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소아 급성 백혈병 환자에서 *FOXP3* 유전자 다형성과 동종 조혈모세포 이식 후 간정맥폐색성질환과 사이토메갈로바이러스감염의 위험성

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협동과정 종양생물학전공

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FOXP3 polymorphism increases risk of hepatic veno-occlusive disease and CMV infection after allogeneic hematopoietic stem cell transplantation in pediatric acute leukemia patients

by

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ABSTRACT

FOXP3 polymorphism increases risk of hepatic veno-occlusive disease and CMV infection after allogeneic hematopoietic stem cell transplantation in pediatric acute leukemia patients

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Introduction: Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative therapeutic option for high-risk acute leukemia, but it can cause severe complications. Homeostasis of immune function plays a key role in the development of some of these complications, and regulatory T cells (Tregs) are known to have an important role in maintaining the immune homeostasis.

Expression of *Forkhead BOX P3* (*FOXP3*) is essential for the development of Tregs. The main aim of this study was to evaluate the potential influence of *FOXP3* rs3761548 polymorphisms of donor on the outcomes of the HSCT.

Methods: A total of 171 patients were enrolled in this study and genotyping was done with PCR and direct sequencing using the post-transplant samples, which showed the complete replacement of hematopoietic cells by donor. We grouped the patients according to the genotype and compared the clinical outcomes using the Log-rank test and Gray test.

Results: The patients who received HSCT from the donor with rs3761548 CC genotype had higher incidence of hepatic veno-occlusive disease (HVOD) and cytomegalovirus (CMV) infection compared to those of AA or AC genotype group (24.9% vs. 8.5%, P=0.011 and 66.3% vs. 47.6%, P=0.023). Treatment-related mortality (TRM) rate of patients with AA or AC genotype was lower than that of the other patients with CC genotype (3.4% vs. 14.2%, P=0.044) resulting in the difference of overall survival. There was no difference in graft-versus-host disease (GVHD), relapse or blood stream infection (BSI) according to the rs3761548

genotype. In multivariate analysis, CC genotype maintained its statistical significance in the development of HVOD (HR=3.97, 95% CI=1.47−10.74) and CMV infection (HR=1.88, 95% CI=1.19−2.97), showing lower overall survival.

Conclusions: This is the first report on *FOXP3* rs3761548 SNP in allogeneic HSCT and this SNP can be considered as a candidate marker for predicting the development of HVOD and CMV infection after allogeneic HSCT.

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Keywords: FOXP3, rs3761548, hematopoietic stem cell transplantation, acute leukemia

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List of abbreviations and symbols

BSI blood stream infection

CMV cytomegalovirus

FOXP3 Forkhead BOX P3

GVHD graft-versus-host disease

HSCT hematopoietic stem cell transplantation

HVOD hepatic veno-occlusive disease

OS overall survival

PCR polymerase chain reaction

SNP single nucleotide polymorphism

Treg regulatory T cell

TRM treatment-related mortality

Introduction

Leukemia is one of the hematological malignancies and is the most common cancer in Korean pediatric patients[1]. It is classified into four main groups according to cell types: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML). Acute leukemia is the highest incidence malignancy in children and adolescents[2]. The improvement of treatment on leukemia is associated with intensification, better supportive therapies and a risk-based treatment approach[3]. Therefore, taking the intensive treatment to the high-risk patients who classified according to prognostic factors is very important. With the further understanding of the mechanism of leukemia, the prognostic factors transformed from clinical characteristic into cytogenetic abnormalities[3, 4]. Table 1 and 2 were listed the prognostic factors for ALL and AML.

Despite significant improvement in the treatment outcomes of these diseases over the past several decades, the prognosis for certain high-risk groups of leukemia and for relapsed disease remains poor[5] and allogeneic hematopoietic stem cell transplantation (allo-HSCT) may be the only curative option for high-risk pediatric acute leukemia patients[6].

