Association of aplastic anemia and FoxP3 gene polymorphisms in Koreans

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\textbf{ABSTRACT}

\textbf{Objectives:} Aplastic anemia (AA) is characterized by pancytopenia and bone marrow failure, and most acquired AA is an immune-mediated disorder. Regulatory T cells (T\textsubscript{reg}) suppressing autoreactive T cells were decreased in AA patients. FoxP3 is a major regulator for the development and function of T\textsubscript{reg}. Polymorphism in FoxP3 was shown to be associated with various autoimmune diseases, however, has not yet been studied in AA. In this study, we examined the association between FoxP3 polymorphisms and AA in Korean patients.

\textbf{Methods:} The study population consisted of 94 patients diagnosed by bone marrow examination in Seoul National University Hospital (SNUH) during 1997–2012 and 195 healthy controls. FoxP3 polymorphisms (rs5902434 del/ATT, rs3761548 C/A, rs3761549 C/T, rs2232365 A/G) were analyzed by PCR-sequencing method. We analyzed differences of genotype and allele frequencies between patients and controls. We also compared differences of genotype and allele frequencies between responder and non-responder in patients treated with immunosuppressive therapy (IST). For the statistical analysis, the chi-square test and Fisher’s exact test were used and \( P < 0.05 \) was regarded as statistically significant.

\textbf{Results:} There was no significant difference in the genotype frequencies of FoxP3 polymorphisms between patients and controls. With regards to the allele frequencies, rs3761548 C allele was significantly higher in AA patients than in controls (87.4% vs. 79.7%, \( P = 0.047 \)). In patients treated with IST, rs3761549 C allele was significantly higher in non-responder patients than in responders (89.6% vs. 66.7%, \( P = 0.036 \)) and female rs3761549 C/C genotype carriers were associated with greater risk for non-response to IST (84.2% vs. 16.7%, \( P = 0.006 \)).

\textbf{Conclusion:} Polymorphisms in rs3761548 and rs3761549 of FoxP3 in our population were associated with disease susceptibility and response to IST, respectively. This study suggests an association between FoxP3 polymorphisms and AA in Korean patients and will be useful in further understanding the genetic basis of disease susceptibility and response to IST in AA patients.

\textbf{Introduction}

Aplastic anemia (AA) is a bone marrow failure disorder characterized by peripheral pancytopenia and marrow hypoplasia. The incidence of AA is two per million population in the West and two- to threefold higher in Asia. The distribution by age is biphasic, with peaks at 15–30 years and >60 years. There is no significant difference in incidence between men and women [1]. Acquired AA is known to result from chemical agents, drugs, or viral infections; however, the majority of cases are idiopathic. In subsequent studies, it was revealed that most cases of acquired AA are immune-mediated [2]; the best evidence is the strong response to immunosuppressive therapies (IST). Laboratory experiments have provided insights into the disease including the observation that there was an increase in the number of hematopoietic stem cell colonies in cell cultures generated from the bone marrow lymphocytes of AA patients, and normal bone marrow was suppressed by marrow lymphocytes of patients with AA [3]. In addition, T cells producing cytokines such as interferon-\( \gamma \) and tumor necrosis factor-\( \alpha \) were increased [4] and CD3+/CD4+/IL17-producing Th17 cells were also increased in AA patients [5].

CD4+/CD25+/FoxP3+ regulatory T cells (T\textsubscript{reg}) are major regulators of self-tolerance and immune homeostasis, and also known to be associated with AA [6,7]. It is well known that FoxP3 plays a key role in the differentiation of T cells into T\textsubscript{reg} [8]. The FoxP3 gene is located on chromosome Xp11.23 and polymorphisms in FoxP3 are known to be associated with various conditions such as autoimmune diseases [9–11], allergies [12], feto-maternal rejection [13], and allograft rejection [14]. No previous study, however, has analyzed the association between FoxP3 polymorphisms and AA. In addition, the genetic predisposition associated with
the response to IST has been reported including HLA-DR15 [15,16], IFN gamma, and TGF beta gene polymorphisms [17] in patients with AA. In the present study, we evaluate the association of FoxP3 polymorphisms with disease susceptibility and response to IST in Korean patients with AA.

