Basic Medical Research

Association between interleukin-2 (rs2069762) gene polymorphism and FVIII inhibitor development in Indonesian patients with severe hemophilia A

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ABSTRACT

BACKGROUND Factor VIII (FVIII) inhibitors in hemophilia A (HA) patients render FVIII replacement therapy ineffective. Although its development cause is unclear, it has been classified into therapeutic and genetic-related etiologies. Single nucleotide polymorphisms (SNPs) in several cytokine genes, including interleukin (IL)-2, could increase the risk of FVIII inhibitor development. This study aimed to evaluate the association between IL-2 (rs2069762) gene SNP and FVIII inhibitor development in Indonesian patients with severe HA.

METHODS The IL-2 (rs2069762) gene SNP was examined in 119 HA patients. The presence of FVIII inhibitors was quantified using an enzyme-linked immunosorbent assay, with a titer of <0.28 ng/ml considered negative. Patients were divided into two groups: 59 with FVIII inhibitors (positive group) and 60 without inhibitors (negative group). The genotype of the subjects was determined using peripheral blood mononuclear cells and tetra-primer amplification refractory mutation system-polymerase chain reaction.

RESULTS There was no association between IL-2 (rs2069762) gene polymorphism and FVIII inhibitor development on genotypes ($p = 0.138$) and allele frequencies ($p = 0.780$).

CONCLUSIONS IL-2 (rs2069762) gene polymorphism is not a risk factor in the development of FVIII inhibitors in Indonesian patients with severe HA. Thus, further polymorphism studies in other cytokine genes are required to gain a comprehensive understanding of the FVIII inhibitor development.

KEYWORDS antibodies, factor VIII, hemophilia A, interleukin-2, single nucleotide polymorphism

Hemophilia A (HA) is a rare hematologic disease due to factor VIII (FVIII) deficiency.[1] Of the 320,000 HA cases worldwide, only 25% of patients receive adequate FVIII concentrate therapy.[2] HA is a complex disorder to treat, and chronic treatment with FVIII concentrate may cause the development of alloantibodies that inhibit FVIII. These inhibitors are found in approximately 15% of the HA population who have never received treatment and in 30% of those who previously have received FVIII concentrate.[3]

The development of FVIII inhibitors causes FVIII concentrates on being ineffective, leading to poor clinical outcomes.[4] Factors that increase the risk of FVIII inhibitor development include a family history of the inhibitor, race, human leukocyte antigen (HLA) haplotype, FVIII gene mutation, and single nucleotide

polymorphisms (SNPs) in several cytokine genes.[5,6] A study found 53 SNPs as the risk factor for FVIII inhibitors, demonstrating the immunological intricacy of inhibitor development.[7] Although HLA haplotypes were not a significant factor in FVIII inhibitor development, polymorphisms in several cytokine genes seemed to play a role.[8]

Inflammatory cytokines regulate FVIII inhibitor development.[9–11] Interleukin (IL)-2 plays a significant role in activating and regulating the immune system, shown in the pathogenesis of several autoimmune diseases.[12] The IL-2 gene is located in the 4q27 chromosomal region, has five exons, and contains a restriction site at 2069762 (rs2069762).[13] While a study in the Iranian population did not find a significant association between SNP in the IL-2 (rs2069762) gene and the risk of FVIII inhibitor development, results might vary by race.[14] However, no data were available in the Indonesian population. Thus, this study aimed to evaluate the association between IL-2 (rs2069762) gene SNP and FVIII inhibitor development in Indonesian patients with severe HA.

METHODS

This study was a 2-year case-control study conducted from April 2017 to May 2019 at the hematology polyclinic of Dr. Moewardi General Hospital and the biomolecular laboratory of Universitas Sebelas Maret, Surakarta, Indonesia. The inclusion criteria were males aged 18 years or older who received FVIII concentrate therapy for more than 9 days of exposure.[15] This study only included Javanese patients due to the focus on the SNP aspect and the study's effectiveness. The ethnicity was confirmed by lineage trace of their Javanese parents and grandparents. The exclusion criteria included bleeding disorders and other comorbidities of organ systems. The sample size was determined by Kelsey-Fleiss' method from OpenEpi (https://www.openepi.com) calculation with a two-sided 95% confidence interval (CI), 80% chance of detecting, a ratio of controls to cases 1, the hypothetical proportion of controls with exposure of 79.81, and the least extreme odds ratio (OR) of 0.31. Thus, the minimum sample size was 46 for each group.