Allo-HSCT is a curative therapy for various diseases such as hematologic malignancies, bone marrow failure and congenital immunologic diseases. It is a high-risk procedure and can have severe complications such as graft failure, graft-versus-host disease (GVHD), hepatic veno-occlusive disease (HVOD) and infection. According to hematopoietic stem cells sources, HSCT can be divided bone marrow transplantation (BMT), peripheral blood stem cell transplantation (PBSCT) and cord blood transplantation (CBT). BMT has been reported to correlate with fewer cases of GVHD and treatment-related mortality (TRM), as well as with improved survival, but with an increased risk of relapse. While PBSCT correlate with faster engraftment and immune reconstitution and better disease control by an enhanced graft-versus-leukemia (GVL) effect, which simultaneously increases the risk of GVHD and TRM[7]. Compare to BMT, CBT has the lower rates of acute and chronic GVHD, but had high rates of TRM[8].

With the developments in medicine, the incidences of complications have decreased markedly, but significant numbers of patients still suffer from the complications. Homeostasis of immune function plays a key role in the development of some of these complications such as graft failure and GVHD, and regulatory T

cells (Tregs) are known to have an important role in maintaining the immune homeostasis. Tregs belong to a $CD4^+$ T-cell population and account for 5~10% of circulating $CD4^+$ T-cells[9, 10]. Tregs are essential in the maintenance of self-tolerance and immune homeostasis[11], and play a decisive role in transplantation tolerance[12].

FOXP3 is a member of transcription factor winged-helix family and positioned at the Xp11.23 locus on the X-chromosome[13, 14]. Expression of FOXP3 is essential for the development of Tregs and their suppressor function[15]. Continued expression of FOXP3 in mature Tregs is necessary for suppressive function and loss of FOXP3 expression in Tregs is associated with autoimmune pathology[16].

FOXP3 has a size of 14,392 base pairs with 12 exons[17]. Until now, 90 single nucleotide polymorphisms (SNP) of *Homo sapiens* have been registered in the SNP database. Among these SNPs, rs3761548 (NG_007392.1:g.8048A>C) polymorphism is located on the promoter region of *FOXP3* and is one of the most commonly seen SNPs in the population. These SNPs located in the promoter region of *FOXP3* are known to have an effect on the expression as well as transcriptional activity the protein[18] and its presence results in lack of Treg function[19]. Previous studies of rs3761548

polymorphisms have shown that it was associated with the development of lung cancer[19], breast cancer[20], and colorectal cancer[21] as well as autoimmune diseases, recurrent spontaneous abortion[22], autoimmune thyroid diseases[23], vitiligo[24], systemic sclerosis[25] and allergic rhinitis[26].

A few studies have demonstrated a role of Tregs in the outcome of allogeneic HSCT[27], but the effect of rs3761548 polymorphism on the outcomes of allo-HSCT has not been investigated. In this study, we evaluated the clinical impact of *FOXP3* rs3761548 polymorphism in the donor on the outcomes of allo-HSCT in Korean pediatric patients.

Table 1 Prognostic features for childhood ALL

Favorable prognostic features	Unfavorable prognostic features
Clinical	Clinical
Age >1<10 years	Age <1 year or Age ≥10 years*
WBC $<50\times10^9$/L	WBC $\geq50\times10^9$/L
NCI SR	NCI HR
Caucasian, Asian, Pacific Islander	Black, Native American, Alaskan Native, Hispanic
CNS1	CNS3
Response to chemotherapy	Response to chemotherapy
PGR	PPR
Day 7 BM M1/M2 by morphology	Day 7 BM M3 by morphology
Day 14 BM M1 by morphology	Day 14 BM M2/M3 by morphology
End induction BM M1 by morphology	End induction BM M2/M3 by morphology
Day 8 PB MRD <0.01%	Day 8 PB MRD ≥0.01%
End induction BM MRD <0.01%	End induction BM MRD ≥0.01%
End consolidation BM MRD <0.1% (T-ALL only)	End consolidation BM MRD ≥0.1% (T-ALL only)
Blast biology	Blast biology
B-cell immunophenotype	T-cell immunophenotype †
ETV-RUNX1	*BCR-ABL* ‡
high hyperdiploid	*MLL-r*
trisomy +4,+10	*TCF3-HLF*
	iAMP21
	hypodiploid <44 chromosomes
	BCR-ABL1-like (Ph^+-like) expression profile
	CRLF2r §
	IKZF1 mutation or deletion
	TP53 mutation