Materials and methods

Population

A total of 94 patients who were diagnosed as having AA based on bone marrow exams at Seoul National University Hospital from 1997 to 2012 were included in this study. As controls, 195 healthy subjects were included [18]. Patients were classified into the severe aplastic anemia (SAA) group if they had at least two of the following three peripheral blood findings: (i) absolute neutrophil count (ANC) <0.5 × 10⁹/L, (ii) platelet count <20 × 10⁹/L, or (iii) corrected reticulocyte count <1%, and (iv) no treatment or follow-up loss. IST included: rabbit anti-thymocyte globulin (ATG) (Thymoglobulin®, SangStat, Lyon, France) alone (n = 34), (ii) cyclosporin A (CsA) alone (n = 6), or (iii) combination of rabbit ATG or horse anti-lymphocyte globulin (ALG) (Lymphoglobulin®, SangStat, Lyon, France) with CsA (n = 34) (four patients with ALG and 30 patients with ATG). The response to IST was evaluated at 6 months. The patients with complete or partial response to IST were defined as responders. Complete responders were the patients that achieved all of the three following conditions: (i) hemoglobin greater than 10 g/dL; (ii) ANC greater than 1 x 10⁹/L; and (iii) platelet count >100 × 10⁹/L without transfusion. Partial responders were attained improvement in any of the following: (i) no need for red blood cell transfusions, (ii) ANC increment by ≥0.5 × 10⁹/L above the baseline, or (iii) platelet count increment by ≥30 × 10⁹/L above the baseline. Non-responders were those not meeting the criteria for complete or partial response [19,20].

Specimen collection and DNA extraction

A total of 289 DNA samples extracted from the peripheral blood of patients and controls using the LaboPass Genomic DNA Extraction Kit (COSMO, Seoul, Korea) or QuickGene DNA whole blood kit (Fujifilm, Tokyo, Japan) and kept in −80°C were used for these analyses.

Genotyping of FoxP3 polymorphisms

Genotyping of FoxP3 polymorphisms (rs5902434 del/ATT, rs3761548 C/A, rs3761549 C/T, and rs2232365 A/G) was performed using the polymerase chain reaction (PCR)-sequencing method [12,21] (Table 1). PCR was performed in a volume of 40 µL, containing 40 ng DNA, 0.2 µM each of the forward and reverse primers, 0.8 µL of 10 mM dNTP, 1.0 U Taq DNA polymerase, and 4 µL reaction buffer. PCR reactions included an initial denaturing step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Sequencing PCR was performed with a total of 10 µL reaction mixture containing 1 µL purified PCR product, 1 µL forward or reverse primers (F1, R2, F3, and F4) and 4 µL BigDye Terminator Ready Reaction Mix (Life technologies, Grand Island, NY, U.S.A) with distilled water. DNA sequencing was performed on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, U.S.A) and sequences were analyzed using Sequencher Software (Gene Codes, Ann Arbor, MI, U.S.A).

HLA-DR typing

Low-resolution HLA-DR typing for all DNA samples from patients and controls was performed using the Dynal RELI sequence specific oligonucleotide HLA Test kit (Dynal Biotech, Wirral, UK) or WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan) according to manufacturer’s instructions.

Statistical analysis

All four polymorphisms were tested for Hardy-Weinberg equilibrium. The genotype and allele frequencies of FoxP3 polymorphisms were obtained using the direct counting method in patients and controls. We also investigated the differences in genotype and allele frequencies between responders and non-responders to IST. Since the FoxP3 gene is located on the X chromosome, genotype frequencies were analyzed according to sex and allele frequencies were analyzed including men and women together. The significance of differences in genotype and allele frequencies were evaluated using the chi-square test or Fisher’s exact test and P < 0.05 was regarded as statistically significant. SPSS for Windows version 22.0 (SPSS, Inc., Chicago, IL, U.S.A) was used for statistical analysis.

Results

Population characteristics

The demographic characteristics of AA patients are summarized in Table 2. Among the 94 patients, 45 were male (47.9%) and 49 were female (52.1%). Forty-four patients (nine patients with NSAA and 35 with SAA) were treated with IST and among them, 29
patients (65.9%) showed complete or partial response. The 195 control individuals consisted of 95 (48.7%) men and 100 (51.3%) women.

**Association with FoxP3 polymorphism and AA**

All four polymorphisms were shown to be in Hardy-Weinberg equilibrium. Table 3 provides the distribution of FoxP3 polymorphisms in AA patients and controls. There were no differences in genotype frequencies of FoxP3 polymorphisms between patients and controls in each sex. In allele frequency analysis, the rs3761548 C allele frequency was significantly higher in the patients than in the controls (87.4% vs. 79.7%, \( P = 0.047, \text{OR} = 1.8, 95\% \text{ CI} 1.0–3.1\)).

