or microbe infected cells, helping these cells to identify and eliminate microbial pathogens (Janeway CA Jr, 2002). Most Fc receptor and Fc receptor-like genes are located in chromosome 1 and a few of them are in chromosome 11, and 19. Especially, Fc gamma receptors are the most studied and variants in these genes are associated with autoimmune disease such as SLE, RA, and inflammatory bowel disease (IBD), and so on (Jostins L, 2012; Bentham J, 2015). Furthermore, genome-wide association study (GWAS) found that single nucleotide polymorphism (SNP) in Fc gamma receptor II A (*FCGR2A*) is significantly associated with KD patients in Taiwan, Korea and China (Khor CC, 2011). However, it is difficult to study the genetic association in Fc receptor genes, because the Fc receptor genes have a very high homology to each other (Van der Heijden J, 2012).

In addition to immunoglobulin Fc receptors, glycosylation of the Fc binding region of immunoglobulins is critical for causing a proper immune response (Daeron M, 2014). Up to now, glycosylation of immunoglobulin gamma (IgG) is well described. Glycosylation of IgG is structurally important for stability of the antibody and thought to have regulatory functions for the modulatory activity of IgG (Wahl A, 2018). Its glycosylation also can diminish Fc receptor and complement dependent inflammatory reactions. Mutation in glycosylation related genes can lead to various diseases, such as RA and different type of autoimmune disease. A first GWAS on immunoglobulin glycosylation identified nine genes. Four of them (*ST6GAL1, B4GALT1, FUT8*, and *MCAT3*) participate directly IgG glycosylation and encoding glycosyltransferases. Five genes have not been implicated in glycosylation protein, but they have been reported to be related to autoimmune and inflammatory conditions (Lauc G, 2013).

Previous GWAS of KD identified that inositol-trisphosphate 3-kinase C (*ITPKC*), caspase-3 (*CASP3*), B lymphoid tyrosine kinase (*BLK*), cluster of differentiation 40 (*CD40*), human leukocyte antigen (*HLA*) region and *FCGR2A* are KD susceptible loci (Onouchi Y, 2010; Onouchi, 2012; Lee YC, 2012, Khor CC, 2011) (Table 1). In this study, we sought to investigate the genetic variant of immunoglobulin Fc receptor genes for KD susceptibility and the association of IVIG resistance in immunoglobulin G glycosylation genes or immune related genes, respectively. In order to perform this study, following specific aims were developed: 1) Identify the candidate coding SNPs in each group, and 2) Validate the candidate

coding SNPs in a large independent sample set for the association of KD and the resistance of IVIG treatment, respectively.

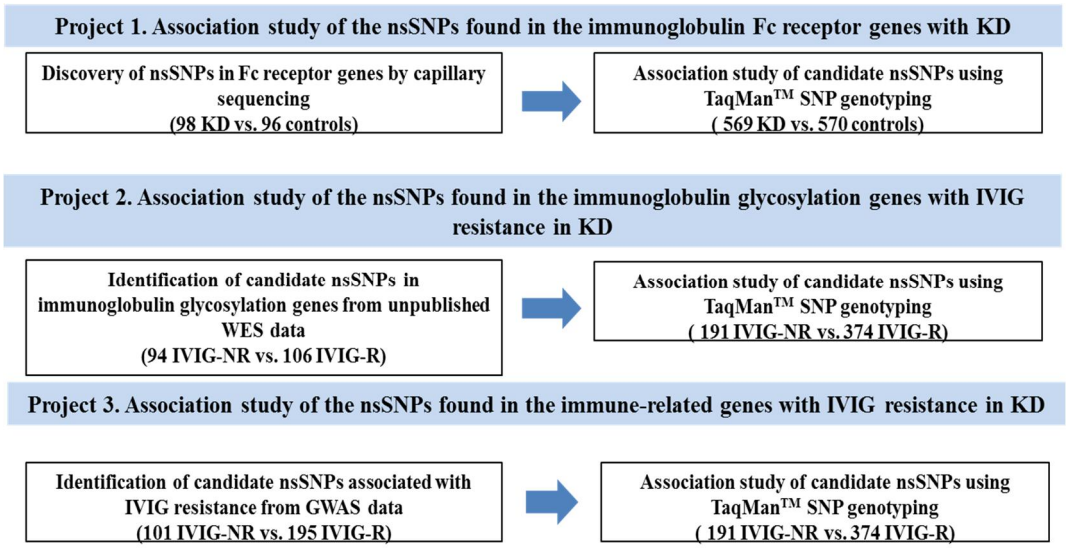

Figure 1. Overall workflow of this study. Part 1 is association study of the nonsynonymous SNPs (nsSNPs) in the immunoglobulin Fc receptor genes with KD. Candidate nsSNPs were selected capillary sequencing using 98 KD cases and 96 controls. Part 2 is association study of the nsSNPs found in the immunoglobulin glycosylation genes with IVIG resistance in KD. Candidate nsSNPs were selected by unpublished our lab whole exome sequencing (WES) data (94 IVIG-NR vs. 106 IVIG-R). The validation of the selected candidate nsSNPs was performed by TaqMan™ genotyping assays. Part 3 is association study of the nsSNPs found in the immune-related genes with IVIG resistance in KD. Candidate nsSNPs were selected from our previous GWAS data (101 IVIG-NR vs. 195 IVIG-R) (Kim JJ, 2019). GWAS, genome wide association study; IVIG-NR; intravenous immunoglobulin non-responder, IVIG-R; intravenous immunoglobulin responder; KD, Kawasaki disease; WES, whole exome sequencing; nsSNP, nonsynonymous SNP

HYPOTHESIS AND SPECIFIC AIMS

KD is characterized by systemic vasculitis, and mostly affects cardiovascular dysfunction in children. The only treatment of the KD is injection of the single high dose of the IVIG. To date, the working mechanism of the IVIG is little known. But, one of the proposed mechanisms of action of IVIG depends on the functional interaction with Fc receptors (especially IgG and Fc gamma receptors). Fc receptors act a major role in maintaining the homeostatic balance in human immune system. Through the studies of polymorphisms in the human immunoglobulin Fc receptor genes, immunoglobulin glycosylation genes, and immune related genes, we supposed the contribution of these variants to the pathogenesis, progression or of treatment outcome. They affect susceptibility to bacterial and viral pathogens, and impact the development of many autoimmune conditions. GWAS has identified an association between KD and *FCGR2A* locus with 131H variant contributing elevated disease risk (Khor CC, 2011; Onouchi Y, 2012). GWAS, also, has identified nine genome-wide significant loci ($P < 2.27 \times 10^{-9}$) associated with glycans attached to human immunoglobulin G in 4,095 individuals (Lauc G, 2013). Although the etiology and immune pathogenesis of KD are still unclear, if we understand the effects of immunoglobulin related genes (such as Fc receptor genes and IgG glycosylation genes) with the susceptibility of KD and IVIG resistance, we are expecting the better understanding the cause of disease and the prognosis of IVIG treatment.

The hypotheses to be tested in this proposal are that ***immunoglobulin related genes, such as Fc receptors genes and immunoglobulin glycosylation genes, are associated with the susceptibility to KD and/or the response of IVIG treatment***.

CHAPTER II

LITERATURE REVIEW

1. Kawasaki disease (KD)

1) Clinical features and diagnosis

Diagnosis of KD follows the criteria of American Heart Association published in 2004 (Newburger JW, 2004). According to American Heart Association, fever continued at least five days and over four symptoms of following five features: 1. changes in extremities, 2. erythematous rash, 3. bilateral, painless bulbar conjunctival injection without exudate, 4. changes in lips and oral cavity: erythema and cracking of lips, strawberry tongue and 5. cervical lymphadenopathy (1.5 cm in diameter) (Newburger JW, 2004). Types of KD divided into complete KD (cKD) and incomplete KD (iKD). While cKD patient shows over 4 of 5 symptoms, iKD patient shows below three clinical symptoms (Newburger JW, 2004). The stage of KD is classified into three clinical phases: acute, subacute and convalescent. For 1 to 2 weeks, it is called acute KD and patients have fever and other signs of KD. The subacute phase is associated with desquamation of the fingers and toes, thrombocytosis, the development of CAA, the risk of sudden death, and lasts about 3 weeks. Last stage is called the convalescent phase. Clinical signs start to disappear and the ESR becomes normal. Typically, it lasts about 6 – 8 weeks after the onset of the KD (Burns JC, 1996).

KD often accompanied coronary complications such as CAAs. CAAs occur as a consequence of the vasculitis in 10 – 15% of untreated patients. CAAs are classified into three groups, small (< 5 mm internal diameter), medium (5 – 8 mm internal diameter) and giant (> 8 mm internal diameter) according to the size of the aneurysm (Dajani AS, 1994).

2) Epidemiology

Predominantly 85% of KD patients are younger than 5 years old. The highest onset age is 9-11 months of age and decreased with age (Makino N, 2015). The incidence of KD is 1.5 to 1.7 times higher in boys than in girls (Holman RC, 2003). KD is 20 times more common in East Asian than in Caucasian. The incidence of KD patients with less than 5 year-olds was highest in Japan, occurred 308 per 100,000 cases in 2014, and Korea has a second highest incidence, 217.2 per 100,000 cases (Kim GB, 2019). The incidence of KD is 12-19 times higher in Japan and Korea than in Caucasians (Nakamura Y, 2008; Park YW, 2007; Holman RC, 2003). KD also affected seasonally. In Korea, KD occurred more frequently in the

summer (June and July) and winter (December and January) (Park YW, 2011).

3) Treatment

IVIG is the standard treatment for KD with a single infusion of 2 g/kg IVIG, together with aspirin. IVIG treatment within 10 days of onset of fever prevents CAL in patients with KD. Approximately, 10-20% of patients failed to respond to the initial IVIG injection (Burns JC, 1998; Durongpisitkul K, 2003). Failure to treatment usually defined as persistent fever ≥ 36 hours after completion of the initial IVIG infusion (Newburger JW, 2004). Additional treatment with 2 g/kg of IVIG is required. Patients who failed to treatment of IVIG have high risk to CALs (Newburger JW, 2004). The mechanism of IVIG is presumed that regulating the cytokine production, neutralizing the pathogen, inhibiting the production of antibodies, and inhibiting the activating Fc receptors action while activating the inhibitory Fc receptor (Burns JC, 2015).

2. Genetics of KD

1) Genetic influence

The cause of KD is unknown but there are several evidences that a genetically vulnerable individual is more susceptible to KD. The incidence of KD is 12 – 19 times higher in Asian, especially in Japan and Korea, than in Caucasians and it is also valid in Japanese-American in the United States (Nakamura Y, 2008; Park YW, 2007; Holman RC, 2003). KD predominantly occurs in male than female. In Japan, the relative risk of developing the disease for siblings is 10 times higher (Hirata S, 2001). In addition, a child whose parents have a history of KD is two times more likely to have a KD than those whose parents are not in the general population. Furthermore, the frequency of cases in twins and sibling is higher than in general population (Fujita Y, 1989; Harada F, 1986; Uehara R, 2003). All these studies demonstrated that development of KD can be affected by various genetic factors.

2) Genetic studies of KD

KD susceptibility genes: Although a number of studied performed, studies usually

involved in cardiovascular pathology or have insufficient sample size, so further studies are needed. A genome-wide linkage study identified *ITPKC* (rs28493229) and *CASP3* (rs113420705) as KD susceptible genes (Onouchi Y, 2007; Onouchi Y, 2010) (Table 1). After that, SNPs in *FCGR2A* (rs1801274), *BLK* (rs2736340, rs2618476, and rs6993775), *CD40* (rs1569723, rs4813003), *HLA* region (rs2857151), and *NMNAT2* (rs2857151) shows significant association with KD in GWAS (Khor CC, 2011; Lee YC, 2012; Onouchi Y, 2012; Kim JJ, 2017) (Table 1). Among identified genes, *BLK* and *FCGR2A* support that the dysfunction of B cells is related with abnormal immune responses in KD patients.

KD subgroup-specific KD susceptibility genes: In a previous IVIG response-stratified GWAS analysis, a variant in *BCL2L11* (rs3789065) showed KD association in IVIG responder but not in IVIG non-responder patients (Kwon YC, 2018). *FCGR2A* (rs1801274) discovered as common KD susceptibility gene, shows male KD patients specific association, not female KD patients. In addition, by an age-stratified KD association test, *LEF1* (rs4365796) was identified as a new susceptibility gene showing association with KD patients in patients younger than 6 months old (Kim HJ, 2018).

Table 1. KD susceptibility genes identified by genome-wide analysis

Group	Gene*	SNP	Consequence	OR	*P*-value	Population	Reference
KD	*ITPKC*	rs28493229	2 kb upstream	1.52	1.7×10^{-12}	Japanese	Onouchi Y, 2007
	CASP3	rs113420705	5 ‘ UTR	1.51	4.2×10^{-8}	Japanese	Onouchi Y, 2010
	FCGR2A	rs1801274	missense (H167R)	1.32	7.0×10^{-11}	Multi-ethnicity	Khor CC, 2011
				1.30	5.6×10^{-5}	Korean	Kim JJ, 2017
	BLK	rs2736340	7.5 kb upstream	1.54	9.0×10^{-10}	Chinese	Lee YC, 2012
		rs2618476	intron	1.96	2.0×10^{-9}	Chinese	Lee YC, 2012
		rs6993775	intron	1.52	2.5×10^{-11}	Korean	Kim JJ, 2017
	CD40	rs1569723	5 kb downstream	1.42	5.7×10^{-9}	Chinese	Lee YC, 2012
		rs4813003	16 kb upstream	1.41	4.8×10^{-8}	Japanese	Onouch Y, 2012
	HLA-DOB	rs2857151	19 kb downstream	1.47	3.3×10^{-5}	Japanese	Onouch Y, 2012
	NMNAT2	rs2078087	intron	1.33	1.2×10^{-6}	Korean	Kim JJ, 2017
KD-IVIG responder	*BCL2L11*	rs3789065	intron	1.55	4.4×10^{-11}	Korean	Kwon YC, 2018
KD-male	*FCGR2A*	rs1801274	missense (H167R)	1.40	9.3×10^{-5}	Korean	Kwon YC, 2017
KD- < 6 months	*LEF1*	rs4365796	missense (T269M)	3.07	1.1×10^{-5}	Korean	Kim HJ, 2018

**ITPKC* and *CASP3* were identified by genome-wide linkage analysis, while the rest is identified by GWAS.

IVIG, intravenous immunoglobulin; KD, Kawasaki disease; OR, odds ratio, UTR, untranslated region

3. Immunoglobulin Fc receptor genes

1) Immunoglobulins: ligand of the Fc receptors

Immunoglobulins consist of IgA, IgD, IgE, IgG, and IgM. Through the early B cell development, rearranged variable domains (VH and VL) are expressing the μ heavy chain to produce IgM, and then IgD by alternative splicing (Harry WS, 2009). After that, in response to stimulation of antigen and cytokine, isotype switching does occur to produce other isotype of immunoglobulins (IgA, IgE and IgG) (Harry WS, 2009). Isotypes have different feature including size, complement attachment, Fc receptor binding and the reaction to antigen (Harry WS, 2009). In case of IgD, it is found in the serum at very low levels with a short half-life. The function of circulating IgD is not known to participate in the major antibody effector mechanisms (Rogentine GN Jr, 1966). So, we are focusing with other four isotypes.

IgA – IgA plays a crucial role in protecting mucosal surfaces from virus and bacteria by neutralization or preventing binding of pathogens to the mucosal surface (Woof JM, 2005). The most prevalent form is dimeric IgA and is also called secretory IgA (sIgA) (Harry WS, 2009). sIgA is mainly found in mucous secretions, such as tears, saliva, sweat, and secretions from the genitourinary tract, gastrointestinal tract, prostate, and so on (Delacroix DL, 1982). It termed secretory IgA associated with a J-chain and another polypeptide chain, the secretory component. IgA has two subtypes, IgA1 and IgA2, whose structures differ in their hinge regions (IgA1 has a longer hinge region than IgA2) (Harry WS, 2009). IgA2 predominates in the many mucosal secretions, whereas more than 90% of serum IgA is in the form of IgA1 (Delacroix DL, 1982).

IgE – IgE is the lowest serum concentration with the shortest half-life (0.002% and 2 days of half-life) (Winter WE, 2000). IgE has a potent role in hypersensitivity and allergic reactions as well as the reaction to parasitic infections (Harry WS, 2009). IgE binds to the Fc-epsilon receptor expressed on mast cells, basophils, and so on (Novak N, 2001).