M1: <5% lymphoblasts by morphology. M2: 5–25% lymphoblasts by morphology. M3 > 25% lymphoblasts by morphology. Prednisone good response (PGR): $<1\times10^9$ leukemic blasts/l in peripheral blood after a 1 week prednisone prophase. Prednisone poor response (PPR): ≥ 1×10^9 leukemic blasts/l in peripheral blood after a 1-week prednisone prophase. Other definitions and abbreviations are provided in main text. Some of these features lose prognostic value in multivariate analysis. MRD is a continuous variable, and some cooperative groups use different cut-offs to define risk. The thresholds provided in the Table are ones confirmed and used by multiple independent groups.

*Age ≥ 16 years worse than 10–15.

† ETP phenotype particularly poor outcome.

‡ Improved survival with addition of TKIs.

§ Only unfavorable prognosis for *CRLF2r* if NCI HR.

Table 2 Risk group classification according to the presence of the most frequent cytogenetic abnormalities

Risk group	Cytogenetic abnormalities
Favorable prognosis	CBF leukemia
	t(8;21)(q22;q22)
	inv 16(p13;q22)
	t(15;17)
Intermediate prognosis	Other abnormalities
Adverse prognosis	Monosomy of chromosome 5 and 7,
	t(9;22),
	abn (5q),
	abn (3q),
	MLL rearrangement in t(9;11) with additional aberrations,
	MLL rearrangements other than t(9;11) and t(11;19),
	t(6;9)(p23;q34),
	Aberrations involving 12p without favorable genetics,
Adverse prognosis	complex karyotypes (≥5 abnormalities) in the absence of favorable-risk features

CBF: Core-binding factor; MLL: Mixed lineage leukemia.

Patients and Methods

Patients

Patients enrolled in this study underwent HSCT between January 2002 and May 2014 at Seoul National University Hospital. Peripheral blood or bone marrow aspiration samples stored in the human-derived sample storage (IRB No. 1102-085-353) were selected based on the following inclusion criteria: 1. patients who were diagnosed with acute leukemia and received allo-HSCT; 2. patients who had post-transplant samples showing complete replacement of hematopoietic cells by cells from the donor (short tandem repeat (STR) test on patients' DNA < 0.8%), so that the genotype of donors could be determined.

After quality control, 171 post-transplant samples were included for genotyping. This study was approved by the Institutional Review Board for Human Research of Seoul National University (IRB No. 1411-008-621).

Transplantation Protocol

Donors selected before 2005 were based on human leukocyte antigen (HLA) serologic typing performed for class I antigens and

HLA molecular typing for the DR loci. From 2006, typing for the DQ loci was included. HLA-A, -B, -C, -DR, and -DQ were confirmed by a high-resolution molecular method for both recipients and donors.

The major conditioning regimens used were BuFluVP and TBIAcFlu. BuFluVP consisted of drugs busulfan, fludarabine (40 mg/m2 once daily *i.v.* on days -8 ~ -3) and etoposide (20 mg/kg once daily *i.v.* on days -4 ~ -2). Busulfan (120 mg/m2 for patients ≥ 1 year and 80 mg/m2 for < 1 year) was administered once daily with the first dose on day -8, and subsequent doses of busulfan was adjusted according to the therapeutic drug monitoring results from day -7 ~ -5[28]. TBIAcFlu composed of total body irradiation (300cGy once daily on days -9 ~ -6) and administration of arabinosylcytosine (3g/m2 twice daily *i.v.* on days -5 and -4) and fludarabine (50mg/ m2 once daily *i.v.* on days -5 ~ -2).

Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine plus prednisolone for related HSCT, cyclosporine plus mycophenolate mofetil for CBT, or tacrolimus plus methotrexate for unrelated BMT/PBSCT. HVOD and infection prophylaxis were administered according to the guidelines for HSCT of our center[29]. Patients received lipo-PGE1 (alprostadil, Eglandin®; Welfide,

Osaka, Japan) at a dose of 1 mcg/kg/day through continuous infusion for prophylaxis of VOD with or without low molecular weight heparin (nadroparine calcium, Fraxiparine®; GlaxoSmithKline, United Kingdom). Patients received ciprofloxacin, itraconazole or micafungin, and acyclovir as a prophylaxis for infection. Intravenous immune globulin (0.5 g/kg/dose) was infused every 2 weeks until day 100 then monthly until day 180. Sulfamethoxazole/trimethoprim was discontinued 3 days before HSCT and restarted after engraftment.

Chimerism analysis

Serial analysis of short tandem repeats (STR) in bone marrow (BM) aspirates were performed to evaluate the hematopoietic chimerism in patients at 1, 3 and 6 months and 1 year after the allo-HSCT excluding patients who underwent CBT. For patients who underwent CBT, the chimerism analysis was performed more frequently beginning at 1, 2 and 3 weeks from peripheral blood and 1, 3, 6 months and 1 year from BM aspirate. The chimerism analysis was based on the quantitative amplification of specific STR regions in the patient and donor by using AmpFlSTR profiler polymerase chain reaction (PCR) amplification kit (Applied

Biosystems, Foster City, CA, USA)[30].

DNA extraction

Genomic DNA was extracted from peripheral blood or bone marrow samples by using Qiagen kit (QIAamp® DNA Blood Mini Kit, QIAGEN, Hilden, Germany). Pipet 40 μl QIAGEN protease in to the bottom of a 1.5 ml microcentrifuge tube and add 400 μl sample to the tube. Add 400 μl Buffer AL to the sample. Mix by pulse-vortexing for 15 s. incubate at 56℃ for 10 min. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid and add 400 μl ethanol (96 ~ 100%) to the sample, mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid. Carefully apply the mixture sample to the QIAamp Mini spin column without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. Open the QIAamp Mini spin column and add 700 μl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 8000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube. Add 700 μl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed

for 3 min. Place the column in a new 2 ml collection tube and centrifuge at full speed for 1 min. Place the column a clean 1.5 ml tube add 60 μl Buffer AE. Incubate at room temperature for 1 min, and then centrifuge at 8000 rpm for 1 min. DNA purity and concentration were determined by spectrophotometric measurements of absorbance at 260 nm and 280 nm.

FOXP3 gene amplification and genotyping

FOXP3 gene amplification was performed via polymerase chain reaction (PCR). The sequences of forward and reverse primers used are 5'-GCCCTTGTCTACTCCACGCCTCT-3' and 5'-CAGCCTTCGCCAATACAGAGCC-3' respectively. Twenty-five μl PCR mixture (Solg™ 2X Taq PCR Pre-Mix, SolGent, Daeieon, Korea) containing 50~100 ng of genomic DNA was coupled with 1 μl of forward primer and 1 μl of reverse primer. The parameters for the PCR cycles were set at: initial denaturation at 95℃ for 2 min followed by 35 cycles of 95℃ for 20 s, 68℃ for 40 s, 72℃ for 60 s, with the final cycle of extension at 72℃ for 5 min.

PCR products were purified with PCR purification kit (QIAquick® PCR Purification Kit, QIAGEN, Hilden, Germany) and genotyped by direct sequencing using BigDye(R) Terminator v3.1 Cycle

Sequencing kits (Applied Biosystems, Foster City, USA) on the ABI PRISM 3730XL Analyzer (Applied Biosystems, Foster City, USA).