**Association with FoxP3 polymorphism and response to IST in AA patients**

Among 44 patients treated with IST, we performed FoxP3 gene polymorphism analysis between responders and non-responders (Table 4). In female patients, genotype frequency of rs3761549 C/C was significantly higher in non-responders than in responders (84.2% vs. 16.7%, \( P = 0.006, \text{OR} = 26.7, 95\% \text{ CI} 2.2–317.1\)). There were no differences in the allele frequencies in male patients. The frequency of the rs3761549 C allele was significantly higher in non-responders than in responders (89.6% vs. 66.7%, \( P = 0.036, \text{OR} = 4.3, 95\% \text{ CI} 1.2–15.7\)).

**Association with FoxP3 gene polymorphism and response to IST in AA patients according to HLA-DR15 typing**

When the HLA-DR15 alleles were analyzed among 44 patients treated with IST, 20 patients (45.5%) were positive for HLA-DR15 and 24 patients (54.5%) were negative. The allele frequency of HLA-DR15 was significantly higher in responders than in non-responders.

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**Table 1. Primers used in the FoxP3 genotyping by PCR-sequencing.**

| Polymorphisms       | Primer | Length (bps) | AT (°C) |
|---------------------|--------|--------------|---------|
| rs5902434 (del/ATT) | F1     | 5′-CTGCTCTCCCTACCAAGATG-3′ | 196     | 56 |
|                     | R1     | 5′-CCCTGCCCATGCTTAAGTA-3′ |          |     |
| rs3761548 (C/A)     | F2     | 5′-TGCTCTACCTCAAGGTCCTCC-3′ | 373     | 60 |
|                     | R2     | 5′-TGCTCTATACCAACCGG-3′     |          |     |
| rs3761549 (C/T)     | F3     | 5′-GTCTCTCCACAAACCTCTAA-3′ | 250     | 60 |
|                     | R3     | 5′-CAAGATTTTTCCGCCATGAC-3′ |          |     |
| rs2232365 (A/G)     | F4     | 5′-GGAGCTTTCAGTGAGGA-3′     | 371     | 60 |
|                     | R4     | 5′-GGAGGTTGGATAGGTCAGG-3′   |          |     |

F: forward primer; R: reverse primer; AT: annealing temperature.

**Table 2. Clinical characteristics of aplastic anemia patients (n = 94).**

| Characteristics                  | Patients (%) |
|----------------------------------|--------------|
| Sex (male/female)                | 45:49        |
| Age (median)                     | 22           |
| Severity (NSAA/SAA)              | 19:75        |
| Treatment                        |              |
| Oxymetholone alone               | 5 (5.3)      |
| Immunosuppressive therapy        |              |
| ATG alone                        | 4 (4.3)      |
| ATG/ALG + CsA                    | 34 (36.2)    |
| CsA alone                        | 6 (6.4)      |
| HSCT                             | 20 (21.3)    |
| No treatment or F/U loss         | 25 (26.6)    |

NSAA: non-severe aplastic anemia; SAA: severe aplastic anemia; ATG: anti-thymocyte globulin; ALG: anti-lymphocyte globulin; CsA: cyclosporin A; HSCT: hematopoietic stem cell transplantation.

**Table 3. Genotype and allele frequencies of Foxp3 polymorphisms in aplastic anemia patients (n = 94) and controls (n = 195).**