IgG – IgG is the abundant isotype in the body with the longest serum half-life (Harry WS, 2009). IgG is the major antibody found in blood and extracellular fluid, protecting body tissues from infections. IgG contribute directly to an immune response including neutralization of toxins and virus. IgG is the only antibody isotype providing protection to the fetus in utero, as it has receptors to facilitate passing the human placenta (Fouda GG,

2018). IgG received from mother through the placenta give humoral immunity until its own immunity develops (Fouda GG, 2018).

IgM – IgM is the first immunoglobulin expressed on the surface of the naïve B cells as monomeric form (Harry WS, 2009). Through the maturation and antigen stimulation, IgM is secreted as pentamer form, the largest of the antibodies. The pentamer is fixed by the J-chain (Mestecky J, 1971). IgM antibodies are associated with a major immune reaction such as opsonizing for destruction and fixing complement. IgM usually used to diagnose acute exposure to pathogen (Harry WS, 2009).

2) Human Fc receptor genes

There are several types of Fc receptor depending on the recognition of antibody. For example, Fc receptor binding to IgA is called Fc alpha receptor (FcαR), Fc receptor binding to IgE is called Fc epsilon receptor (FcεR), Fc receptor binding to IgG is called Fc gamma receptor (FcγR) and Fc receptor binding to IgM is called Fc mu receptor (FcμR). There are two types of receptor act as transport the immunoglobulins, FcγR and polymeric immunoglobulin receptor (pIgR), and a family of protein that resemble Fc receptors, Fc receptor-like molecules (FCRLs) (Table 2).

IgA receptor – *FCAR* encodes for the transmembrane receptor, FcαRI. FcαRI binds IgA antibodies and is on the surface of myeloid cells, such as neutrophils, monocytes, macrophages, and eosinophils (Otten MA, 2004).

IgE receptor – IgE receptors are classified into two groups according to affinity (Fridman WH, 1991). The high affinity IgE receptor, known as FcεRI, is the high-affinity for the Fc region of IgE, involved in the allergy disorder and parasites clearance. On the other hand, low affinity receptor, known as FcεRII, is also involved in the allergy reaction and resistance to parasites, and is main regulator of IgE levels (Fridman WH, 1991). FcεRI consists of one alpha (FcεRIα encoded by *FCERIA*), one beta (FcεRIβ encoded by *MS4A2*), and two gamma chains (FcεRIγ encoded by *FCER1G*). It is expressed on mast cells and basophils (Prussin C, 2006). Degranulation of mast cells or basophils and releasing of inflammatory cytokine is led by crosslinking of the FcεRIγ through IgE-antigen complexes (Fridman WH, 1991).

IgG receptor – As described above, IgG is the most abundant in the serum, about 80% (Harry WS, 2009). The surface glycoproteins encoded by eight genes located in chromosome 1q21-23 constitute an FcγRs that bind to the Fc portion of IgG (Li X, 2014). These FcγRs are divided into three subgroups such as FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) by structural homology and affinity differences (Indik ZK, 1995; Li X, 2009). FcγR functions as activating receptor (FcγRI, FcγRIIa/c, FcγRIII) or inhibitory receptor (FcγRIIb) induced by their signals through the tyrosine activating or inhibitory motifs (ITAM or ITIM) (Li X, 2009). In addition to interacting with IgG, FcγR also functions as a receptor for opsonized complex and provides a connection between innate and acquired immunity (Fridman WH, 1991). Besides Fcγ receptor family, there are other two, DC-SIGN and CD22 that IgG binds (Bournazos S, 2016). Unlike the pro-inflammatory Fcγ receptor family, DC-SIGN acts as anti-inflammatory receptor (Bournazos S, 2016).

IgM receptor –FCMR initially named TOSO, or Fas apoptotic inhibitory molecule 3 (FAIM3), and later it has been identified Fc receptor for IgM (Kubagawa H, 2009). FCMR is expressed on B cells and NK cells and only Fc receptor on T cells (Kubagawa H, 2009). There is another receptor for IgM, FCAMR, binds to both IgM and IgA. FCAMR has high affinity to IgM and middle affinity to IgA (Kubagawa H, 2009). Binding with IgA likely happens when complex formation and related with removal of IgA complexes from circulation (Kubagawa H, 2009).

Ig transport – Polymeric immunoglobulin receptor (pIgR) is a transmembrane protein encoded by the *PIGR* gene located in chromosome 1q32.1. It facilitates the transcytosis of the polymeric immunoglobulin, IgA and IgM (Kaetzel CS, 1997). pIgR only binds to polymeric immunoglobulin and it is not binding to monomeric immunoglobulin (Kaetzel CS, 1997). The J-chain is involved in the binding of pIgR and its ligand (Asano M, 2011). pIgR transports immune complex from the basolateral to the apical mucosal epithelial cell surface (Kaetzel CS, 1997). The other Ig transport is FcRn, encoded by *FCGRT* gene located in chromosome 19q13.33. FcRn gives a passive transfer of IgG from mother to fetus and regulation of serum IgG levels (eliminating the unbinding IgGs) both before and after a birth (Story CM, 1994). Also, FcRn is competing Fcγ receptor to bind IgGs (Harry WS, 2009).

FCRL family – Fc receptor-like molecules (FCRLs) are a group of proteins

resemble the Fc receptors. There is no clear evidence that FCRL binds to the Fc portion, but some FcRLs bind to immunoglobulins, such as FCRL4 is known to bind IgA, FCRL5 binds IgG, and FCRLA binds to IgA, IgG, and IgM (Santiago T, 2011; Wilson TJ, 2012). Their function is unknown and they are preferentially expressed by B lymphocytes (Davis RS, 2007).

Table 2. The classification of the human Fc receptor genes

Group	Fc Receptor gene (alias)	Ligand	Location	Exon count	Expression
IgA receptor	*FCAR*	IgA	19q13.42	7	Restricted expression toward bone marrow
IgE receptor	*FCER1A*	IgE	1q23.2	7	Broad expression in placenta, lymph node
	MS4A2 (FCER1B)	IgE	11q12.1	8	Broad expression in skin, esophagus
	FCER1G	IgE	1q23.3	5	Broad expression in appendix, spleen
	FCER2 (CD23)	IgE/G	19p13.2	12	Biased expression in spleen, lymph node
IgG receptor	*FCGR1A*	IgG	1q21.2	6	Biased expression in appendix, placenta
	FCGR1B	IgG	1p11.2	6	Biased expression in appendix, placenta
	FCGR1CP (pseudo)	-	1q21.1	6	Biased expression in appendix, placenta
	FCGR2A	IgG	1q23.3	11	Broad expression in appendix, placenta
	FCGR2B	IgG	1q23.3	11	Biased expression in placenta, appendix
	FCGR2C	IgG	1q23.3	7	Biased expression in placenta, appendix
	FCGR3A	IgG	1q23.3	7	Biased expression in spleen, lung
	FCGR3B	IgG	1q23.3	7	Biased expression in spleen, appendix
	CD209 (DC-SIGN)	IgG/A	19p13.2	7	Broad expression in placenta, lymph node
	CD22	IgG/M	19q13.12	14	Biased expression in lymph node, ovary
IgM receptor	*FCMR*	IgM	1q32.1	8	Biased expression in lymph node, spleen

	FCAMR	IgA/M	1q32.1	10	Biased expression in kidney, lymph node
Ig transport	*FCGRT*	IgG & albumin	19q13.33	7	Ubiquitous expression in duodenum, small intestine
	PIGR	IgA/M	1q32.1	12	Biased expression in duodenum, colon
FCRL family	*FCRL1*	unknown	1q23.1	12	Biased expression in lymph node, spleen
	FCRL2	unknown	1q23.1	13	Biased expression in lymph node, spleen
	FCRL3	unknown	1q23.1	16	Biased expression in lymph node, spleen
	FCRL4	IgA	1q23.1	12	Biased expression in lymph node, appendix
	FCRL5	IgG	1q23.1	17	Biased expression in lymph node, spleen
	FCRL6	HLA-DR	1q23.2	11	Broad expression in spleen, lung
	FCRLA	IgA/G/M	1q23.3	6	Biased expression in lymph node, spleen
	FCRLB	unknown	1q23.3	9	Broad expression in kidney, appendix

The information for the location, exon count and expression of each gene is extracted from the Gene database of NCBI (https://www.ncbi.nlm.nih.gov/gene/). Ig, immunoglobulin; FCRL, Fc receptor-like

3) Association of Fc receptor genes with human diseases

Fc receptor genes play a significant role in maintaining the immune system and their polymorphisms impact on human immune disease (Li X, 2014). They affect susceptibility to pathogens and contribute as risk factors for immunoglobulin mediated inflammatory diseases (Li X, 2014). Fc receptor genes associated human diseases, especially auto-inflammatory diseases or auto-immune diseases have been identified by GWAS. The IgA receptor gene, *FCAR,* is associated with IBD (Jostins L, 2012). One of the IgE receptor genes, *FCER1A,* is associated with increased IgE levels, and the other IgE receptor gene, *FCER1G*, is associated with allergy disease and allergic rhinitis (AR) (Granada M, 2012; Ferreira MA, 2017; Waage J, 2018). However, these associated SNPs are in the untranslated region or introns. It is not changing an amino acid and proteins. Among Fc gamma receptor genes, only rs1801274, in *FCGR2A* is significantly associated with various inflammatory diseases, such as, ankylosing spondylitis (AS), Crohn's disease (CD), Grave's disease (GD), IBD, KD, SLE, ulcerative colitis (UC) (Cortes A, 2013; Liu JZ, 2015; Jostins L, 2012; Korr CC, 2011; Bentham J, 2015; Liu JZ, 2015). One of the Ig transport gene, *FCGRT,* is associated with albumin level (Kanai M, 2018). Among the FCRL family, *FCRL1* is associated with MS, *FCRL3* with GD, and *FCRL5* with adolescent idiopathic scoliosis (AIS) (Beecham AH, 2013; Chu X, 2011; Liu J, 2018) (Table 3).

Through the candidate gene study, two SNPs in *FCAR* gene are associated with IgA levels and susceptibility of myocardial infarction (MI), respectively (Wu J, 2007; Shiffman D, 2008). For IgE receptors, variant in *FCER1A* is identified for SLE risk factor and in *MS4A2* is identified as risk factor of AR and in *FCER2* is associated with IgE levels (Yang J, 2013; Amo G, 2016; Laitinen T, 2000). In the *FCGR2A* gene, rs1801274, also known in GWAS as KD risk factor, has been found to be significantly associated with KD through the candidate gene study (Duan J, 2014). Each mutation associated with AS and SLE in *FCGR2B* has been identified. Locus in *FCGR3A* and haplotype variant in *FCGR3B* is identified as risk factor of SLE (Duan ZH, 2012; Zhu XW, 2016). Among FCRL family genes, two variants in *FCRL3* and one in *FCRL5* are known through a candidate gene study (Gu Z, 2015; Yuan M, 2016; Tang X, 2009; WTCCC, 2007) (Table 4). In particular, KD is significantly associated only in *FCGR2A* in both GWAS and candidate gene study.

Table 3. Association of the human Fc receptor genes with disease from GWAS

Group	Fc Receptor	Diseases / traits	SNP	Consequence[a]	OR/β	*P*-value	Reference
IgA receptor	*FCAR*	IBD	rs11672983	2.5 kb upstream	1.09	7×10^{-11}	Jostins L, 2012
IgE receptor	*FCER1A*	IgE levels	rs2251746	intron	0.087[b]	5×10^{-26}	Granada M, 2012
	MS4A2	-	-	-	-	-	-
	FCER1G	Allergy	rs2070901	29 bp upstream	1.04	1×10^{-11}	Ferreira MA, 2017
		AR	rs2070902	intron	1.05	6×10^{-19}	Waage J, 2018
	FCER2	-	-	-	-	-	-
IgG receptor	*FCGR1A*	-	-	-	-	-	-
	FCGR1B	-	-	-	-	-	-
	FCGR2A	AS	rs1801274	missense (H167R)	1.12	1×10^{-9}	Cortes A, 2013
		CD	rs1801274	missense (H167R)	1.08	9×10^{-11}	Liu JZ, 2015
		IBD	rs1801274	missense (H167R)	1.12	2×10^{-38}	Jostins L, 2012
		KD	rs1801274	missense (H167R)	1.32	7×10^{-11}	Khor CC, 2011
		SLE	rs1801274	missense (H167R)	1.16	2×10^{-14}	Bentham J, 2015
		UC	rs1801274	missense (H167R)	1.19	1×10^{-41}	Liu JZ, 2015
	FCGR2B	-	-	-	-	-	-
	FCGR2C	-	-	-	-	-	-

	FCGR3A	-	-	-	-	-	-
	FCGR3B	-	-	-	-	-	-
	CD209	-	-	-	-	-	-
	CD22	-	-	-	-	-	-
IgM receptor	*FCMR*	-	-	-	-	-	-
	FCAMR	-	-	-	-	-	-
Ig transport	*FCGRT*	albumin level	rs34010237	intron	0.089[b]	2×10^{-40}	Kanai M, 2018
	PIGR	-	-	-	-	-	-
FCRL family	*FCRL1*	MS	rs2050568	intron	1.08	2×10^{-10}	Beecham AH, 2013
	FCRL2	-	-	-	-	-	-
	FCRL3	GD	rs3761959	intron	1.23	2×10^{-13}	Chu X, 2011
	FCRL4	-	-	-	-	-	-
	FCRL5	AIS	rs11264728	intergenic	na	3×10^{-21}	Liu J, 2018
	FCRL6	-	-	-	-	-	-
	FCRLA	-	-	-	-	-	-
	FCRLB	-	-	-	-	-	-

The information in this table is extracted from the GWAS Catalog database (https://www.ebi.ac.uk/gwas/). The variants pass the significance *P*-value threshold of GWAS association test (*P*-value < 5×10^{-8}).

[a]Amino acid is represented in a single letter code.

[b]These are β value.

AIS, adolescent idiopathic scoliosis; AR, allergic rhinitis; AS, ankylosing spondylitis; CD, Crohn's disease; GD, Grave's disease; IBD, inflammatory bowel disease; KD, Kawasaki disease; MS, multiple sclerosis; na, not available; OR, odds ratio; SLE, systemic lupus erythematosus; UC, ulcerative colitis

Table 4. List of coding variants of the Fc receptor genes associated with human diseases from candidate gene study

Group	Fc Receptor	Disease/trait	SNP	Amino acid change	OR	*P*-value	Number (case / control)	Population	Reference
IgA receptor	*FCAR*	IgA levels	rs16986050	S248G	na	0.019	190 / 222	American	Wu J, 2007
		MI	rs11666735	D92N	1.68	0.02	28/149	Caucasian	Shiffman D, 2008
IgE receptor	*FCER1A*	SLE	rs2298804	K84R	2.22	< 0.001	150/315	Chinese	Yang J, 2013
	MS4A2	AR	rs569108	E237G	3.85	0.017	515 / 526	European	Amo G, 2016
	FCER1G	-	-	-	-	-	-	-	-
	FCER2	IgE levels	rs2228137	R62W	na	< 0.03	316 / 244	European	Laitinen T, 2000
IgG receptor	*FCGR1A*	-	-	-	-	-	-	-	-
	FCGR1B	-	-	-	-	-	-	-	--
	FCGR2A	KD	rs1801274	H167R[b]	1.29	0.012	428/493	Chinese	Duan J, 2014
	FCGR2B	AS	rs10917661	Q57X	1.72	0.02	306 / 300	Chinese	Duan ZH, 2012
		SLE	rs1050501	I232T	1.75	1×10^{-7}	5,741 / 6,530	International	Zhu XW, 2016
	FCGR2C	-	-	-	-	-	-	-	-
	FCGR3A	SLE	rs396991	F176V[b]	1.26	9×10^{-5}	5,741 / 6,530	International	Zhu XW, 2016
	FCGR3B	SLE	rs200688856	NA1/NA2/SH[c]	1.18	1×10^{-3}	5,741 / 6,530	International	Zhu XW, 2016
			rs448740						

			rs5030738						
			rs147574249						
			rs2290834						
	CD209	-	-	-	-	-	-	-	-
	CD22	-	-	-	-	-	-	-	-
IgM	*FCMR*	-	-	-	-	-	-	-	-
	FCAMR	-	-	-	-	-	-	-	-
Ig transport	*FCGRT*	-	-	-	-	-	-	-	-
	PIGR	-	-	-	-	-	-	-	-
FCRL family	*FCRL1*	-	-	-	-	-	-	-	-
	FCRL2	-	-	-	-	-	-	-	-
	FCRL3	AR	rs7522061	N28D	1.30	2×10^{-8}	540 / 600	Chinese	Gu Z, 2015
		MS	rs2282284	N721S	1.75	0.019	120 / 240	Chinese	Yuan M, 2016
	FCRL4	-	-	-	-	-	-	-	-
	FCRL5	AS	rs12036228	V269I	1.66	0.003	169 / 184	Chinese	Tang X, 2009
			rs6427384	V466L	1.43	1×10^{-5}	1,000 / 1,500	Caucasian	WTCCC, 2007
	FCRL6	-	-	-	-	-	-	-	-
	FCRLA	-	-	-	-	-	-	-	-

FCRLB	-	-	-	-	-	-	-	-

[a]Amino acid is represented in a single letter code.