Peripheral blood mononuclear cells (PBMCs) specimens were collected from 125 severe HA patients with FVIII levels of <1% or <1 U/dl at the hematology-oncology clinic of Dr. Moewardi General Hospital from 2019 to 2021. The BIOENZY® (Indonesia) human inhibitor factor VIII enzyme-linked immunosorbent assay kit (DiaPharma Group, Inc., USA) was used to measure the FVIII inhibitor titer (ng/ml), and the titer of ≥0.28 ng/ml indicated to have FVIII inhibitors.[15] Six patients were excluded from the study because they never received FVIII concentrate replacement therapy. A total of 59 patients had FVIII inhibitors, and 60 had no inhibitors. All subjects in both groups had FVIII concentrate therapy for at least 9 days of exposure.

Approximately 50 ng/µl DNA was extracted from the PBMCs using the GF-1 viral nucleic acid extraction kit (Vivantis, Malaysia) based on the laboratory user manual. The primer to detect the rs2069762 SNP was designed using primer1.soton.ac.uk. Gene sequence validity product was evaluated using SnapGene software (Dotmatics, USA) and the basic local alignment search tool database (Table 1). The tetra-primer amplification refractory mutation system-polymerase chain reaction (T-ARMS-PCR) amplification method was applied to perform IL-2 (rs2069762) gene genotyping with an annealing temperature of 63.44°C. T-ARMS-PCR amplification products were then run in the 2.00% agarose gel electrophoresis and visualized via an ultraviolet transilluminator using blue light emitting diode light with a wavelength of 470 mm. The 1 kb DNA ladder was used as a marker because it was suitable for normal-sized gels and would separate within a short distance of 200-1,000 base pairs. The genotype was then determined by the product size of the agarose gel.

Table 1. T-ARMS-PCR design for IL-2 (rs2069762) gene

Primer	Primer sequence 5' to 3'	Product size (bp)
Forward inner (G allele)	TGCTATTCACATGTTCAGTGTAGTTTCAT	179
Reverse inner (T allele)	AAAGTAACTCAGAAAATTTTCTTTGCCC	289
Forward outer	TAGCGTTAAACAGTACCTCAAGCTCAAT	409
Reverse outer	GATGTAGGTGAAATCCCTCTTTGTTACA	-

bp=base pair; IL-2=interleukin-2; rs=restriction site; T-ARMS-PCR=tetra-primer amplification refractory mutation system-polymerase chain reaction

The genotype and allelic frequencies of the SNP were compared between the FVIII inhibitor positive and negative groups using a chi-square test. A logistic regression test was performed to estimate polymorphism risk in the FVIII inhibitor development, using 95% CI and OR. A *p*-value <0.05 was considered significant. SPSS software version 22.0 (IBM Corp., USA) for Windows was used for the statistical analysis. This study was approved by the Health Research Ethics Committee of Dr. Moewardi General Hospital (No: 210/II/HREC/2020).

RESULTS

A total of 119 HA patients were obtained following the inclusion and exclusion criteria (Table 2). The IL-2 (rs2069762) SNP genotyping in severe HA patients with and without FVIII inhibitors was conducted (Figure 1). We found an insignificant statistical difference between genotype (p = 0.138) and allele frequencies (p = 0.780) for the IL-2 gene in the FVIII inhibitor positive group, compared with the negative group (Table 3).

Table 2. Demographic and clinical profiles of the HA patients

Variables	n (%) (N = 119)
FVIII inhibitors	
Positive	59 (49.6)
Negative	60 (50.4)
Age (years)	
18–30	49 (41.2)
30–45	38 (31.9)
45–60	32 (26.9)
HA duration (years)	
<1	83 (69.7)
1–3	20 (16.8)
>3	16 (13.5)
Total duration of FVIII concentrate therapy (days)	
10–19	79 (66.4)
20–29	21 (17.6)
≥30	19 (16.0)
Medication received*	
Desmopressin	92 (45.3)
Methylprednisolone	60 (29.6)
NSAIDs (except aspirin)	51 (25.1)

FVIII=factor VIII; HA=hemophilia A; NSAIDs=nonsteroidal anti-inflammatory drugs
All patients were male, Javanese, and had FVIII inhibitor positive
*One patient may receive more than one or combined medication