| Polymorphisms       | Patients (%) | Controls (%) | \( P \) value | Odds ratio |
|---------------------|--------------|--------------|---------------|------------|
| rs5902434 Genotype (F) |              |              |               |            |
| del/del             | 18 (36.7)    | 34 (34.0)    | 0.288         |            |
| del/ATT             | 25 (51.0)    | 43 (43.0)    |               |            |
| ATT/ATT             | 6 (12.2)     | 23 (23.0)    |               |            |
| Genotype (M) del    | 32 (71.1)    | 65 (68.4)    | 0.747         |            |
| ATT                 | 13 (28.9)    | 30 (31.6)    |               |            |
| Alleles C del       | 65.0         | 59.7         | 0.279         |            |
| ATT                 | 35.0         | 40.3         |               |            |
| rs3761548 Genotype (F) |              |              |               |            |
| C/C                 | 35 (71.4)    | 60 (60.0)    | 0.308         |            |
| C/A                 | 13 (26.5)    | 34 (34.0)    |               |            |
| A/A                 | 1 (2.0)      | 6 (6.0)      |               |            |
| Genotype (M) C      | 42 (93.3)    | 81 (85.3)    | 0.172         |            |
| T                   | 3 (6.7)      | 14 (14.7)    |               |            |
| Alleles C           | 87.4         | 79.7         | 0.047         | 1.8 (1.0–3.1) |
| A                   | 12.6         | 20.3         |               |            |
| rs3761549 Genotype (F) |              |              |               |            |
| C/C                 | 31 (63.3)    | 62 (62.0)    | 0.966         |            |
| C/T                 | 16 (32.7)    | 33 (33.0)    |               |            |
| T/T                 | 2 (4.1)      | 5 (5.0)      |               |            |
| Genotype (M) C      | 35 (77.8)    | 79 (83.2)    | 0.445         |            |
| T                   | 10 (22.2)    | 16 (16.8)    |               |            |
| Alleles C           | 79.0         | 80.0         | 0.811         |            |
| T                   | 21.0         | 20.0         |               |            |
| rs2232365 Genotype (F) |              |              |               |            |
| A/A                 | 18 (36.7)    | 34 (34.0)    | 0.288         |            |
| A/G                 | 25 (51.0)    | 43 (43.0)    |               |            |
| G/G                 | 6 (12.2)     | 23 (23.0)    |               |            |
| Genotype (M) A      | 32 (71.1)    | 65 (68.4)    | 0.747         |            |
| G                   | 13 (28.9)    | 30 (61.3)    |               |            |
| Alleles A           | 65.0         | 59.7         | 0.279         |            |
| G                   | 35.0         | 40.3         |               |            |

F: female; M: male
Table 4. Comparison of genotype and allele frequencies for FoxP3 polymorphisms between non-responders (n = 15) and responders (n = 29) in aplastic anemia patients treated with IST (n = 44).

| Polymorphisms | Non-responder (%) | Responder (%) | P value | Odds ratio |
|---------------|------------------|--------------|---------|------------|
| rs5902434     |                  |              |         |            |
| Genotype (F)  |                  |              |         |            |
| del/del       | 7 (36.8)         | 1 (16.7)     | 0.643   |            |
| del/ATT       | 10 (52.6)        | 4 (66.7)     |         |            |
| ATT/ATT       | 2 (10.5)         | 1 (16.7)     |         |            |
| Genotype (M)  |                  |              |         |            |
| del           | 8 (80.0)         | 6 (66.7)     | 0.628   |            |
| ATT           | 2 (20.0)         | 3 (33.3)     |         |            |
| Alleles del   |                  |              |         |            |
| ATT           | 33.3             | 42.9         |         |            |
| rs3761548     |                  |              |         |            |
| Genotype (F)  |                  |              |         |            |
| C/C           | 11 (57.9)        | 5 (83.3)     | 0.507   |            |
| C/A           | 7 (36.8)         | 1 (16.7)     |         |            |
| A/A           | 1 (5.3)          | 0 (0.0)      |         |            |
| Genotype (M)  |                  |              |         |            |
| C             | 9 (90.0)         | 8 (88.9)     | 1.000   |            |
| T             | 1 (10.0)         | 1 (11.1)     |         |            |
| Alleles C     |                  |              |         |            |
| C             | 79.2             | 90.5         | 0.321   |            |
| A             | 20.8             | 9.5          |         |            |
| rs3761549     |                  |              |         |            |
| Genotype (F)  |                  |              |         |            |
| C/C           | 16 (84.2)        | 1 (16.7)     | 0.006*  | 26.7 (2.2–317.1) |
| C/T           | 2 (10.5)         | 5 (83.3)     |         |            |
| T/T           | 1 (5.3)          | 0 (0.0)      |         |            |
| Genotype (M)  |                  |              |         |            |
| C             | 9 (90.0)         | 7 (77.8)     | 0.528   |            |
| T             | 1 (10.0)         | 2 (22.2)     |         |            |
| Alleles C     |                  |              |         |            |
| C             | 89.6             | 66.7         | 0.036   | 4.3 (1.2–15.7) |
| T             | 10.4             | 33.3         |         |            |
| rs2223265     |                  |              |         |            |
| Genotype (F)  |                  |              |         |            |
| A/A           | 8 (42.1)         | 0 (0.0)      | 0.155   |            |
| A/G           | 9 (47.4)         | 5 (83.3)     |         |            |
| G/G           | 2 (10.5)         | 1 (16.7)     |         |            |
| Genotype (M)  |                  |              |         |            |
| A             | 8 (80.0)         | 6 (66.7)     | 0.628   |            |
| G             | 2 (20.0)         | 3 (33.3)     |         |            |
| Alleles A     |                  |              |         |            |
| A             | 68.7             | 52.4         | 0.193   |            |
| G             | 31.3             | 47.6         |         |            |

F: female; M: male.