Definitions

The diagnosis of HVOD was based on the modified Seattle criteria [31] (Table 3), while acute graft-versus-host disease (aGVHD) and chronic graft-versus-host disease (cGVHD) were diagnosed according to standard criteria[32, 33] (Table 4 and 5). Treatment-related mortality (TRM) was defined as death not attributed to relapse after HSCT. Blood stream infection (BSI) was defined as the isolation of a bacterial or fungal pathogen from at least 1 blood culture. Cytomegalovirus (CMV) infection was defined as the presence of CMV pp65 antigen positive cells at a frequency of $\geq 1/2 \times 10^5$cells and the diagnosis of CMV disease was made by histopathological examinations or ophthalmoscopy exam showing characteristic retinal changes. Neutrophil engraftment was defined as achievement of absolute neutrophil counts >500/mm^3 on at least 3 consecutive days after HSCT.

Statistical analysis

Statistical calculations were performed with SPSS statistical

software (version 22) and R statistical software (version 3.1.2). The Kaplan-Meier method and Log-rank test were used for univariate survival analysis. Cumulative incidences (CI) were analyzed with Gray test. Competing events were defined as death without aGVHD for aGVHD; death without cGVHD for cGVHD; death without HVOD for HVOD; death without relapse for relapse; death without BSI for BSI; death without cytomegalovirus (CMV) infection for CMV infection. Reconstitution of T cells were represented as box-plot, and t-test was used to compare between genotypes at specified time intervals. Factors that gave a statistically significant difference upon univariate analysis were included in multivariate analysis. Differences were considered significant if P value <0.05.

Table 3 modified Seattle criteria

diagnostic criteria
1. Serum bilirubin > 34 umol/L (> 2mg/dL)
2. Hepatomegaly with right upper quadrant pain
3. > 2% weight gain from baseline due to fluid retention
At least two of the following, occurring within 20d of transplantation:

Table 4 Acute GVHD criteria

Stage	Skin	Liver (bilirubin)	Gut (stool output/day)
0	No GVHD rash	< 2 mg/dl	< 500 ml/day or persistent nausea
1	Maculopapular rash< 25% BSA	2 – 3 mg/L	500–999 ml/day
2	Maculopapular rash< 25 – 50% BSA	3.1 – 6mg/dl	1000–1500 ml/day
3	Maculopapular rash> 50% BSA	6.1 – 15mg/dl	Adult: >1500 ml/day
4	Generalized erythroderma plus bullous formation	> 15 mg/dl	Severe abdominal pain with or without ileus
Grade			
I	Stage 1 – 2	None	None
II	Stage 3 or	Stage 1 or	Stage 1
III	–	Stage 2 – 3 or	Stage 2 –4
IV	Stage 4 or	Stage 4	–

GVHD, graft–versus–host disease; BSA, body surface area

Table 5 chronic GVHD criteria

Organ or site	Diagnostic (sufficient to establish the diagnosis of chronic GVHD)	Distinctive (Seen in Chronic GVHD, but Insufficient Alone to Establish a Diagnosis of Chronic GVHD)	Other Features*	Common (Seen with Both Acute and Chronic GVHD)
skin	Poikiloderma Lichen planus–like features Sclerotic features Morphea–like features Lichen sclerosus–like features	Depigmentation	Sweat impairment Ichthyosis Keratosis pilaris Hypopigmentation Hyperpigmentation	Erythema Maculopapular rash Pruritus
Nails		Dystrophy Longitudinal ridging, splitting, or brittle features Onycholysis Pterygium unguis Nail loss (usually symmetric; affects most nails) †		
Scalp and body hair		New onset of scarring or nonscarring scalp alopecia (after recovery from chemoradiotherapy) Scaling, papulosquamous lesions	Thinning scalp hair, typically patchy, coarse, or dull (not explained by endocrine or other causes) Premature gray hair	
Mouth	Lichen–type features Hyperkeratotic plaques Restriction of mouth opening from sclerosis	Xerostomia Mucocele Mucosal atrophy Pseudomembranes † Ulcers †		Gingivitis Mucositis Erythema Pain
Eyes		New onset dry, gritty, or painful eyes ‡ Cicatricial conjunctivitis Keratoconjunctivitis sicca ‡ Confluent areas of punctate keratopathy	Photophobia Periorbital hyperpigmentation Blepharitis (erythema of the eyelids with edema)	
Genitalia	Lichen planus–like features Vaginal scarring or stenosis	Erosions † Fissures † Ulcers †		
GI tract	Esophageal web Strictures or stenosis in the upper to mid third of the esophagus †		Exocrine pancreatic insufficiency	Anorexia Nausea Vomiting Diarrhea Weight loss Failure to thrive (infants and children)
Liver				Total bilirubin, alkaline phosphatase >2 upper limit of normal † ALT or AST >2 upper limit of normal †