[b]H167R in *FCGR2A* is identical to H131R and F176V in *FCGR3A* also referred to as F158V by counting from the N-terminus of the mature protein after cleavage of the signal peptide.

[l]NA1/NA2/SH in *FCGR3B* is consisted with 6 nucleotide sites.

AR, Allergic rhinitis; AS, Ankylosing spondylitis; KD, Kawasaki disease; MI, Myocardial infarction; MS, Multiple sclerosis; na, not available; OR, odds ratio; SLE, Systemic lupus erythematosus

4. Immunoglobulin glycosylation genes

1) IgG glycosylation and its impact on antibody activity and anti-inflammatory activity

IgG glycosylation is important for the immunomodulatory activity of IgG (Lauc G, 2013). Compared to most immunoglobulin isotypes, such as IgA, IgM, and IgE containing multiple N-linked sugar moieties, IgG has a single sugar domain attached to the IgG Fc fragment at the asparagine 297 (N297) residue in the CH2 domain (Lauc G, 2013; Kobata A, 2008). Both monomeric IgG as well as IgG immune complex can reduce Fc receptor and complement dependent inflammatory reactions (Nimmerjahn F, 2007; Nimmerjahn F, 2010). Also, IgG modulates B and T cell responses, mainly involved in the organization and maintenance of the immune responses (Nimmerjahn F, 2007; Nimmerjahn F, 2010).

Several studies have been described that the removal of sugar moiety of IgG by genetically or enzymatically, it causes a loss of both the pro- and anti-inflammatory activities of IgG (Kaneko Y, 2006; Albert H, 2008). For example, the capacity of IVIG to suppress inflammatory arthritis was lost in the absence of IgG glycosylation or sialylation, respectively (Aschermann S, 2010). Also, lacking form of sialic acid and galactose (IgG-G0 form) at terminal residue of IgG is increasing during acute inflammation, which can be caused by autoimmune diseases such as RA (Parekh RB, 1985; Aschermann S, 2010). A various role for sialylated IgG in maintain immune homeostasis is reported that autoimmune-prone mice (MRL-lpr, K/BxN mice) and patients with RA, CD, UC and tuberculosis showed that increased levels of IgG glycoforms without terminal sialic acid and galactose residues (Kaneko Y, 2006; Parekh RB, 1985; van de Geijn FE, 2009; Dube R, 1990; Bond A, 1990; Mizuochi T, 1990; Nakajima S, 2011). Also, treatment of IVIG which is removed terminal sialic acid residues diminished IVIG activity to that of aglycosylated IVIG (Kaneko Y, 2006). In contrast to this, the enrichment of terminal sialic acid residues resulted in a tenfold increased anti-inflammatory activity. So, it suggests that IgG glycosylation may be involved in a role on the anti-inflammatory and immunomodulatory activity.

2) Association study of the immunoglobulin glycosylation genes with human disease

The first GWAS of analyzing glycans attached to immunoglobulin G using 4,095

individuals was performed and nine genetic loci were showed genome wide significance (Lauc G, 2013). Of them, four genes (*ST6GAL1*, *B4GALT1*, *FUT8*, and *MGAT3*) encoding glycosyltransferases, while the remaining five loci (*IKZF1, IL6ST-ANKRD55, ABCF2-SMARCD3, SUV420H1-CHKA* and *SMARCB1-DERL3*) is not encoding glycosylation protein, but they showed glycosylation traits and the most of them reported that it is related to autoimmune and inflammatory conditions (Lauc G, 2013) (Table 5). The variant in *ST6GAL1* is associated with type 2 diabetes in South Asian. The variant in *FUT8* is associated with MS. Especially *IKZF1* was found that it is associated with multiple IgG N-glycans and this gene has been implicated in several diseases, such as SLE, acute lymphoblastic leukemia (ALL), CD, systemic sclerosis (SS). The variant in *IL6ST-ANKRD55* locus is associated with RA (Lauc G, 2013) (Table 6). Other GWAS study reported that genetic loci, *RUNX3,* which encodes for a transcription factor of the runt domain-containing family, has been showed strong influences on decreased galactosylation (Wahl A, 2018). The variants of *RUNX3* are involved in not only glycosylation, but also, B-cell maturation as well as T-cell differentiation during immune response and apoptosis (Wahl A, 2018).

Table 5. Loci associated with immunoglobulin G glycosylation which are identified by GWAS

Gene	SNP	Effect size	*P*-value	Ancestry	Reference
ST6GAL1	rs11710456	0.64	6.12×10^{-75}	European	Lauc G, 2013
B4GALT1	rs12342831	-0.24	2.70×10^{-11}	European	Lauc G, 2013
MGAT3	rs909674	0.34	9.66×10^{-25}	European	Lauc G, 2013
FUT8	rs11847263	-0.31	1.08×10^{-22}	European	Lauc G, 2013
IKZF1	rs6421315	0.23	1.87×10^{-13}	European	Lauc G, 2013
SMARCB1-DERL3	rs2186369	0.35	8.63×10^{-17}	European	Lauc G, 2013
IL6ST-ANKRD55	rs17348299	0.29	6.88×10^{-11}	European	Lauc G, 2013
ABCF2-SMARCD3	rs1122979	0.31	2.10×10^{-10}	European	Lauc G, 2013
SUV420H1	rs4930561	0.19	8.88×10^{-10}	European	Lauc G, 2013

SNP, single nucleotide polymorphism.

Table 6. Disease association of the genes involved in immunoglobulin G glycosylation

Gene	Disease	SNP	Risk allele	*P*-value	Ancestry	Reference
ST6GAL1	T2D	rs16861329	G	3.00×10^{-8}	South Asian	Kooner JS, 2011
FUT8	MS	rs8007846	-	9.00×10^{-6}	American	Baranzini SE, 2010
IKZF1	SLE	rs2366293	G	2.33×10^{-9}	European	Cunninghame DS, 2011
	ALL	rs4132601	C	1.00×10^{-19}	European	Papaemmanuil E, 2009
	CD	rs1456893	A	5.00×10^{-9}	European	Barrett JC, 2008
	SS	rs1240874	-	1.00×10^{-6}	European	Gorlova O, 2011
SMARCB1-DERL3	GGT	rs2739330	T	2.00×10^{-9}	European	Chambers JC, 2011
IL6ST-ANKRD55	RA	rs6859219	C	1.00×10^{-11}	European	Stahl EA, 2010

ALL, acute lymphoblastic leukemia; CD, Crohn's disease; GGT, gamma-glutamyl-transferase; MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, systemic sclerosis; T2D, type 2 diabetes

CHAPTER III

ASSOCIATION STUDY OF THE NONSYNONYMOUS SNPs FOUND IN THE IMMUNOGLOBULIN Fc RECEPTOR GENES WITH KD

ABSTRACT

KD is an acute febrile syndrome in children, affecting mainly who is younger than 5 years old. Without proper treatment for disease can be accompanied by the CALs in approximately 20% of the children in KD. Treatment of IVIG for KD patients leads to a decrease of complications to 5-16%. In this study we evaluated the involvement of Fc receptor genes as KD susceptibility genes by performing the association study of nsSNPs in the genes coding for the Fc receptors with KD cases and a cohort of healthy controls. We used pre-made ABI primer sets for the identification of potential coding variants, covering the coding region of the 15 Fc receptor genes, due to high homology between the Fc receptor genes. Through the primary capillary sequencing test using 98 DNA samples from KD patients, 20 nsSNPs were found in the coding regions of 15 Fc receptor genes. Of them, seven candidate nsSNPs were selected and eight nsSNPs were excluded according to our exclusion criteria 1) minor allele frequency (MAF) < 0.01 in case, 2) OR ≤ 1.5, and 3) *P*-value > 0.2 obtained from association study using discovery sample set (98 cases vs. 96 controls). Seven candidate nsSNPs were tested for large scale case-control test by TaqMan^{TM} SNP genotyping with 569 KD cases and 570 controls. There was no significant association for candidate nsSNPs in Fc receptor genes with KD.

INTRODUCTION

KD is a systemic vasculitis mainly occurring in the children under 5 year old. High dose of IVIG is used as the primary treatment for KD (Burns JC, 1998). It is expected that the interaction of immunoglobulin and its receptor may play an important role for the susceptibility of KD as well as IVIG treatment response. Fc receptors are receptors for the constant region of immunoglobulins (Ravetch JV, 2001). The central role of Fc receptors in supporting an appropriate humoral immune system has been demonstrated in immune system, such as clearance and processing of immune complexes or antibody opsonized particles (Janeway CA Jr, 2002). Thus, functional polymorphisms of the Fc receptor genes may significantly influence effector cell functions, thus providing diversity in host responses properly to many infectious, inflammatory and autoimmune diseases, including KD (Bentham J, 2015). Therefore, they form a crucial connection between innate and adaptive immunity, as well as the connection between the humoral and cellular parts of the immune system (Deo YM, 1997). Recent years, a number of studies have been published on the association of polymorphisms in the Fc receptor genes and a variety of human diseases, including infectious as well as autoimmune diseases (Biezeveld M, 2007). Furthermore, KD is significantly associated with a nsSNP (rs1801274, H167R) of the *FCGR2A* gene (Khor CC, 2011). Since the KD is triggered by an infectious agent in genetically predisposed children, in this study, we investigated the possible association of coding variants found in the 15 Fc receptor genes with KD.

SUBJECTS AND METHODS

Subjects

KD patients were recruited from 12 hospitals in Korea that are currently participating in the Korean Kawasaki Disease Genetics Consortium from June 2008 through February 2019. All KD patients were diagnosed by pediatricians according to the diagnostic criteria of the American Heart Association (Newburger JW, 2004). Control samples with no history of KD were acquired from national institutes of health in Korea. The study protocol was approved by the Institutional Review Board of the involved institutions, and written informed consent was obtained from the parents of all KD patients.

PCR amplification and DNA sequencing

To initially identify functional coding variants of a total of 15 Fc receptor genes, we sequenced the coding region of the human Fc receptor genes in DNA samples of 98 KD cases and 96 controls using pre-made PCR primers purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA) (Table 7). Each fragment amplified by PCR was sequenced with an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA, USA). DNA polymorphisms were identified using the PolyPhred program (http://droog.gs.washington.edu/polyphred/).

TaqMan™ SNP genotyping

To test candidate nsSNPs in association study, we used a custom-made TaqMan™ probe sets or pre-designed TaqMan™ probe sets (Applied Biosystems, Foster City, CA, USA). TaqMan™ genotyping assays were performed according to the standard protocol on a 7900HT Real-Time PCR System using Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analyses were performed using the PLINK (version 1.07) (http://pngu.mgh.harvard.edu/~purcell/plink/). Allelic association test was performed for

genetic association study obtaining OR and *P*-value. Also, the nsSNPs with a Hardy-Weinberg Equilibrium (HWE) *P*-value < 10^{-4} were excluded in genetic association study. In addition, Haploview was used for analysis and visualization of haplotype block (http://www.broad.mit.edu/mpg/haploview/). To study the significance of differences in the clinical variables in each group, SPSS programs (version 18) (SPSS, Chicago, IL, USA) were used. The functional effects of candidate variants were predicted by Polyphen and SIFT programs. HaploReg v4.1 program was searched for exploring annotations of the associated SNPs (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php).

Table 7. Primer information for amplifying the coding regions of the Fc receptor genes

Gene	Target exon	Assay ID	Primer sequence (5′ → 3′)	Product size (bp)
FCGR1A	3	Hs00400124_CE	F: M13F-TAACCTTGCACTGTGAGGTGC	546
			R: M13R-GATCTAGGTTTTGGGTGGGAGGAAGAT	
	4	Hs00641220_CE	F: M13F-GTCTCCAGCAGAGTCTTCACG	250
			R: M13R-ATTCCAATACAATACCTTTCACAGTGACA	
	5	Hs00516099_CE	F: M13F-GGCTCTCTGCTGGAAGTAAAAAG	453
			R: M13R-CTTCCCGTCATTCTCACTCAC	
FCGR1B	1	Hs00813950_CE	F: CATAAAATAAGCTCTAATAAACAGTGGAAGCAC	238
			R: CTTCAATATCTTGCATGTTACAGATTTCACTAC	
	2	Hs00516100_CE	F: TCTCTGCTTTCTCTATATGCTCTTCT	408
			R: CAACTTGTATCTGTGCTTCCACT	
	3	Hs00247303_CE	F: CCAGATAAATCGACACACACACACATC	509
			R: CTGGCTTTTGTAGGTTTCCACTTG	
	5a	Hs00411678_CE	F: TGGCTTCCCGTCATTCTCACCA	517
			R: GGTATCCTCAGTAGGACTCAGCAA	
	5b	Hs00247300_CE	F: CACAATCCCTCTGCCTCAGTTAAAC	504
			R: TTCAAGGGTTTTTGGCCCAGACAA	
	6	Hs00247299_CE	F: GTCCAGATCGATGGCCACCA	531
			R: GGCCCAGCAATTAAGCTCTTCCA	
FCGR2A	1	Hs00597754_CE	F: M13F-TGGTTAGCAGAAACAGAATTGAGGA	274
			R: M13R-CCACACTGGTACCTGGAGTC	
	2	Hs00609450_CE	F: M13F-AACAGGATCTTGAGATGGGTCCT	272
			R: M13R-CATGCTGAACCCAAGCTTGATTTT	
	3	Hs00293326_CE	F: M13F-CTACAATAGGACTCTTGGGTGCCT	500
			R: M13R-GGGAATGAATGAGCAACTCACAAC	
	4	Hs00692008_CE	F: M13F-CTCTGAGACTGAAAAACCCTTGGAA	274
			R: M13R-TGTTTCCTGTGCAGTGGTAATCA	

	5	Hs00755658_CE	F: M13F-CTGGGACATAGCATTGGAGGTG	272
			R: M13R-GGGCCAAATGGAAACTGATAACAAAA	
	6	Hs00637943_CE	F: M13F-AGGCCATATTTACTTAGCCCTTGG	274
			R: M13R-ATTAACAAAGGCAAAAATGAGAGGAAACAG	
	7a	Hs00293330_CE	F: M13F-AGGTGAGGATCTGGTATTTCCTGGA	507
			R: M13R-ATAGTTCAGTCTACACCCCAATCAA	
	7b	Hs00785077_CE	F: M13F-TTACCTCCCCGGTGTATTGAATTATC	274
			R: M13R-TCCACTCAGCAAGCTGAGAGTA	
FCGR2B	3	Hs00293368_CE	F: M13F-CCTACAGGTGCTTTTTTGTCTGA	509
			R: M13R-ATCAGAACTGAGGCAAAAACAACCA	
	4	Hs00395871_CE	F: M13F-TGTATCCTGAACACCTATTATGTGCTA	555
			R: M13R-TCCTTACATCTTGGCAGACTCCG	
	6	Hs00594299_CE	F: M13F-ACAGGAAACATCTGCCAGAGTG	274
			R: M13R-CAGCTGAACTTGCACAAGCC	
	7	Hs00649897_CE	F: M13F-ATCCCACTAATCCTGATGAGGCT	256
			R: M13R-TTCTTCTCAAATCCCAATGCAAGACA	
	8a	Hs00293373_CE	F: M13F-GAGTCATCTACCTGAGTCCTGAA	526
			R: M13R-CACTCTACATTCTAAATCCCCATCTCT	
	8b	Hs00293375_CE	F: M13F-GGGATTTAGAATGTAGAGTGAGTGC	541
			R: M13R-GGGAGAATAGCAGAGCAGGACAT	
FCGR2C	3	Hs00293349_CE	F: M13F-CAGGTGCTTTTTTGTCTGAGATTCA	508
			R: M13R-TTCAATCAGGACTGAGGCAAAGCA	
	4	Hs00293350_CE	F: M13F-GGGTCTCTTGTGTTCTTCCTGA	504
			R: M13R-AAGTTGGGATCCGAACGGG	
	5	Hs00293353_CE	F: M13F-TAGCTCCCAGCTCTTCACCGATG	510
			R: M13R-CCCTGAGAACTTCTCCAACTGCGT	
	6	Hs00293354_CE	F: M13F-AGCTGTCTGGTTTGCTTCTAGGAA	400
			R: M13R-AACAAAGGCAAAAAGAGAGGAAACAG	
	7	Hs00293355_CE	F: M13F-GTTCCAAAAGAGAAGGACTCTTCC	543