* C/C vs. C/T+T/T (84.2% vs. 16.7%, P = 0.006, OR = 26.7, 95% CI 2.2–317.1).

(66.7% vs. 34.5%, P = 0.042, OR = 3.8, 95% CI 1.0–14.2).

In patients with HLA-DR15 there was no significant difference in allele frequency of rs3761549 C between non-responders and responders, whereas the rs3761549 C allele frequency was significantly higher in non-responders compared to responders among HLA-DR15-negative patients (93.8% vs. 62.5%, P = 0.046, OR = 9.0, 95% CI 1.2–68.1).

### Discussion

Tregs expressing the IL-2 receptor α-chain (CD25) on the cell surface account for approximately 5–10% of CD4+ T cell populations and actively suppress autoreactive T cells [22]. Decreased number and function of Tregs were observed in AA patients [6,7]. The function of Tregs is achieved through the FoxP3 protein, and Fontenot et al. [8] reported the essential role of the FoxP3 gene in development and activation of Tregs. High expression of FoxP3 is necessary to maintain the suppressive function of Tregs, and FoxP3 act as both a transcriptional activator and a repressor by interacting with other transcriptional factors such as NFAT, RUNX1, and ROR-γt [23,24].

In this study, we observed the association between FoxP3 rs3761548 C allele and AA. The associations between rs3761548 polymorphism and various diseases have been described in many studies with controversial results [9]. Systemic lupus erythematosus patients in Taiwan carrying rs3761548 A allele showed lower anti-dsDNA levels [10] and genotype frequency of rs3761548 C/A+A/A were higher in Chinese allergic rhinitis patients [25], which implicate the risk of C alleles and are similar with our results. In contrast, Foxp3 rs3761548 C/A+A/A genotypes were associated with increased risk of psoriasis in Chinese [26]. Rs3761548 A/A genotype was related with unexplained recurrent spontaneous abortion [13]. Allele frequency of rs3761548 A and genotype frequency of rs3761548 C/A+A/A were higher in Chinese vitiligo patients [27]. Polymorphisms of FOXP3 gene promoter may alter the binding specificity of transcription factors and are relevant to initiating transcription, therefore, might affect the function or quantity of Treg [28], resulting in various autoimmune diseases. The reason why the impacts are various in different disease and ethnic groups should be further elucidated.

There may be other causes of reduced Tregs in AA, including various genetic reasons as well as rs3761548 polymorphism. NFAT1 related to activity of T cells have been reported to be reduced in patients with AA, followed by reduced expression of FoxP3 [6,23]. Further studies on other genes which can affect the function of Treg including NFAT1 might be needed.

In addition, the FoxP3 rs3761549 polymorphism was associated with response to IST. In Korean females with AA, the frequency of the FoxP3 rs3761549 C/C genotype was higher than that in controls. The rs3761549 C allelic frequency was significantly higher in patients with AA than in controls, and was about four times more at risk. Increased risk of rs3761549 C allele was suggested in patients with hepatocellular carcinoma [29,30], non-small cell lung cancer [30], and female psoriasis vulgaris patients [31]. In Japanese patients with Hashimoto’s disease (HD), rs3761549 C was not associated with development of HD, however, was significantly associated with severity of HD [32]. Weaker binding of transcriptional factor Yin-Yang-1 with FoxP3 might cause stronger autoimmune response and lower response to IST.
In AA, HLA-DR15 is the most well-known genetic predisposition associated with a good response to IST [33] and in this study, the frequency of HLA-DR15 was also significantly higher in responders than in non-responders. With regards to the FoxP3 rs3761549 polymorphism, the frequency of the rs3761549 C allele was significantly higher in responders than in non-responders among HLA-DR15-negative patients, which might suggest a role of rs3761549 polymorphism in addition to HLA-DR15 in the responsiveness to IST. Our study has several limitations, such as small number of patients, retrospective design, and heterogeneity of treatment protocols. Further studies in larger number of patients and other ethnic groups are required to confirm our findings.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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