Lung	Bronchiolitis obliterans diagnosed with lung biopsy	Bronchiolitis obliterans diagnosed with PFTs and radiology ‡		BOOP
Muscles, fascia, joints	Fasciitis Joint stiffness or contractures secondary to sclerosis	Myositis or polymyositis †	Edema Muscle cramps Arthralgia or arthritis	

GVHD, graft-versus-host disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BOOP, bronchiolitis obliterans- organizing pneumonia; PFTs, pulmonary function tests; AIHA, autoimmune hemolytic anemia; ITP, idiopathic thrombocytopenic purpura.

*Can be acknowledged as part of the chronic GVHD symptomatology if the diagnosis is confirmed.

† In all cases, infection, drug effects, malignancy, or other causes must be excluded.

‡ Diagnosis of chronic GVHD requires biopsy or radiology confirmation (or Schirmer test for eyes).

Results

Characteristics of patients

Patient characteristics are summarized in Table 6. Patients received allo-HSCT in complete remission (CR) 1, CR2, CR >2 or in advanced disease were 119 (69.6%), 34 (19.9%) and 18 (10.5%), respectively. Related and unrelated donors were 43 (25.1%) and 128 (74.9%) respectively, including 100 (58.5%) males and 71 (41.5%) females.

HSCT complications

Thirty-two (CI 19.2%) patients developed HVOD. CI of aGVHD was 41.7% with 44 patients developing grade II aGVHD, 20 patients with grade III and 5 patients with grade IV aGVHD. Forty (CI 26.2%) patients developed cGVHD; BSI occurred in 56 patients with 33.7% of CI. A hundred and ten patients (CI 70.0%) developed CMV infection and 8 of them were diagnosed as having CMV disease.

The CI of HVOD was classified according to each genotype (AA, AC and CC) as shown in Figure 1-A. Curves for AA, AC and CC were sequentially arranged and it was observed that the AC curve was very similar to the AA curve. Therefore, AA and AC genotypes together will be considered a group for some of the following

analyses. The AA or AC genotype group was shown to have lower CI of HVOD than the CC genotype group (8.5% vs. 24.9%, P=0.011, Figure 1-B).

Similarly, the three genotypes curves were arranged sequentially for CMV infection (Figure 2-A) and it was observed that the AA or AC group had lower CI in the case of CMV infection as compared to the CC genotype group (47.6% vs. 66.3%, P=0.023, Figure 2-B). However, this difference could not be observed for CMV disease (10.3% vs. 30.8%, P=0.592).

In the case of acute and chronic GVHD (Figure 3), the results showed no statistical differences between AA or AC and CC genotype in aGVHD (39.9% vs. 42.7%, P=0.776, Figure 3-B) or cGVHD (22.5% vs. 28.4%, P=0.486, Figure 3-D).

Events and survival data

Seventeen patients died of TRM (CI 10.5%). Specifically, TRM was caused by aGVHD in 2 patients, cGVHD in 6 patients, infection in 7 patients (6 of them were CMV infection), HVOD in 1 patient and fulminant hepatitis in 1 patient.