			R: M13R-TCACCCTACACTCCAAATCTCCA	
FCGR3A	2	Hs00293347_CE	F: M13F-CATCCCTTCGTGGGAGTCTCAT	537
			R: M13R-TTGAATCTCATCCCCAGGGTCTCA	
	3	Hs00395869_CE	F: M13F-ACACTGTCCTTCTCGAGCACC	549
			R: M13R-ACATCCTGATTCTTGGGAAAATTGG	
	4	Hs00293346_CE	F: M13F-CAGCAGTCCTATATCTACTGTCTGA	509
			R: M13R-CCCTGTCCTCACCCCACATTA	
	5	Hs00395867_CE	F: M13F-ACACCGTGGGTGTGATTAGC	549
			R: M13R-TCGGTGGGTGTTCAAGGAGGAA	
	6a	Hs00293344_CE	F: M13F-CCCTGCTTGAAGATCATGGGCTTTT	499
			R: M13R-CCTACCACTTGCCTGTTGGCTG	
	6b	Hs00485596_CE	F: M13F-ACTTAGCAACTTGGGACAGTGACA	502
			R: M13R-CAACAATACAGGCTAGGGATGGT	
	6c	Hs00293340_CE	F: M13F-ATCCCATACTTCCCATAAGAATCCT	504
			R: M13R-TGTAAAACCACCTTTTCTGCTTCAATA	
FCGR3B	2	Hs00748330_CE	F: M13F-GAACCCAAGGCATCTCAAACTTC	262
			R: M13R-GCGAGACTTCAGATTGAGAAATCAGA	
	3	Hs00293362_CE	F: M13F-ACCACTGTGTGGAATTGTCCTCA	513
			R: M13R-TGCCCCTACTCAATATTTGATTTACTC	
	4	Hs00681335_CE	F: M13F-GTGATTTTCCTCTTCCCCTTCATCA	262
			R: M13R-TGGAGCCTCAATGGTACAGC	
	5	Hs00293360_CE	F: M13F-CCCAACTCAACTTCCCAGTGTGAG	504
			R: M13R-TTCTCTTGCAGGGCCCCATCA	
	6a	Hs00395870_CE	F: M13F-GGATCTGGCTCTGAGTTCTATGTT	547
			R: M13R-TCAAACAAGCAGGAGCCTTTGCTA	
	6b	Hs00293358_CE	F: M13F-TTTCTTTTCCACCCCACCCCCA	503
			R: M13R-ACAAACACTTTTTCTGTCCCAACCA	
	6c	Hs00293356_CE	F: M13F-ATTCACCCTCCTATCCCATACTTC	501
			R: M13R-TCTGCTTCAATGTCTAGTTCCTGTA	

FCRL1	1	Hs00833476_CE	F: TCTGAGCACCCCTGAGCATTA	274
			R: CTGAAGGGTAACTTCCGATATCAACTTC	
	2	Hs00415221_CE	F: CACGTGTCAGGTGCAGAGACTGA	506
			R: TGTGTTGTTCTGTGGGCAGGTG	
	3	Hs00639803_CE	F: TTGCCTGAATGGAAACAGAGTGAA	250
			R: GGGTGGCCTGTGTGTCCAAC	
	4	Hs00289472_CE	F: ATGCCCCAGGGAAACTTTGTGT	510
			R: CCCACCTCACCCAAATTAAGCTATA	
	5	Hs00289480_CE	F: CACAGTGGCACCAGCAAAACTCT	508
			R: TCACAAACTGGGCTCCACATTTC	
	6	Hs00722189_CE	F: TTGCAGTATACATGTCACAATTTCAGC	274
			R: CACCCAAGATGTCTGCTCTCTAAG	
	7	Hs00608436_CE	F: TCAGGAGTGAGTTCTGGGTATAATAAAGC	266
			R: CACAATGGGCATCTGAGCATG	
	8, 9	Hs00422728_CE	F: CACAGTGATGCTCACAGCCTTCTG	586
			R: TGTCACAGGCTTCTCTCCTTCAGTG	
	10	Hs00809225_CE	F: GAAAGAAAGAACATGGAAGAAAGAACAATAGAG	246
			R: TCTGGCTACTAAGGGAATCACATTTG	
	11a	Hs00289469_CE	F: TTTTGGATGTGGTATAGGTTGGC	543
			R: GGGCATTAGCTTTCCAGTATACC	
	11b	Hs00485610_CE	F: CAGCAGGAGTGCCATAATATGC	517
			R: TTTTAAAATAACCAGGCACTCACTCT	
	11c	Hs00485611_CE	F: ATTCATAAGAGTGAGTGCCTGGTT	544
			R: GGATCATCGAGTTTCCACCTAAAA	
FCRL2	1	Hs00289465_CE	F: ATGCTTAATGGATGTGCTGCAAA	421
			R: TCTGGCATTGAGAGGATCAGCTATA	
	2	Hs00719562_CE	F: TGTCAGCCTAGAGGGCAGTAATAT	274
			R: AGGGAAATAGAAAAGAAAGGGAAAATGGA	
	3	Hs00445088_CE	F: CCTCTCACCCATCATGGCTCC	573

			R: CCCTCAGGAAACATGGGATGAA	
	4	Hs00289462_CE	F: AGGAGTTGAACAAAGAAACAGACTAG	509
			R: AAAGGAGCCATGATGGGTGAGA	
	5	Hs00289461_CE	F: CCTGAAATTTCCTCAGGACACAT	511
			R: CCCTACCTCCATAGGAATGCTTATA	
	6	Hs00289460_CE	F: CCTCTTCACACACAGCAGACATCTC	501
			R: CTGTTCTAGTGGGAATGTGGGAAT	
	7	Hs00811556_CE	F: TTCAATCTATATCAGTAGTGGAAAGGTCATAGC	246
			R: AGCATTTATAAGAAGGGCCCAAGTC	
	8	Hs00785145_CE	F: AATTACATAGCTTCAGTCTCAACGTACAA	246
			R: ACTCTTCTATATCTTATGTCTTACCAGCTGATT	
	9	Hs00636177_CE	F: GTGTCTACACCACCCACCTAATG	256
			R: CTCTCTCCTCAGCCAGTCTCC	
	10	Hs00751544_CE	F: ACAATGCATCACTTTCCTGTGAATATG	230
			R: GGTTGAGAGGAAGGATATAATCATTAAGCC	
	11a	Hs00719562_CE	F: ATGATGCCCCATCCTTGCTG	274
			R: TCTGGGAGTGAGGCCTAATTAAGA	
	11b	Hs00460019_CE	F: GGAAGGGCTCCTGCCTAACTCA	446
			R: GCTTGAGGCTTATCACCTGCCA	
	11c	Hs00460031_CE	F: TGCAATTTACCCATATAACACACCCA	472
			R: TCAACTTCAGCGCTTGATATTGGTC	
FCRL3	1, 2	Hs00289452_CE	F: GCAGAGATAGAAGCTCCTTGAGTT	505
			R: CCAAAGGCTGTGTGAAGACAAGGTA	
	3	Hs00681144_CE	F: TAGTAGCAGATGTGACCCCAGA	274
			R: GCTCTGGATGATATAAAAAGGGAGCA	
	4	Hs00452562_CE	F: GCATCATGCTGCTGCCCTATC	526
			R: CCACCCTGGTCCTGACTGGAA	
	5	Hs00289449_CE	F: ATTCCCACTGATAAACATCTTCCCTTT	500
			R: GTAGGGCAAGGAAGAATCAGGTA	

	6	Hs00285817_CE	F: TGTTATGTCCACCATCCTAGGG	495
			R: GATGGTTCCTTCTGCATGCTTCTC	
	7	Hs00285816_CE	F: CCGTGCCTCTTTTAGGAGACCTT	511
			R: TTCTTATTTCCTACTTTGGGCTTCTG	
	8	Hs00285815_CE	F: GGGAAGTCTATTGGGATATCAACCA	499
			R: GGAAACAGAAACAATCAAGGGACAA	
	9	Hs00285813_CE	F: GCATATGAAAGGTATTCTCCAGCAT	509
			R: AGTGAACTATTTTTCCTCCCTCAGT	
	10	Hs00649406_CE	F: TAGGGTGCACCAGACAACTTC	274
			R: ACACTCACCCTACTTGCATGATTTT	
	11	Hs00615818_CE	F: ATTGTGTTTAAGTAGAGACAGTGAATATCGTT	274
			R: ATTTACACAGCTCACTTACCACCAA	
	12, 13	Hs00285809_CE	F: ATAACTCTGATCAAAGACCCCTTGC	496
			R: CTCTCCCCCTCTGTCACAGTCA	
	14, 15	Hs00285807_CE	F: TAACACAGTGCTTGCTCAGAGGCT	499
			R: TTTGCTACTCCTACCCAACAGCTA	
FCRL4	1	Hs00781931_CE	F: TCTTTCCTTTTTGTTCATTTTCTGCTGTT	274
			R: TTACTGAGCTCACTGCTGGTG	
	2	Hs00643885_CE	F: CTACATCCCTGCAGCACTCAG	274
			R: GGATGGATGGAATGGAACGTGT	
	3	Hs00285803_CE	F: GGAATGAAAGACCCCAGTTCGT	494
			R: AGCTCACCTGCAACCTGGGTAA	
	4	Hs00285802_CE	F: CCCGTAAATTCTTTCCTTACATGCT	500
			R: GTACCTGAAGCATGTTACACTGAT	
	5	Hs00285801_CE	F: CTGCTACATAGTCCCCATGCCTACA	501
			R: AAGAAAACTGTGATAGAGGGCTTGA	
	6	Hs00467423_CE	F: TTATCACAGTGGAGCATGGCAAA	549
			R: TGGCCACAATGCTAGTGCCTG	
	7	Hs00834449_CE	F: ACTAAGGCTGTCTGCATGCTG	268

			R: GTTACCCAACCAAGCTGAGGAA	
	8	Hs00815282_CE	F: GTGAAAATGGAAGTATCTAGCCACAGG	274
			R: GACTGGGCAGGTGTTTGAATTG	
	9	Hs00786008_CE	F: CATTTCCCAGGGCTGAAAGC	256
			R: TCCAAATCCTCTTTCTCCTCTGC	
	10	Hs00641052_CE	F: AATGAATATAGTTTGCAGCCAGAATCTCT	240
			R: AAATCCAGATATTCCCAGATAATTGTTCCATT	
	11	Hs00750668_CE	F: CATGAGTAGGACGTTCTCGTAACTTTT	274
			R: CCCATAGTCCAGTGATTCTCTTTCTG	
	12	Hs00285792_CE	F: GACAGTGTAAGCTGTGCAGGC	509
			R: GAGAACGTCCTACTCATGTGATTTC	
	12	Hs00285791_CE	F: AGTAACTCAAAGACACAGTTCCTACA	524
			R: GAGACTCACTTGCATCAGTTTGTT	
FCRL5	1	Hs00285778_CE	F: AATTGTTGTGGTTATTGTGGTTTCC	501
			R: TTTTTTTCTTTGATCATCGGGTAGGA	
	2	Hs00692461_CE	F: AAGAAAACAGGAGATATTGGAGATCTCAG	240
			R: TTTTTAAAGGGACAAGTAATACATGCATGG	
	3	Hs00285770_CE	F: AATTGTCCACTCATGAAGGGTCT	495
			R: TGAGCTATCAGTGACCCTCCCA	
	4	Hs00285787_CE	F: GGAATGTGTCCTGTGGTTTAGTG	483
			R: TAAGAGTTCTTTTTCCCAACCCATTT	
	5	Hs00467331_CE	F: TGCACAACTCCCATTCTCACAGG	588
			R: GGACACATTCCTGCAAGGAGCA	
	6	Hs00285785_CE	F: GGATACTGGAAGGTATATGCTGACT	504
			R: AGGTAAGGGTGTGTGGTTCTGATG	
	7	Hs00285784_CE	F: AGTGGAGGAGGATATTAGGTTGTTT	468
			R: ATGAAAACTACAGCAGGAGGCAGAA	
	8	Hs00485613_CE	F: CTAGTTAGTTGAGGAGGAAGCCAAGAA	510
			R: TGTACTGGAGACTGGGCATCTGC	

	9	Hs00395844_CE	F: TCACACAGCAAATGACTCTAGAAAC	409
			R: TGTCCCTAACTCATTTTCTCTCTCA	
	10	Hs00285781_CE	F: GCTCTGTGGCTCCATTTTCAGC	511
			R: TCTGGAGATCTGAAAATATTGCTGAGT	
	11	Hs00285777_CE	F: CCCAACGCCATCACTTCAGAA	497
			R: CAAATATGTTAGAAGCAGAAACTGCTG	
	12	Hs00285779_CE	F: GATGAGCTCTTGCGAGCCTGG	493
			R: GCAAGAGCGCCACTGACTTTAGA	
	13	Hs00788156_CE	F: GTACAACAGGTAGTGGCCACA	274
			R: CCTTCAGTGACAGCAACTCCT	
	14, 15	Hs00285776_CE	F: CTGTCTGGCTCAGATCTGCTGA	496
			R: AGCTACTTTGTCTGGATTCCTAAGA	
	16	Hs00720411_CE	F: GGCTGAAACAGCAGTTGGAG	262
			R: TCCACAACTGAACACTTTCCTCTTT	
	17a	Hs00285773_CE	F: ATCCACCCAAAGCAGGAACTCT	506
			R: TACTCTGAAGTTAAGGTGGCGTCAA	
	17b	Hs00285772_CE	F: ATGCCAGTTTGATTACCCACAGT	505
			R: GGGTCTTACCACACAAAGGGAGAGT	
	17c	Hs00285771_CE	F: CTGGTGGTGCTGTGGCCAAA	498
			R: GGGTGCAGACAGAACACATACTG	
	17d	Hs00285770_CE	F: CCCTGTCTATCTGGGCAAGGC	495
			R: TAGGTCTCAGCAGAGGCCCATG	
	17e	Hs00285769_CE	F: GTGGAGTTTGTTCTGCCACTGT	515
			R: CCACGGCCCTGAAATAAAATCCT	
	17f	Hs00415246_CE	F: TGTGTACTCACATGCACCCATGA	599
			R: CAGCAGCAAGCATAGGCTCAGG	
FCRL6	2	Hs00643658_CE	F: CTATAGCACCACCCAGCTCAG	274
			R: CTTGAGCTCCAGCCACAAAC	
	3	Hs00790454_CE	F: ATGGGTGTAAGTCCCCATTGC	236

			R: CTAGTCTGGTGCTGCTCTGC	
	4	Hs00289751_CE	F: ATCCCCAGCAGGAGGCCTTA	489
			R: CCCACACATTGGCCCAGCAT	
	5	Hs00289752_CE	F: GGATGAAACTGCCTCCCATCGG	501
			R: GACCCTTCCCCCAACTCCTTTAGT	
	6	Hs00289753_CE	F: GGATCCCCTGGGACTAAAGGAGTT	495
			R: CTGCCATGAGTCCAGCACTGTG	
	7	Hs00676677_CE	F: CAGGCTAGAATGCATTTCCGAATTTT	274
			R: CCTGTCAGACTCTGGCTTTCC	
	8	Hs00697194_CE	F: AGCGGCCTCCTTGTCATTC	262
			R: TCCTGAGATACAGGGCTCTGG	
	9	Hs00722063_CE	F: TATCCCCACATTAGTGACCAACC	274
			R: TTGTAGGTCCAAAACTTCTGTTTGC	
	10, 11	Hs00289757_CE	F: TAGAGGAAGATAGCCAGCCAGGA	511
			R: GGTTGGTCTTCTGCTAAGAAAAGAA	
FCRLA	1	Hs00293376_CE	F: TTCAAACAACATCCCACCTTCTGT	501
			R: AAAGCTCCATTCCCACACCAATAT	
	2, 3	Hs00293377_CE	F: AATCTGATGTCTCCCTTTCATTCCT	506
			R: GGTGGAAGGGTTCACTGAAAGT	
	4	Hs00293379_CE	F: AGAGTTTACCTGGACCCTCAGAGAA	498
			R: ATTTGCCAGCAACCTGCTCCATA	
	5	Hs00293378_CE	F: AGTATGACTCCCCAAGTCTCTTC	496
			R: CCCCTCTGTGTCCCTTCTTAACC	
	6a	Hs00293376_CE	F: GAACAAGAAAGATTGGCCAGGGAT	501
			R: TCTAACAAAACACTGGGACAACTT	
	6b	Hs00293383_CE	F: AGTTGTCCCAGTGTTTTGTTAGAAT	544
			R: CCAGCTAAAGACTGGTATGTCAGTA	
	6c	Hs00293384_CE	F: ATGAAACCCTAGGTGTTGGGCTCT	511
			R: CATAAACTCAGTCATGTGGCAGAGG	

FCRLB	3, 4	Hs00293385_CE	F: CATCCACTGGCCACATTCTTTCTC	508
			R: AGTGAGGAGTCTACTTGAAAAGGT	
	5	Hs00293387_CE	F: CTAAGATTTCCCAAACTCAGGTCGT	496
			R: GTCCCTACCTGCTCCAGAGACT	
	6	Hs00293388_CE	F: AGAAAGCTGGAAGGCTGTGAGAGT	500
			R: AAAGGAGAGGAGACAGCGTCCATAG	
	7	Hs00293389_CE	F: GTCCCTCTTCCTTTCAAGCTTTC	491
			R: CAGCCACGGACTGCGTTTC	
	8a	Hs00639180_CE	F: GCGTGACTGGGCGTAATG	256
			R: CCAGCCGATTGTTCCAAGG	
	8b	Hs00812817_CE	F: CCGAGAAATGCAGCTGCTC	236
			R: GGCTTTGAAAGAAATTCGCGTGA	
	8c	Hs00293393_CE	TGCAGGCTCATTCCTCCTTGGT	495
			R: GGAGGCCCACACAAACAACTTTC	

Assay ID is the catalog ID of Thermo Fisher Scientific commercial products (https://www.thermofisher.com/kr/ko/home.html).