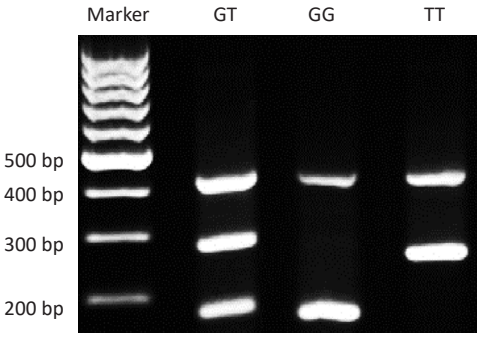

Figure 1. Electrophoresis arrangement results of T-ARMS-PCR for the IL-2 (rs2069762) gene polymorphism. IL-2=interleukin-2; rs=restriction site; T-ARMS-PCR=tetra-primer amplification refractory mutation system-polymerase chain reaction

DISCUSSION

IL-2, an antibody-based pro-inflammatory cytokine, has a role in several hypersensitivities and autoimmune conditions, including FVIII inhibitor development.[16] Some SNPs in the IL gene have been linked to antibody development in autoimmune diseases.[17] However, the immune response phenotype based on race/ethnicity can have different effects on the therapy using exogenous FVIII concentrate.[15] A study showed that SNPs in the IL-2 gene (rs2069762 and rs4833248) were related to autoimmune diseases such as multiple sclerosis, hematological-bleeding disorders, Addison's disease, and type 1 diabetes mellitus.[18]

The contribution of rs2069762 SNP to FVIII inhibitor susceptibility in the Indonesian population is yet to be determined. The results of this study indicated that the polymorphism of the IL-2 (rs2069762) gene had no significant association with the risk of developing FVIII inhibitors. Similarly, FVIII inhibitor development was not related to IL-2 SNPs of rs2069762 (p = 0.321) and allele frequency (p = 0.217) in the Iranian population.[14] This lack of association may be due to the functional SNPs of transforming growth factor-β1 (TGF-β1) at several sites.[19] TGF-β1 gene structure has been linked to functional implications, as it binds to transcription factors, leading to decreased protein production in several autoimmune diseases.[20] This interference is related to eight TGF-β1 SNPs, including rs2317130, rs11466313, rs1800468, rs1800469, rs11466314, rs1800471, rs1800470, and rs11466316.[19]

Table 3. Association of IL-2 (rs2069762) gene polymorphism in severe HA patients with and without FVIII inhibitors

Polymorphism	FVIII inhibitor positive, n (%) (N = 59)	FVIII inhibitor negative, n (%) (N = 60)	OR (95% CI)	*p**
Genotypes				0.138
GT	18 (30.5)	29 (48.3)	0.466 (0.180–1.206)	
GG	25 (42.4)	19 (31.7)	0.987 (0.379–2.570)	
TT	16 (27.1)	12 (20.0)	1.00	
Alleles			1.076 (0.644–1.797)	0.780
G	68 (57.6)	67 (55.8)		
T	50 (42.4)	53 (44.2)		

CI=confidence interval; FVIII=factor VIII; HA=hemophilia A; IL-2=interleukin-2; rs=restriction site; OR=odds ratio
*Chi-square test, $p<0.05$ indicated significant association

Of all studies on polymorphism cytokine, the polymorphism of the IL-5 (rs2069812) gene promoter was significantly associated with the risk of FVIII inhibitor development in Turkish patients with severe HA.[21] The product of the IL-5 gene is involved in B cell antibody synthesis and stimulates B cell differentiation, leading to eosinophil activation. Thus, the IL-5 gene (rs2069812) is considered a key factor in the pathogenesis of hypersensitivities such as FVIII inhibitor development.[22]

This study showed that the SNP of the IL-2 (rs2069762) gene is not associated with the risk of FVIII inhibitor development in Indonesian patients with severe HA. However, it must be noted that this study had a small sample size. The SNP detection was performed using T-ARMS-PCR, which is not the most advanced method. Hence, conducting further studies with a larger number of subjects and using the latest direct sequencing method with a cohort study design is recommended. Evaluating other SNP sites or cytokine genes, such as TGF-β1 (rs1982037), IL-2 (rs4833248), IL-4 (rs2243267, rs2243282), IL-10 (rs3024496), and interferon-γ (rs2069705), and their associated alleles are also suggested. The studies are expected to identify potential significant factors and the pathogenesis of FVIII inhibitor development in HA patients.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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