M13F= TGTAAAACGACGGCCAGT/ M13R= CAGGAAACAGCTATGACC

F, forward primer sequence; R, reverse primer sequence

RESULTS

1. Discovery of 20 nsSNPs in 15 Fc receptor genes by capillary sequencing using Thermo Fisher Scientific primer sets

A total of 136 primer sets covering coding regions of the 15 human Fc receptor genes were purchased from Thermo Fisher Scientific and tested for PCR amplification using 2 DNA samples. The PCR amplification condition for Fc receptor genes is as follows: one cycle of 5 minutes at 94 ℃, thereafter 35 cycles of 45 seconds at 94 ℃, 30 seconds at 55 ℃, 1 minute 30 seconds at 72 ℃ and final extension step for 10 minutes at 72 ℃. All primer sets were successfully PCR-amplified, except 3 primer sets (primers for exon 3 of *FCGR1A*, exon 4 of *FCGR2A*, and exon 7 of *FCRLB*) (Figure 2).

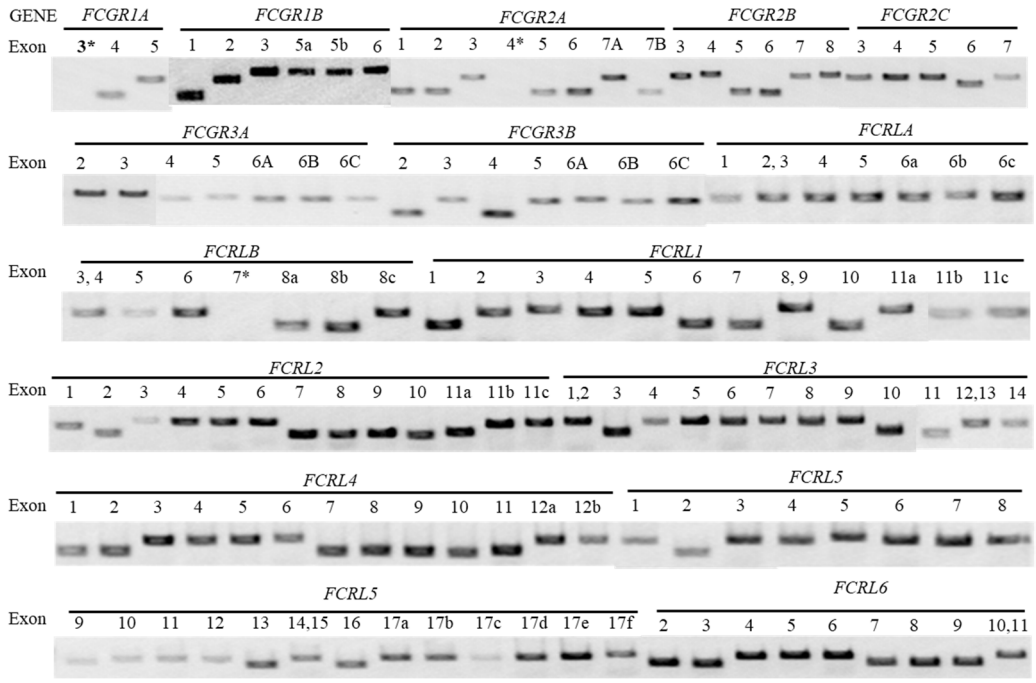

Figure 2. Agarose gel electrophoresis of PCR-amplified products. Primers for exon 3 of *FCGR1A*, exon 4 of *FCGR2A*, and exon 7 of *FCRLB* did not work.

By capillary sequencing, a total of 20 nsSNPs were found in the coding regions of the 15 Fc receptor genes using 50 or 98 DNA samples collected from KD patients (Table 8). The functional effects of discovered coding variants were predicted by PolyPhen and SIFT programs. After excluding five very rare variants having MAF < 0.01 and quality control threshold (HWE *P*-value < 10^{-3}, and missing rate), a total of 15 nsSNPs were selected for next pilot association test.

Table 8. List of 20 nsSNPs in the human Fc receptor genes identified by capillary sequencing using DNA samples of KD cases (n = 50 or 98).

Gene	SNP	Allele A1/A2	Amino Acid change	Polyphen / SIFT	No. of case	Genotype (11/12/22)	No. of missing	MAF	MAF in East Asian	primer set	TaqMan probe availability
Candidate nsSNPs for validation:											
FCGR2A	rs199502630	A / C	A224E	- / -	98	0/3/95	0	0.02	0.0002	Hs00755658_CE	Yes
FCGR2C	rs138747765	T / C	T118I	PrD / D	98	2/31/64	1	0.18	0.1691	Hs00293349_CE	No
FCGR3A	rs396991	G / T	F176V	- / -	98	4/41/51	2	0.26	0.3273	Hs00395867_CE	Yes
FCRL3	rs7522061	C / T	N28D	- / -	98	16/51/30	1	0.43	0.4048	Hs00452562_CE	Yes
	rs79895668	C / A	V93G	PoD / D	98	0/11/87	0	0.06	0.0916	Hs00452562_CE	Yes
	rs2282284	C / T	N721S	- / -	98	3/9/86	0	0.07	0.0600	Hs00285807_CE	Yes
FCRL4	rs80105432	T / C	R78Q	- / -	98	1/11/86	0	0.07	0.0301	Hs00285803_CE	Yes
FCRL5	rs6679793	G / A	Y267H	- / -	98	1/20/75	2	0.11	0.0970	Hs00467331_CE	Yes
	rs12036228	T / C	V269I	- / -	98	1/8/88	1	0.05	0.0819	Hs00467331_CE	Yes
	rs2012199	C / T	G418V	- / -	98	1/22/74	1	0.12	0.1284	Hs00285784_CE	Yes
	rs6427384	C / T	V466L	- / -	98	6/33/58	1	0.23	0.2228	Hs00285784_CE	Yes
	rs73011568	G / C	R551P	- / -	98	1/19/77	1	0.11	0.0946	Hs00485613_CE	No
	rs199706458	G / A	S798P	- / -	98	0/2/96	0	0.01	0.0046	Hs00285777_CE	Yes
FCRL6	rs4301626	C / T	X414R	na / na	98	8/24/66	0	0.20	0.1941	Hs00289757_CE	Yes

FCRLA	rs11746	A / G	R372Q	- / -	98	6/31/59	2	0.22	0.2600	Hs00293376_CE	Yes
Excluded nsSNPs for validation due to low frequency (MAF ≤0.01):											
FCRL3	rs115596233	A / G	R293Q	- / -	50	0/1/49	0	0.01	0.0030	Hs00285816_CE	Yes
	rs12041673	T / C	L307F	PrD / D	50	0/1/49	0	0.01	0.0123	Hs00285816_CE	Yes
	rs181476479	T / G	G604X	na / na	50	0/1/49	0	0.01	0.0001	Hs00649406_CE	Yes
FCRL4	rs148814087	T / C	R88Q	- / -	50	0/1/49	0	0.01	0.0051	Hs00285803_CE	Yes
	rs750459940	T / C	T293I	na / na	50	0/1/49	0	0.01	0	Hs00467423_CE	No

Benign and tolerated prediction of coding variants by PolyPhen or SIFT are shown in '-'.

Amino acid is presented in a single letter code.

A1, reference allele; A2, alternative allele; AF; allele frequency; D, deleterious; MAF, minor allele frequency; na, not available; nSNP, nonsynonymous SNP; OR, odds ratio; PoD, possibly damaging; PrD, probably damaging; SNP, single nucleotide polymorphism.

2. Selection of seven candidate nsSNPs found in the human Fc receptor genes for replication study

We pre-screened the selected 15 candidate nsSNPs by performing genetic association analysis with 98 KD samples and 96 control samples (Table 9). Eight out of 15 were excluded for the large scale test based on the 1) MAF < 0.01 in case, 2) odds ratio (OR) ≤ 1.5, and 3) *P*-value > 0.2 obtained from association study using discovery sample set. So, finally seven candidate nsSNPs in Fc receptor genes were selected for large scale case-control test using TaqMan^{TM} SNP genotyping.

Table 9. Genetic association result of 15 identified SNPs using 98 cases and 96 controls.

Gene	SNP	Allele 1/2	Amino Acid Change	Polyphen / SIFT	Genotype (11-12-22)		MAF		Association		Call rate		HWEP	
					case	control	case	control	OR	*P*	case	control	case	control
Candidate nsSNPs for replication study														
FCGR2A	rs199502630	A / C	A224E	- / -	0/3/95	0/0/96	0.0153	0	na	0.0853	1	1	1	1
FCGR3A	rs396991*	G / T	F176V	- / -	4/41/52	2/30/64	0.2552	0.1771	1.59	0.0629	0.98	1	0.2914	0.7281
FCRL3	rs7522061*	C / T	N28D	- / -	16/51/30	1/45/401	0.4278	0.3490	1.40	0.1119	0.99	1	0.5379	0.8257
FCRL4	rs80105432	T / C	R78Q	- / -	1/11/86	1/18/77	0.0663	0.1042	0.61	0.1816	1	1	0.3465	1
FCRL5	rs12036228	T / C	V269I	- / -	1/8/88	0/18/76	0.0516	0.0957	0.51	0.0975	0.99	0.98	0.2165	1
FCRL6	rs4301626	C /T	X414R	na / na	8/24/66	15/25/54	0.2041	0.2926	0.62	0.0446	1	0.98	0.0242	0.0009
FCRLA	rs11746	A /G	R372Q	- / -	6/31/59	6/42/47	0.2240	0.2842	0.73	0.1761	0.98	0.99	0.5548	0.6127
Excluded nsSNPs for replication study due to MAF < 0.01, OR<1.5 or P-value > 0.2														
FCGR2C	rs138747765	T / C	T118I	PrD / D	2/31/64	1/32/63	0.1804	0.1771	1.02	0.9320	0.99	1	0.7296	0.2896
FCRL3	rs79895668	C / A	V93G	PoD / D	0/11/87	0/12/83	0.0561	0.0632	0.88	0.7703	1	0.99	1	1
	rs2282284*	C / T	N721S	- / -	3/9/86	1/9/83	0.0765	0.0591	1.32	0.5000	1	0.97	0.0090	0.2692
FCRL5	rs6679793	A / G	Y267H	- / -	1/20/75	4/13/78	0.1146	0.1105	1.04	0.9002	0.98	0.99	1	0.0124

rs2012199	C/ T	G418V	- / -	1/22/74	2/24/67	0.1237	0.1505	0.80	0.4469	0.99	0.97	1	1
rs6427384*	C /T	V466L	PoD / -	6/33/58	6/33/53	0.2320	0.2446	0.93	0.7736	0.99	0.96	0.5816	0.7794
rs73011568	G / C	R551P	- / -	1/19/77	3/13/78	0.1082	0.1011	1.08	0.8187	0.99	0.98	1	0.0459
rs199706458	G /A	S798P	- / D	0/2/96	0/0/92	0.0102	0	na	0.1695	1	0.96	1	1

Benign or tolerated prediction of coding variants by PolyPhen or SIFT are shown in '-'.

*rs396991, risk allele of SLE; rs7522061, risk allele of AR; rs2282284, risk allele of MS; rs6427384, risk allele of AS.

A1, reference allele; A2, altered allele; D, deleterious; MAF, minor allele frequency; na, not available; OR, odds ratio; P, P-value; HWEP, Hardy-Weinberg Equilibrium *P*-value; PoD, possibly damaging; PrD, probably damaging.

3. Association study of the selected seven candidate nsSNPs with KD using TaqMan™ SNP genotyping

Though the discovery phases, seven candidate nsSNPs found in Fc receptor genes were selected (Table 9). All TaqMan probes were working properly except one TaqMan probe (rs199502630 in *FCGR2A)*. Of tested six nsSNPs, one SNP (rs396991) in the *FCGR3A* showed a marginal significant association with KD susceptibility at replication stage (P = 0.0254) (Table 10). However, in the combined analysis of discovery data and replication data, we did not find any significant association between coding variants of immunoglobulin Fc receptor genes and KD susceptibility (Table 10). Additionally, we performed subgroup association study between IVIG non-responders and IVIG responders for six candidate nsSNPs. However, we could not find any significance with IVIG responsiveness and candidate nsSNPs found in Fc receptor genes (Table 11).

Table 10. Summary association results for six candidate nsSNPs in the replication study by TaqMan^{TM} SNP genotyping using 569 KD cases and 570 controls

Gene	SNP	Allele 1 / 2	Amino acid change	Polyphen/ SIFT	Discovery			Replication			Combined		
					MAF (case/control)	OR	*P*	MAF (case/control)	OR	*P*	MAF (case/control)	OR	*P*
FCGR3A	rs396991	G / T	F176V	-/-	0.2526/0.1771	1.57	0.0711	0.2346/0.2754	0.81	**0.0254**	0.2372/0.2613	0.88	0.1518
FCRL3	rs7522061	C / T	N28D	-/-	0.4278/0.3490	1.40	0.1119	0.3849/0.4246	0.85	0.0537	0.3913/0.4137	0.91	0.2392
FCRL4	rs80105432	T / C	R78Q	-/-	0.0663/0.1042	0.61	0.1816	0.058/0.0597	0.97	0.8669	0.0592/0.0667	0.89	0.4658
FCRL5	rs12036228	T / C	V269I	-/-	0.0516/0.0957	0.51	0.0975	0.0861/0.118	0.8318	0.2008	0.081/0.1009	0.78	0.0735
FCRL6	rs4301626	C / T	X414R	na/na	0.2041/0.2926	0.62	**0.0446**	0.2223/0.2113	1.07	0.5225	0.2196/0.2228	0.98	0.8440
FCRLA	rs11746	A / G	R372Q	-/-	0.2240/0.2842	0.73	0.1761	0.2496/0.2667	0.92	0.3509	0.2459/0.2692	0.89	0.1692

Allele1 is minor allele.

Amino acid is presented in single letter code. Significant *P*-values are shown in bold.

Benign and tolerated prediction by PolyPhen and SIFT are shown in '-'.

MAF; minor allele frequency; na, not available; OR, odds ratio; P, P-value

Table 11. Association results between IVIG non-responders and IVIG responders for six candidate nsSNPs

Gene	SNP	Allele 1 / 2	Amino acid change	Polyphen/ SIFT	Discovery			Replication			Combined		
					MAF (case/control)	OR	*P*	MAF (case/control)	OR	*P*	MAF (case/control)	OR	*P*
FCGR3A	rs396991	G / T	F176V	-/-	0.1538/0.2712	0.73	0.3627	0.2329/0.2347	0.99	0.9614	0.2277/0.2391	0.94	0.7154
FCRL3	rs7522061	C / T	N28D	-/-	0.4744/0.3966	1.37	0.2828	0.3836/0.3867	0.99	0.9414	0.4152/0.3878	1.12	0.444
FCRL4	rs80105432	T / C	R78Q	-/-	0.0385/0.0848	0.43	0.2025	0.0343/0.0602	0.55	0.2063	0.0357/0.0628	0.55	0.1141
FCRL5	rs12036228	T / C	V269I	-/-	0.0256/0.0678	0.36	0.1892	0.089/0.0847	1.06	0.8607	0.0670/0.0829	0.79	0.4241
FCRL6	rs4301626	C / T	X414R	na/na	0.2308/0.1864	1.31	0.451	0.2123/0.2255	0.93	0.7215	0.2188/0.2213	0.99	0.9329
FCRLA	rs11746	A / G	R372Q	-/-	0.1538/0.2712	0.49	0.053	0.2192/0.2531	0.83	0.3769	0.1964/0.255	0.71	0.0632

Allele1 is minor allele.

Amino acid is presented in single letter code. Significant *P*-values are shown in bold.

Benign and tolerated prediction by PolyPhen and SIFT are shown in '-'.

MAF; minor allele frequency; na, not available; OR, odds ratio; P, P-value

DISCUSSION

The cause of KD is still unknown. Possible causes proposed for KD are infection, autoimmune disease, and genetic susceptibility. Many studies have evaluated the coding H131R polymorphism in *FCGR2A* gene with the risk for the several human immune diseases (Nimmerjahn F, 2007; Asano M, 2011). Recently, a few studies reported the possible association between H131R polymorphism in *FCGR2A* gene and KD (Khor CC, 2011; Kim JJ, 2017). So, in this study, we tested the hypothesis that immunoglobulin Fc receptors genes are associated with the susceptibility to KD. We initially tried to identify all coding variants of 15 human Fc receptor genes by capillary sequencing with 50 DNA samples of KD cases. Due to high homology between Fc receptor genes, we used 136 pre-made primer sets covering the coding regions of 15 Fc receptor genes from Thermo Fisher Scientific. To distinguish the target sequence from homologous gene sequences, individual nsSNP was examined with HWE-test and sequence was confirmed by BLAST alignment search tool. All 136 primer sets were successfully polymerase chain reaction (PCR)-amplified, except 3 primer sets (primers for exon 3 of *FCGR1A*, exon 4 of *FCGR2A*, and exon 7 of *FCRLB)* (Figure 2), indicating that the quality of primer sets provided by the commercial company are good. In this study, we found a total of 20 nsSNPs in Fc receptor genes and tested six common nsSNPs for genetic association with KD in large case-control samples. However, we could not find any significance with KD, suggesting that Fc receptor genes are not associated with KD except previously confirmed H167R polymorphism of *FCGR2A* gene.

A potential limitation of this study is that we used pre-made primer sets, we could only test with exons covered by these primer sets. All exons of the all Fc receptor genes were not covered in this study. Because of the high homology sequence in Fc receptor genes, the exact target sequences are not analyzed even if all coding sequences are not amplified or amplified by PCR. Also, it was difficult to confirm that the tested genes were related to KD susceptibility because the genetic association study for rare coding variants was not conducted. Lastly, although the sample size might be small and the impact of genetic statistics is weak, but increasing the sample size does not guarantee for higher significance.

In conclusion, the coding variants of Fc receptor genes are not associated with KD, suggesting that Fc receptor genes may not play a crucial role in the pathogenesis of KD except previously confirmed *FCGR2A* gene.

CHAPTER IV

ASSOCIATION STUDY OF THE NONSYNONYMOUS SNPs FOUND IN THE IMMUNOGLOBULIN G GLYCOSYLATION GENES WITH IVIG RESISTANCE IN KD

ABSTRACT

KD is the leading cause of pediatric acquired heart disease in the Korean children. A single high dose of IVIG is the standard treatment for KD and it results in resolution of fever in most KD patients and reduces the rate of CAA. Despite of IVIG treatment, 10-20% of children will show persistent fever after their first infusion of IVIG. These patients have an increased risk of developing coronary artery abnormalities. Identification of patients with IVIG-resistant would help the use of additional therapies for prevention of coronary artery damage. The glycosylation of immunoglobulin play an important role in the immune diseases and the immunoglobulin therapy. To search genetic variants influencing IVIG-resistance in KD, we initially selected candidate nsSNPs in immunoglobulin glycosylation genes from unpublished our WES data with 94 IVIG non-responders and 106 IVIG responders. Through the WES association study, we selected seven candidate nsSNPs in immunoglobulin G glycosylation genes among known nine genes associated with immunoglobulin G glycosylation. For validation, another independent sample set with a total of 191 IVIG non-responders and 374 IVIG responders were used for association study. However, there was no significant association for seven candidate nsSNPs in the immunoglobulin glycosylation genes. The results may suggest that immunoglobulin G glycosylation genes do not play a role for the IVIG resistance in KD.

INTRODUCTION

KD is an acute, self-limited vasculitis of unknown etiology that especially affects infants and children. Standard treatment for KD is 2g/kg of IVIG and aspirin (Newburger JW, 1991). This regimen has shown effective prevention of developing of CAA, the most severe complication of KD, occurring in about 25% of untreated KD patients. The mechanism of IVIG is still unclear. Recently, several studies have reported a crucial role for IgG glycosylation and IgG glycoform-specific Fc gamma receptors for the efficacy of IVIG treatment (Lauc G, 2013; Wahl A, 2018). The capacity of IVIG to suppress inflammatory response was lost in the absence of IgG glycosylation or sialylation, respectively (Kaneko Y, 2006). Glycosylation of the antibodies, especially in the Fc region of IgG, has been extensively studied in disease (Daeron M, 2014). The N-glycans in the Fc regions have been known to be critical for maintaining structure, interacting with the Fc receptor and the downstream immunological response (Wahl A, 2018). Although the most of patients respond to single dose of IVIG with diminishing of fever and improvement in clinical symptoms and laboratory inflammatory markers, 10-20% of children have shown IVIG resistance. The identification of genetic markers that determine the IVIG resistance in KD would allow to prevent the increased risk of coronary complications. So, in this study, we sought to investigate the genetic association of immunoglobulin glycosylation genes for affecting the resistance of IVIG treatment in KD.

SUBJECTS AND METHODS

Subjects

KD patients were recruited from 12 hospitals in Korea that are currently participating in the Korean Kawasaki Disease Genetics Consortium from June 2008 through February 2019 as described in Subjects and Methods of Chapter III. Briefly, all KD patients were diagnosed by pediatricians according to the diagnostic criteria of the American Heart Association (Newburger JW, 2004). The study protocol was approved by the Institutional Review Board of the involved institutions, and written informed consent was obtained from the parents of all KD patients.

Selection of candidate nsSNPs found in immunoglobulin glycosylation genes of our unpublished WES data for association study with IVIG resistance in KD.

To test the possible association of immunoglobulin glycosylation genes with IVIG resistance in KD, we initially searched candidate nsSNPs using previous our unpublished WES data generated with 200 KD cases (94 IVIG non-responders and 106 IVIG responders). From the association test of WES data, seven candidate nsSNPs in immunoglobulin glycosylation genes were selected for validation

TaqMan™ SNP genotyping

To test seven candidate nsSNPs selected in immunoglobulin glycosylation genes from WES data, we used a custom-made TaqMan™ probe sets or pre-designed TaqMan™ probe sets (Applied Biosystems, Foster City, CA, USA). TaqMan™ genotyping assays were performed according to the standard protocol on a 7900HT Real-Time PCR System using Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analyses were performed using the PLINK (version 1.07) (http://pngu.mgh.harvard.edu/~purcell/plink/). The functional effects of candidate variants were predicted by Polyphen and SIFT programs. HaploReg v4.1 program was searched for

exploring annotations of the associated SNPs (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php).

RESULT

Association study of nsSNPs in immunoglobulin glycosylation genes with IVIG resistance in KD

We selected candidate nsSNPs in the immunoglobulin glycosylation genes using our unpublished WES data (94 IVIG non-responder KD cases and 106 IVIG responder KD cases) for association with IVIG resistance in KD. Among the known 9 genes/loci associated with immunoglobulin G glycosylation previously identified by GWAS (Lauc G, 2013), seven candidate nsSNPs in 6 immunoglobulin glycosylation genes were selected (Table 12) and tested using TaqMan^{TM} SNP genotyping in 191 IVIG non-responder KD cases versus 374 IVIG responder KD cases. However, all of nsSNPs did not show any significant association with IVIG resistance in KD (Table 13).

Table 12. List of candidate nsSNPs selected from immunoglobulin glycosylation genes from our unpublished WES data

Gene	SNP	Risk Allele	Amino acid change	Polyphen/ SIFT	Genotype		MAF			OR	*P*
					case	control	case	control	East-Asian		
DERL3	rs148001527	A	R138L	- / -	0/1/93	0/0/106	0.0053	0	0.0023	na	0.2877
FUT8	rs78083604	G	L153V	- / D	0/6/88	0/2/104	0.0319	0.0094	0.0078	3.46	0.1090
ST6GAL1	rs776908493	G	I23M	na / na	0/1/93	0/0/106	0.0053	0	0.0009	na	0.2877
B4GALT1	rs145978864	T	V173I	- / -	0/1/93	0/0/106	0.0053	0	0.0002	na	0.2877
	rs774489888	C	N160D	na / na	0/1/93	0/0/106	0.0053	0	0.0004	na	0.2877
B4GALT4	rs3764779	G	Q116E	- / -	9/42/43	21/54/31	0.3191	0.4528	0.3507	0.57	**0.0062**
ANKRD55	rs201139565	C	D39H	PoD / D	0/1/93	0/4/102	0.0053	0.0189	0.0073	0.28	0.2235

Amino acid is represented in a single letter code. Benign and tolerated prediction using PolyPhen and SIFT are shown in '-'.

Allele frequency data of East Asian were extracted from gnomAD browser database (https://gnomad.broadinstitute.org/).

D, deleterious; MAF, minor allele frequency; na, not available; OR, odds ratio; P, P-value; PoD, possibly damaging; PrD, probably damaging

Table 13. Summary association results for seven candidate nsSNPs in the replication study by TaqMan^{TM} SNP genotyping using 191 IVIG non-responders and 374 IVIG responders

Gene	SNP	Allele 1/2	Amino acid change	Discovery				Replication				Combined			
				Genotype		OR	*P*	Genotype		OR	*P*	Genotype		OR	*P*
				case	control			case	control			case	control		
DERL3	rs148001527	A / C	R138L	0/1/93	0/0/106	na	0.2877	0/3/187	2/9/361	0.45	0.6436	0/4/280	2/9/467	0.51	0.2387
FUT8	rs78083604	G / C	L153V	0/6/88	0/2/104	3.46	0.1090	0/7/184	0/24/349	0.56	0.1782	0/13/272	0/26/453	0.84	0.6035
ST6GAL1	rs776908493	G / C	I23M	0/1/93	0/0/106	na	0.2877	0/1/190	0/0/366	**na**	0.1661	0/2/283	0/0/472	na	0.0686
B4GALT1	rs145978864	T / C	V173I	0/1/93	0/0/106	na	0.2877	0/1/190	0/0/374	**na**	0.1615	0/2/283	0/0/480	na	0.0663
	rs774489888	C / T	N160D	0/1/93	0/0/106	na	0.2877	0/0/191	0/1/364	0	0.4692	0/1/284	0/1/470	1.65	0.7194
B4GALT4	rs3764779	C / G	Q116E	9/42/43	21/54/31	0.57	**0.0062**	29/78/84	40/176/156	1.05	0.6906	38/120/127	61/230/187	0.90	0.3377
ANKRD55	rs201139565	G / C	D39H	0/1/93	0/4/102	0.28	0.2235	0/2/189	0/7/366	0.56	0.4587	0/3/282	0/11/468	0.46	0.2172

Allele1 is minor allele.

Amino acid presented in single letter code..

D, deleterious; MAF, minor allele frequency; na, not available; OR, odds ratio; P, P-value

DISCUSSION

We evaluated the hypothesis that the polymorphisms in immunoglobulin glycosylation genes are associated with the IVIG resistance in KD. Initially, we identified seven candidate nsSNPs in immunoglobulin glycosylation genes associated with IVIG resistance in KD from WES data with 94 IVIG non-responders and 106 IVIG responders. In replication phase using 191 IVIG non-responders and 374 IVIG responders and combined association, we did not find any significant association between genetic variants in IgG glycosylation genes and IVIG resistance in KD patients.

A coding variant in *B4GALT4* (rs3764779, Q116E; OR = 0.57, $P = 0.0062$) showed significance in discovery phase. Other candidate variants showed no significance in discovery phase, but they were selected because they are involved in immunoglobulin glycosylation. Although the genetic variant in *ST6GAL1I* (rs776908493) and *B4GALT1* (rs145978864) did not show significance, the polymorphisms in these genes having very low frequency showed only in IVIG non-responders in discovery and replication study, respectively. A genetic variant in *ST6GAL1* are associated with autoimmune and inflammatory conditions, such as, atherosclerotic coronary artery disease in adults (Lauc G, 2013; Ogata S, 2013). *B4GALT1* which codes the galactosyltransferase is responsible for the addition of galactose to IgG glycans (Lauc G, 2013). Also, SNPs at loci on chromosomes 9 (*B4GALT1* region) and 3 (*ST6GAL1* region) are known that both influenced the percentage of sialylation of galactosylated fucosylated structures in the same direction (Lauc G, 2013).

The limitation of this study is that we focused on coding nsSNPs with low minor allele frequencies in the IgG glycosylation genes, leading to weak statistical power. In summary, we tested seven candidate nsSNPs in immunoglobulin glycosylation genes for association of IVIG resistance in KD. We found no significant differences in candidate genetic variant of IgG glycosylation levels between IVIG non-responders and responders. Therefore, we it is likely that glycosylation of IgG does not appear to play a major role in response to IVIG treatment in KD.

CHAPTER V

ASSOCIATION STUDY OF THE NONSYNONYMOUS SNPs FOUND IN THE IMMUNE-RELATED GENES WITH IVIG RESISTANCE IN KD

ABSTRACT

KD is an acute, self-limited vasculitis, mainly affecting children younger than 5 years old, with accompanying fever and signs of mucocutaneous inflammation. IVIG is the standard treatment for KD; however, approximately 15% of patients are resistant to IVIG treatment. To identify protein coding genetic variants influencing IVIG resistance, we re-analyzed our previous GWAS data from 296 patients with KD, including 101 IVIG non-responders and 195 IVIG responders. Five nsSNPs in five immune-related genes, including a previously reported *SAMD9L* nsSNP (rs10488532; p.Val266Ile), were significantly associated with IVIG non-response (OR = 1.89–3.46, $P = 0.0109–0.0035$). In a replication study of the four newly-identified nsSNPs, only one in the interleukin 16 (*IL16*) gene (rs11556218, p.Asn1147Lys) showed a trend of association with IVIG non-response (OR = 1.54, $P = 0.0078$). The same *IL16* nsSNP was more significantly associated with IVIG non-response in combined analysis of all data (OR = 1.64, $P = 1.25 \times 10^{-4}$). Furthermore, risk allele combination of the *IL16* CT and *SAMD9L* TT nsSNP genotypes exhibited a very strong effect size (OR = 9.19, $P = 3.63 \times 10^{-4}$). These results implicate IL16 as involved in the mechanism of IVIG resistance in KD.

INTRODUCTION

KD is an acute vasculitis affecting young children, primarily aged younger than 5 years (Newburger JW, 2004). There is no specific test for KD, which is diagnosed based on prolonged fever along with common clinical symptoms including erythema of the oral mucosa and lips, and strawberry tongue; polymorphous rash; erythema and edema of the hands and feet; and cervical lymphadenopathy (Newburger JW, 2004). Patients presenting with fever for more than 5 days with more than four clinical signs are diagnosed with complete KD, while those with less than four clinical signs are diagnosed with incomplete KD (Newburger JW, 2004). IVIG is the standard therapy for KD and is effective in reducing the duration of fever, systemic inflammation, and the development of CALs (Burns JC, 2015); however, 10–20% of patients with KD do not respond to IVIG and have persistent or recurrent fever and a high risk of coronary complications, including myocardial infarction, ischemic heart disease, or sudden death (Burns JC, 1996; Durongpisitkul K, 2003). IVIG treatment is also widely used as an anti-inflammatory agent for autoimmune diseases, such as immune thrombocytopenia, RA, SLE, and multiple sclerosis (Nimmerjahn F, 2007). The immunology underlying IVIG resistance is unknown, and the mechanism of action of IVIG is not fully understood. Many studies have reported several clinical risk factors for IVIG resistance in patients with KD, including age, duration of fever, WBC count, Hb and albumin concentrations, CRP concentration, ESR, neutrophil ratio, and AST and ATL levels (Egami K, 2006; Kobayashi T, 2006; Sano T, 2007). Nevertheless, no consistent criteria have been established that can identify KD patients likely to exhibit IVIG treatment resistance. Classification of patients according to the risk of IVIG resistance could inform decisions to administer more aggressive initial treatment, with the aim of reducing the risk of coronary artery lesion development.

To identify genes involved in susceptibility to IVIG non-response, we re-analyzed our previous GWAS data, focusing on coding variants. Interestingly, by re-analysis of our GWAS data and a replication study, we found that a nsSNP in *IL16* (rs11556218; c.3441T>G, p.Asn1147Lys) was significantly associated with IVIG non-response in

patients with KD.

SUBJECTS AND METHODS

Subjects

Patients with KD were recruited from 12 hospitals in Korea that are currently participating in the Korean Kawasaki Disease Genetics Consortium from June 2008 through to February 2019. The initial GWAS was performed using samples from 296 patients with KD, as previously reported (Kim JJ, 2019). A total of 296 KD cases and 1,000 control samples were genotyped using the Illumina HumanOmni1-Quad BeadChip following the manufacturer's instructions (Illumina, San Diego, CA, USA). Of 269 KD cases, 101 IVIG non-responder KD cases and 195 IVIG responder KD cases were included.

Selection of candidate nsSNPs associated with IVIG resistance using previous Illumina HumanOmni1-Quad BeadChip data

All samples had a genotyping call rate of > 98%. To filtering a SNP markers, we also excluded 488 SNP markers with missing call rates > 2%, 43 markers with a HWE *P*-value $< 1 \times 10^{-6}$ in the controls, and 208,625 markers with a MAF < 0.01. After filtering, 721,635 SNPs were included in the GWAS analysis. To select the IVIG-resistance associated nsSNPs, we searched the immune related genes with $P < 0.05$.

TaqMan™ SNP genotyping

To test candidate SNPs, we used a custom-made TaqMan™ probe sets or pre-designed TaqMan™ probe sets (Applied Biosystems, Foster City, CA, USA). TaqMan™ genotyping assays were performed according to the standard protocol on a 7900HT Real-Time PCR System using Genotyping Master Mix (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analyses were performed using the PLINK (version 1.07) (http://pngu.mgh.harvard.edu/~purcell/plink/). Allelic association test was performed for genetic association study obtaining OR and *P*-value of Illumina HumanOmni1-Quad

BeadChip. Also, we used HWE test of discovering candidate nsSNP in genetic association study ($P > 0.0001$). To study the significance of differences in the clinical variables in each group, SPSS programs (version 18) (SPSS, Chicago, IL, USA) were used. The functional effects of candidate variants were predicted by Polyphen and SIFT programs. HaploReg v4.1 program was searched for exploring annotations of the associated SNPs (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php).

RESULTS

A nonsynonymous polymorphism in the *IL16* gene, Asn1147Lys, is associated with IVIG resistance in KD

To identify coding variants associated with IVIG non-response, we re-analyzed our previous GWAS data from 296 patients with KD (Kim JJ, 2019). Initially, we identified five nsSNPs associated with IVIG resistance in KD ([OR] = 1.89–3.46, $P = 0.0109–0.0035$; Table 14); these included rs10488532 in the *SAMD9L* gene, which has previously been identified in our study (Kim JJ, 2019). Among the four newly-identified nsSNPs, only one in the *IL16* gene (rs11556218; c.3441T>G, p.Asn1147Lys) was associated with IVIG resistance in a replication study (OR = 1.54, $P = 0.0078$; Table 14). In combined analysis of GWAS and replication study data, we detected a more significant association with rs11556218 (OR = 1.64, $P_{combined} = 1.25 \times 10^{-4}$; Table 1). Further, although two other variants (rs344560, p.Lys214Glu in *TNFSF14* and rs12479626, p.His446Arg in *NFATC2*) were not replicated in the validation sample, both showed significance on combined analysis (OR = 1.60, $P = 0.0086$ for rs344560 and OR = 1.61, $P = 0.0170$ for rs12479626; Table 14).

Table 14. Analysis of the association of five candidate nsSNPs with IVIG resistance

SNP	Gene	Position	Allele[1] 1 / 2	Stage	No. of samples		RAF		Association	
					IVIG-NR	IVIG-R	IVIG-NR	IVIG-R	OR (95% CI)	*P*
rs11556218	*IL16*	p.Asn1147Lys	T / G	GWAS	101	195	0.847	0.744	1.89 (1.22–2.97)	**0.0042**
				Replication	191	374	0.827	0.758	1.54 (1.10–2.08)	**0.0078**
				Combined	292	569	0.834	0.753	1.64 (1.27–2.13)	**1.25×10^{-4}**
rs344560	*TNFSF14*	p.Lys214Glu	A / G	GWAS	101	195	0.109	0.051	2.26 (1.20–4.25)	**0.0096**
				Replication	161	529	0.098	0.072	1.39 (0.90–2.16)	0.1366
				Combined	262	724	0.102	0.066	1.60 (1.12–2.27)	**0.0086**
rs12479626	*NFATC2*	p.His446Arg	C / T	GWAS	101	195	0.094	0.036	2.79 (1.37–5.69)	**0.0035**
				Replication	191	374	0.076	0.062	1.25 (0.77–2.03)	0.357
				Combined	292	569	0.082	0.053	1.61 (1.09–2.86)	**0.0170**
rs1128127	*DERL3*	p.Ala211Val	A / G	GWAS	101	195	0.089	0.039	2.45 (1.21–4.96)	**0.0109**
				Replication	191	374	0.050	0.063	0.78 (0.45–1.35)	0.3745
				Combined	292	569	0.063	0.055	1.17 (0.77–1.79)	0.4539
Previously tested or reported locus:										
rs10488532	*SAMD9L*	p.Val266Ile	T / C	GWAS	101	195	0.059	0.018	3.46 (1.34–8.92)	**0.0067**
				Replication[2]	161	492	0.056	0.037	1.57 (0.88–2.80)	0.1269

	Combined	262	687	0.057	0.031	1.89 (1.17–3.04)	**0.0083**

GWAS, genome wide association study; IVIG-NR; intravenous immunoglobulin non-responder, IVIG-R; intravenous immunoglobulin responder; RAF, risk allele frequency; 95% CI, 95% confidence interval.

The combined effect of two risk variants in *SAMD9L* (rs10488532) and *IL16* (rs11556218)

To study the combined effect of the two identified risk variants (the previously reported rs10488532 (C > T, risk allele: T) in *SAMD9L* (Kim JJ, 2019) and the newly discovered rs11556218 (T > G, risk allele: T) in *IL16*, we investigated the ORs and significance of all possible combinations of genotypes of these two variants using samples having both genotypes (256 IVIG non-responders and 354 IVIG responders) from two independently performed tests. Using the non-risk genotype (homozygous for the non-risk alleles at both loci; CC-GG) as baseline, the genotype CT-TT was associated with a highly significant increased risk for IVIG non-response, with the highest OR of 9.19 (95% confidence interval [CI] = 2.72–31.09, $P = 3.63 \times 10^{-4}$) in patients with both genotypes (Table 16).

Table 15. ORs for two-locus genotypes (rs10488532 in *SAMD9L*, risk allele T; and rs11556218 in *IL16*, risk allele T) versus the non-risk homozygous genotype (CC-GG) in 256 IVIG non-responders and 354 IVIG responders

Two-locus genotype		Number of IVG non-responders (%)	Number of IVG responders (%)	Allelic test	
SAMD9L (rs10488532)	*IL16* (rs11556218)			OR (95% CI)	P
CC	GG	8 (3.1)	21 (5.9)	1	na
CC	GT	58 (22.7)	130 (36.7)	0.85 (0.36–2.04)	0.722
CC	TT	161 (62.9)	189 (53.4)	2.24 (0.96–5.19)	0.061
CT	GG	0 (0)	0 (0)	na	na
CT	GT	7 (2.7)	8 (2.3)	2.30 (0.63–8.44)	0.210
CT	TT	21 (8.2)	6 (1.7)	9.19 (2.72–31.09)	**3.63×10^{-4}**
TT	GG	0 (0)	0 (0)	na	na
TT	GT	0 (0)	0 (0)	na	na
TT	TT	1 (0.4)	0 (0)	na	na

Significant value is shown in bold.

OR and *P*-values were computed using logistic regression analysis.

95% CI, 95% confidence interval; n, number; na, not available; OR, odds ratio; *P*, *P*-value

Comparison of Clinical Data between IVIG Non-responders and IVIG Responders

In the clinical data analysis between IVIG non-responders and responders, compared to IVIG responders, IVIG non-responders showed a significant differences in longer duration of fever (mean 7.27 days vs. 5.74 days, $P = 1.36 \times 10^{-18}$) and severe inflammation with higher levels of CRP (mean 7.95 mg/dL, vs. 5.52 mg/dL, $P = 3 \times 10^{-7}$), ratio of neutrophils (71.8 % vs. 62.6%, $P = 2.81 \times 10^{-15}$), concentration of AST, ALT, but lower level of platelet (304.79 × 109/L vs. 330.98 × 109/L, $P = 0.002$) and albumin (3.67 vs. 3.77, $P = 0.02$) (Table 16). Interestingly, in this study, IVIG non-responders showed less severe Bacillus Calmette-Guérin (BCG) vaccine reactions (20.9% BCG + vs. 30.2% BCG +, $P = 0.0073$) (Table 16).

Additionally, we examined the associations of *IL16* rs11556218 (risk allele: T) genotype with clinical variables of patients with KD in the IVIG subgroups. But only the duration of fever showed the significant association with this variant (TT genotype: 6.33 ± 1.4 days, GT genotype: 6.16 ± 1.4 days, GG genotype: 5.47 ± 1.5 days; $P = 0.015$; Table 17)

Table 16. Comparison of clinical data between IVIG non-responder and responders

	All (n=861)	IVIG-NR (n= 292)	IVIG-R (n= 569)	*P*
Age of onset (years)	2.17 ± 2.26	2.27 ± 2.20	2.12 ± 2.29	0.260
Male (n (%))	533 (61.9)	185 (63.4)	348 (61.2)	0.579
Incomplete KD (n (%))	159 (18.6)	50 (17.2)	109 (19.3)	0.502
Family history (n (%))	19 (2.2)	4 (1.4)	15 (2.6)	0.342
Duration of fever (days)	6.23 ± 1.41	7.27 ± 1.48	5.74 ± 1.32	**1.36×10^{-18}**
Recurrence (n (%))	60 (7)	25 (8.7)	35 (6.2)	0.227
BCG + (n (%))	203 (26.8)	57 (20.9)	146 (30.2)	**0.007**
CAL (n (%))	221 (25.8)	80 (27.5)	141 (24.9)	0.454
CRP (mg/dL)	6.25 ± 2.88	7.95 ± 2.45	5.52 ± 3.04	**3×10^{-7}**
ESR (mm/hr)	60.80 ± 28.78	60.69 ± 27.02	60.87 ± 29.71	0.933
WBC (x 10^9/L)	13.22 ±1.55	13.60 ± 1.54	13.03 ± 1.55	0.173
Neutrophil (%)	65.74 ± 16.31	71.77 ± 15.03	62.60 ±16.08	**2.81×10^{-15}**
Platelet (x 10^9/L)	321.81 ± 1.44	304.79 ± 1.49	330.98 ± 1.41	**0.002**
Hb (g/L)	11.38 ± 1.10	11.33 ± 1.10	11.40 ± 1.10	0.413
AST (IU/L)	52.84 ± 2.67	73.45 ± 3.01	44.59 ± 2.38	**5.29×10^{-11}**
ALT (IU/L)	46.54 ± 3.67	66.33 ± 3.9	38.75 ± 3.42	**2.74×10^{-8}**
Albumin (mg/dL)	3.73 ± 1.16	3.67 ± 1.16	3.77 ± 1.14	**0.020**
Total protein (mg/dL)	6.61 ± 1.11	6.59 ± 1.13	6.62 ± 1.10	0.505
Ig				
IgG (mg/dL)	1771.74 ± 1.96	1642.99 (±2.19)	1847.30 (±1.82)	0.231
IgA (mg/dL)	85.74 ± 1.89	91.92 (±1.91)	82.47 (±1.88)	0.207
IgM (mg/dL)	137.56 ± 1.53	143.71 (±1.53)	134.29 (±1.53)	0.235
IgE (KU/L)	64 ± 3.74	66.14 (±3.54)	62.86 (±3.86)	0.777

"BCG +" indicates KD patients with redness or swelling at BCG injection site. Of the 861 KD samples, only 757 KD samples with BCG data were used. The difference between the groups was tested by χ^2 -test for the categorical variables. The results for the categorical variables are presented in percentages in round brackets. For continuous variables, differences between two groups were analyzed using Student's t-test. Values are presented as means ± standard deviation (SD) for quantitative variables. Significant values are shown in bold.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCG vaccine, Bacillus Calmette–Guérin vaccine; CAL, KD patients with coronary artery lesions (≥5mm); CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; IVIG, intravenous immunoglobulin; IVIG-NR, IVIG non-responder; IVIG-R, IVIG responder; KD, Kawasaki disease; *P*, *P*-value; WBC, white blood cell

Table 17. Associations of IL16 rs11556218 (risk allele: T) genotype with clinical variables of patients with KD in the IVIG subgroups

Clinical variable	All (n = 861)				IVIG non-responder (n = 292)				IVIG responder (n = 569)			
	TT (n = 530)	GT (n = 284)	GG (n = 47)	*P*	TT (n = 206)	GT (n = 75)	GG (n = 11)	*P*	TT (n = 324)	GT (n = 209)	GG (n = 36)	*P*
Age of onset (years)	2.17 ± 2.22	2.16 ± 2.29	2.23 ± 2.49	0.964	2.34 ± 2.07	2.02 ± 2.56	2.83 ± 2.04	0.25	2.07 ± 2.31	2.21 ± 2.2	2.08 ± 2.62	0.676
Male (n (%))	328 (61.9)	175 (61.6)	30 (63.8)	0.959	129 (62.6)	50 (66.7)	6 (54.5)	0.681	199 (61.4)	125 (59.8)	24 (66.7)	0.73
Incomplete KD, n (%)	92 (17.6)	57 (20.1)	10 (21.3)	0.605	34 (16.6)	13 (17.3)	3 (27.3)	0.657	58 (18.2)	44 (21.1)	7 (19.4)	0.716
Family history, n (%)	10 (1.9)	6 (2.1)	3 (6.4)	0.133	4 (2)	0 (0)	0 (0)	0.427	6 (1.9)	6 (2.9)	3 (8.3)	0.069
Duration of fever (days)	6.33 ± 1.4	6.16 ± 1.4	5.47 ± 1.5	**0.015**	7.41 ± 1.47	7.07 ± 1.49	6.12 ± 1.41	0.218	5.72 ± 1.29	5.86 ± 1.34	5.29 ± 2.11	0.117
Recurrence, n (%)	37 (7)	20 (7.1)	3 (6.4)	0.985	16 (7.8)	7 (9.6)	2 (18.2)	0.465	21 (6.5)	13 (6.2)	1 (2.8)	0.678
BCG +, n (%)	113 (24.1)	76 (31)	14 (32.6)	0.095	38 (19.7)	19 (27.1)	0 (0)	0.107	75 (27.2)	57 (32.6)	14 (42.4)	0.135
CAL, n (%)	130 (24.6)	81 (28.6)	10 (21.3)	0.356	55 (26.7)	25 (33.8)	0 (0)	0.058	75 (23.3)	56 (26.8)	10 (27.8)	0.605
CRP (mg/dL)	6.26 ± 2.87	6.10 ± 2.96	7.03 ± 2.58	0.695	7.72 ± 2.52	8.56 ± 2.4	8.25 ± 1.53	0.691	5.47 ± 3.03	5.41 ± 3.09	6.7 ± 2.89	0.555
ESR (mm/hr)	61.25 ± 28.45	60.91 ± 29.03	55.02 ± 30.92	0.395	60.81 ± 27.41	59.14 ± 26.34	69.9 ± 24.87	0.496	61.55 ± 29.17	61.6 ± 30.05	50.52 ± 31.48	0.118
WBC ($\times 10^9$/L)	13.48 ± 1.53	12.75 ± 1.59	13.31 ± 1.45	0.229	13.91 ± 1.56	12.84 ± 1.52	13.24 ± 1.43	0.386	13.2 ± 1.51	12.72 ± 1.61	13.32 ± 1.47	0.605
Neutrophils (%)	66.27 ± 15.67	64.27 ± 17	68.67 ± 18.53	0.113	71.88 ± 14.72	71.1 ± 15.98	74.21 ± 15.2	0.801	62.61 ± 15.19	61.84 ± 16.72	66.98 ± 19.3	0.208
Platelets ($\times 10^9$/L)	326.06 ± 1.43	316.45 ± 1.46	307.61 ± 1.44	0.371	306.55 ± 1.5	299.3 ± 1.5	307.03 ± 1.32	0.903	339.23 ± 1.36	322.78 ± 1.45	307.19 ± 1.48	0.105
Hb (g/L)	11.37 ± 1.1	11.36 ± 1.1	11.52 ± 1.08	0.638	11.36 ± 1.1	11.26 ± 1.1	11.32 ± 1.06	0.805	11.38 ± 1.1	11.39 ± 1.09	11.58 ± 1.08	0.567
AST (IU/L)	53.02 ± 2.67	53.67 ± 2.74	46.37 ± 2.3	0.636	72.74 ± 2.88	76.07 ± 3.34	69.29 ± 3.68	0.942	43.23 ± 2.89	47.4 ± 2.46	41.01 ± 1.87	0.414
ALT (IU/L)	45.57 ± 3.72	50.28 ± 3.67	36.91 ± 3.03	0.269	65.31 ± 3.95	71.24 ± 3.91	54.64 ± 3.27	0.798	36.12 ± 3.39	44.42 ± 3.51	32.75 ± 2.93	0.117
Albumin (mg/dL)	3.74 ± 1.17	3.72 ± 1.15	3.75 ± 1.14	0.931	3.68 ± 1.21	3.64 ± 1.16	3.68 ± 1.16	0.925	3.78 ± 1.14	3.75 ± 1.15	3.76 ± 1.13	0.840
Total protein (mg/dL)	6.62 ± 1.11	6.58 ± 1.12	6.69 ± 1.09	0.614	6.61 ± 1.12	6.49 ± 1.18	6.75 ± 1.12	0.44	6.62 ± 1.1	6.62 ± 1.1	6.67 ± 1.09	0.905

"BCG +" indicates KD patients with redness or swelling at the BCG injection site. Of the 765 KD samples, only 562 KD samples with BCG data were used. The difference between the groups was evaluated by $\chi 2$-test for categorical variables, which are presented as percentages (in brackets). For continuous variables,

differences between two groups were analyzed using the Student's t-test. Values are presented as means ± standard deviation (SD) for quantitative variables. Significant values are shown in bold.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCG vaccine, Bacillus Calmette‐Guérin vaccine; CAL, KD patients with coronary artery lesions (≥ 5 mm); CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; IVIG, intravenous immunoglobulin; KD, Kawasaki disease; P, P-value; WBC, white blood cell.

DISCUSSION

In this study, we re-analyzed our previous GWAS data, focusing on coding variants in immune-related genes, to identify new susceptibility genes for IVIG resistance in KD. Initially, we identified four new candidate nsSNPs associated with IVIG resistance in KD from GWAS data. After replication analysis, one new susceptibility coding variant (rs11556218: c.3441T>G, p.Asn1147Lys) in the *IL16* gene was validated as associated with IVIG non-response.

The *IL16* gene encodes a pleiotropic cytokine involved in various inflammatory diseases. IL16 is a chemoattractant that modulates T cell activation, localizes to T lymphocyte cytoplasm, and is constitutively expressed in over 70% of CD4 and CD8 T cells (Chupp GL, 1998). Disorders involving the *IL16* gene affect the CD4/CD8 T cell ratio or T cell activation in patients with SLE and multiple myeloma (Matsushita M, 2000; Koike M, 2002). Changes in the CD4/CD8 T cell ratio are mediated by activation of CD8 T cells by the IL16 cytokine. IL16 is also associated with the pathophysiology of chronic immune diseases, such as allergen induced bronchial asthma, RA, and CD (Blaschke S, 2001; Seegert D, 2001). In this study, we identified a new coding variant (rs11556218, p.Asn1147Lys) in the *IL16* gene associated with susceptibility to IVIG non-response in patients with KD. This missense variant was predicted as benign and deleterious by the Polyphen and SIFT programs, respectively, suggesting that this amino acid-altering variant may change the biological function of the IL16 protein.

Interestingly, two other genes, *NFATC2* and *TNFSF14*, which were associated with IVIG resistance in combined analysis of GWAS and replication study data, are also both related to T cell activation, similar to IL16. *NFATC2* is a member of the nuclear factor of activated T cells family and encodes a protein that translocates to the nucleus upon T cell receptor stimulation, where it becomes part of the nuclear factors of activated T cell transcription complex (Rengarajan J, 2002; Köck J, 2014), which induces gene transcription during the immune response. *TNFSF14* encodes a member of the tumor necrosis factor ligand family. The TNFSF14 protein stimulates T cell proliferation and functions as a costimulatory factor for lymphoid cell activation (Tamada K, 2000). The mechanisms underlying IVIG in

KD remain unknown; however, after administration of IVIG, patients with KD exhibit reduced levels of cytokines and chemokines, changes in cell composition (including among lymphocyte subsets), decreased numbers of circulating monocytes, macrophages, neutrophils, and activated T cells, and increased numbers of NK cells (Burns JC, 2015). Overall, the available data suggest that T cells may play an important role in the mechanism underlying IVIG resistance in KD. Furthermore, in contrast to the B cell-dependent pathogenesis of KD (Lindquist ME, 2019), our results suggest that patients with KD who are not responsive to IVIG may be a distinctive subset primarily influenced by T cell-dependent pathogenesis.

When we analyzed the combined effects of the two risk variants (the previously reported rs10488532 in *SAMD9L* and newly discovered rs11556218 in *IL16*), the two-locus genotype, CT-TT, showed the large effect size (OR = 9.19, 95% CI = 2.72–31.09; $P = 3.63 \times 10^{-4}$; Table 15), relative to homozygotes for the non-risk genotype (CC-GG). This combined effect of two risk loci demonstrates that the variants in *SAMD9L* and *IL16* exhibit strong synergistic effects on IVIG resistance. Therefore, patients with KD carrying both risk variants should be selected for more aggressive initial treatment, such as an IVIG plus steroid regimen (Kobayashi T, 2012; Miyata K, 2018).

Analysis of clinical data from IVIG non-responders and responders showed that, compared with IVIG responders, IVIG non-responders exhibited significantly longer fever duration (mean 7.27 vs. 5.74 days; $P = 1.36 \times 10^{-18}$) and more severe inflammation, indicated by higher levels of CRP (mean 7.95 vs. 5.52 mg/dL, $P = 3 \times 10^{-7}$), neutrophil ratio (71.8% vs. 62.6%, $P = 2.81 \times 10^{-15}$), AST (73.45 vs. 44.59 IU/L, $P = 5.29 \times 10^{-11}$), and ALT (66.33 vs. 46.54 IU/L, $P = 2.74 \times 10^{-8}$) levels; however, platelets (304.79 × 109/L vs. 330.98 × 109/L, $P = 0.002$) and albumin (3.67 vs. 3.77, $P = 0.02$) were lower (Table 16), consistent with previous studies (Kobayashi T , 2006; Egami K, 2006; Sano T, 2007). Interestingly, in this study, we also found that IVIG non-responders showed less severe BCG vaccine reactions (20.9% BCG+ in IVIG non-responders vs. 30.2% BCG+ in IVIG responders, $P = 0.0073$) (Table 16), indicating that immunological events in BCG vaccine reaction may be involved in determining IVIG response. We also examined the associations of rs11556218 in *IL16* with clinical variables in patients with KD; however, we found no significant association of this variant with any clinical variables, except for duration of fever (TT genotype: 6.33 ± 1.4 days,

GT genotype: 6.16 ± 1.4 days, GG genotype: 5.47 ± 1.5 days; $P = 0.015$; Table 17).

Several candidate studies and recent GWAS have been conducted to identify genetic markers for IVIG resistance (Weng KP, 2010; Shrestha S, 2012; Kuo HC, 2017); however, the majority of reported results have not been confirmed in any other studies, primarily because of the very small numbers of IVIG non-responder samples used in these studies (< 100 IVIG non-responders). In this study, although we included a relatively large sample of IVIG non-responders (n = 292), we had low statistical power for detection of associations with IVIG resistance, mainly due to the relatively low frequency of the coding variants tested.

In summary, we identified *IL16* as a new susceptibility gene for IVIG resistance in KD, suggesting that *IL16* and/or T cells (as the target cell of *IL16*) may play a role in IVIG non-responsiveness in KD. These findings provide new insights into the involvement of a potential T cell activation mechanism underlying IVIG resistance.

CONCLUSION

In this study, we sought to identify the genetic risk variants of immunoglobulin Fc receptor genes affecting KD susceptibility and the genetic risk variants of immunoglobulin G glycosylation genes for the IVIG treatment resistance, respectively.

First, we sought to identify nsSNPs in immunoglobulin Fc receptor genes influencing KD susceptibility. Through discovery phase a total of 15 immunoglobulin Fc receptor genes sequenced and replication study using TaqMan^{TM} genotyping, we performed the association study for candidate nsSNPs in Fc receptor genes. But the result of this study did not show any significant association with KD susceptibility. Secondly, we performed the association study for IVIG-resistance in KD using seven candidate nsSNPs in the genes involved in glycosylation of immunoglobulin G which are selected from our unpublished WES data. However, there was no significant association of the candidate nsSNPs selected in the immunoglobulin G glycosylation genes with IVIG resistance. Lastly, to identify coding variants associated with IVIG resistance in KD, we re-analyzed our previous GWAS data. Five nsSNPs in five immune-related genes were significantly associated with IVIG non-response (OR = 1.89–3.46, $P = 0.0109–0.0035$). Of them, *IL16* nsSNP was more significantly associated with IVIG non-response in combined analysis of all data (OR = 1.64, $P = 1.25 \times 10^{-4}$). These results indicate that *IL16* are involved in the mechanism of IVIG resistance in KD. We identified nsSNPs (rs11556218: c.3441T>G, p.Asn1147Lys) in the *IL16* gene as risk factors for IVIG resistance. In addition, clinical data analysis of IVIG non-responders and IVIG responders, compared with IVIG responders, IVIG non-responders exhibited significantly longer fever duration and more severe inflammation, indicated by higher levels of CRP, neutrophil ratio, AST, and ALT levels; however, platelets and albumin were lower. We also found that IVIG non-responders showed less severe BCG vaccine reactions. Further, replication studies or meta-analysis will be needed for the identification of consistent risk factors.

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국문요약

가와사끼병에서 면역글로불린 관련 유전자의 질병 연관성 연구

한국에서 소아 후천성 심장 질환의 주요 원인인 가와사키병은 급성, 전신성 혈관염이다. 가와사키병 치료는 정맥 내 면역글로불린과 아스피린이 사용되며, 대부분의 환자에서 열과 관상 동맥류의 발생을 감소시킨다. 면역글로불린 치료에도 불구하고, 가와사키병 환자의 약 10 % 이상이 치료에 반응하지 않고 지속적인 열을 보인다. 본 질병의 발병 원인은 아직 밝혀져 있지 않으며, 특정 감염원에 의한 비정상적인 과민 면역 반응이 유전적으로 특정 소인을 가진 개인에서 혈관의 손상을 일으키는 염증을 유발하는 것으로 추측하고 있다.

본 연구의 목적은 첫째, 면역글로불린 수용체 유전자와 가와사끼병 감수성 간의 연관성을 규명한다. 면역글로불린 수용체와 그 수용체 중 유전자간 상동성이 높은 Fc 감마 수용체 유전자, 그리고 면역글로불린 수용체 유사 유전자의 발현 영역에 존재하는 유전변이형과 가와사끼병 감수성 간의 연관성을 환자-대조군 연관성 연구 방법으로 유의성을 검증한다. 전장 유전체 분석 데이터와 전장 엑솜 분석 데이터가 상동성이 높은 면역글로불린 감마 유전자 모두를 포괄하지 못하기 때문에 면역글로불린 감마 수용체 유전자와 그 유사 수용체 유전자를 capillary sequencing (98 명의 가와사끼병 환자와 96 명의 대조군)을 통해 코딩 부위에 존재하는 기능적으로 중요한 유전변이형을 발굴하고, 가와사끼병의 감수성에 대한 연관성 분석을 수행하였다. 후보로 선별된 가와사끼병의 감수성과 연관된 면역글로불린 수용체 유전변이형의 유의성을 TaqMan genotyping 방법(570 명의 가와사끼병 환자와 570 명의 대조군)을 사용하여 반복성을 검증하였다. 그러나 후보 Fc 감마 수용체 유전자의 유전변이형과 가와사끼병 감수성간의 환자-대조군 연관성 분석 결과에서 유의성을 보이지 않았다.

두번째 연구에서는 면역글로불린 당화 유전자와 가와사끼병의 면역글로불린 치료 저항성 간의 연관성을 분석하였다. 총 94 명의 면역글로불린 치료 저항성 환자 및 106 명의 면역글로불린 치료 반응성 환자의 전장 엑솜 분석 데이터의 연관 연구를 수행하여 가와사끼병의 면역글로불린 치료 저항성에 영향을

미치는 면역 글로불린 당화 유전자의 후보 유전 변이형을 선별하고, 추가적으로 총 191 명의 면역글로불린 치료 저항성 환자 및 374 명의 면역글로불린 치료 반응성 환자를 사용한 반복성 검증 실험을 수행하였다. 그러나, 후보 면역 글로불린 당화 유전자 유전변이형과 면역글로불린 치료 저항성 간의 연관성 분석 결과에서 유의성을 보이지 않았다.

마지막으로, 세번째 연구에서는 면역 관련 유전자와 가와사끼병의 면역글로불린 치료 저항성 간의 연관성을 연구하였다. 면역글로불린 치료 저항성에 영향을 미치는 단백질 코딩 유전자 변이형을 식별하기 위해 101 명의 면역글로불린 치료 저항성 환자 및 195 명의 면역글로불린 치료 반응성 환자를 포함한 가와사끼병 환자 296 명의 전장 유전체 연관 분석 데이터를 재분석하였다. 이전에 보고된 *SAMD9L* nsSNP (rs10488532; p.Val266Ile) (Kim JJ, 2019)를 포함하여 5 개의 면역 관련 유전자에서 발굴된 5 개의 유전변이형은 면역글로불린 치료 저항성 환자군에서 유의한 연관성을 보였다 (OR = 1.89–3.46, P = 0.0109–0.0035). 특히, 3 개의 새로 발견된 유전변이형의 반복성 검증 실험에서, *IL16* 유전자 (rs11556218, p.Asn1147Lys)만이 면역글로불린 치료 저항성 환자군 (OR = 1.54, P = 0.0078)에 유의성이 확인되었다. 특히 동일한 *IL16* 유전변이형은 모든 데이터를 결합한 분석에서 면역글로불린 치료 저항성 환자군에서 더 유의적인 연관성을 보였다(OR = 1.64, $P = 1.25 \times 10^{-4}$). 이러한 결과는 가와사끼병에서 면역글로불린 치료 저항 메커니즘에 *IL16* 이 관여한다는 것을 의미한다.

중심단어: 가와사끼병 / 정맥 면역글로불린 / 면역글로불린 치료 저항성 / 전장 유전체 연관성 연구/ 면역글로불린 Fc 수용체 / *IL16*