TRM rate based on the genotype at the rs3761548 locus is illustrated in Figure 4. Patients with AA, AC and CC genotype

showed 3.7%, 3.1% and 14.2% of TRM rate respectively (Figure 4-A). When the AA and AC genotype were grouped together, TRM rate of patients with AA or AC genotype was lower than that of patients with CC genotype (3.4% vs. 14.2%, P=0.044, Figure 4-B). The patients with CMV infection had higher incidence of TRM than the patients without CMV infection (14.4% vs. 3.3%, P=0.034). Thirty-eight patients relapsed, but the incidence of relapse was not influenced by genotype.

One-year and 5-year OS for all patients was 80.9% and 69.9%, respectively. When classified based on genotype, OS was 54.3%, 83.0% and 63.5% for AA, AC and CC genotypes respectively (Figure 4-C). However, the data indicates a trend for higher OS rate in of AA/AC genotype group than that in CC genotype group (65.7% vs. 63.5%, P=0.043, Figure 4-D).

T cells reconstitution

In our study, we had monitored the changes of $CD3^+$, $CD4^+$ and $CD8^+$ cells 30-, 100- and 180-days after HSCT. As showed in Figure 5, there were no differences between genotypes.

Multivariate analysis

Multivariate analysis to identify predictors of HVOD, CMV infection, TRM and OS was done using Cox regression analysis (Table 7). The influence of rs3761548 and other clinical variables such as gender, age, diseases, pre-SCT status, donor type, donor sex and conditioning regimen was determined. The analysis identified rs3761548 CC genotype as a risk factor for HVOD (HR=3.97; 95%CI=1.47 to 10.74), CMV infection (HR=1.88; 95%CI=1.19 to 2.97) as well as lower OS (HR=2.25; 95%CI=1.10 to 4.62). Multivariate analysis also revealed other clinical variables such as advanced disease before HSCT to have statistical significance in OS (HR=4.30; 95%CI=1.861 to 9.947) and AML as a favorable factor for CMV infection (HR=0.60; 95%CI=0.38 to 0.97). In this multivariable analysis, rs3761548 polymorphism did not influence the risk for TRM.

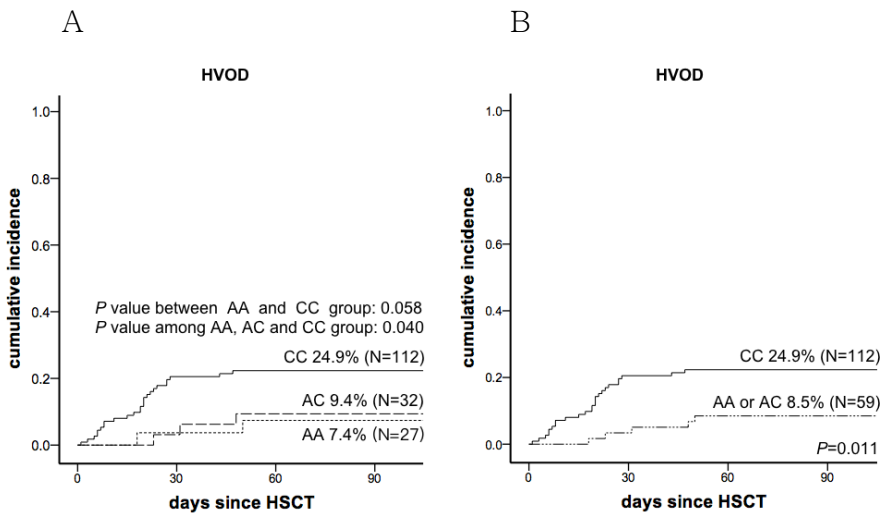

Figure 1. The CI of HVOD by AA, AC and CC genotype groups and the CI of HVOD were 7.4%, 9.4% and 24.9%, respectively (A). There was statistical significant difference among the three genotypes groups, though it was not shown that CC genotype group had higher incidence of HVOD than AA genotype group, but the CC genotype group showed an increasing trend in HVOD incidence; AA and AC genotype groups were grouped together and compared to CC genotype group (B), and AA or AC genotype group was shown to have lower incidence of HVOD than the CC genotype group. CI, cumulative incidence; HVOD, hepatic veno-occlusive disease.

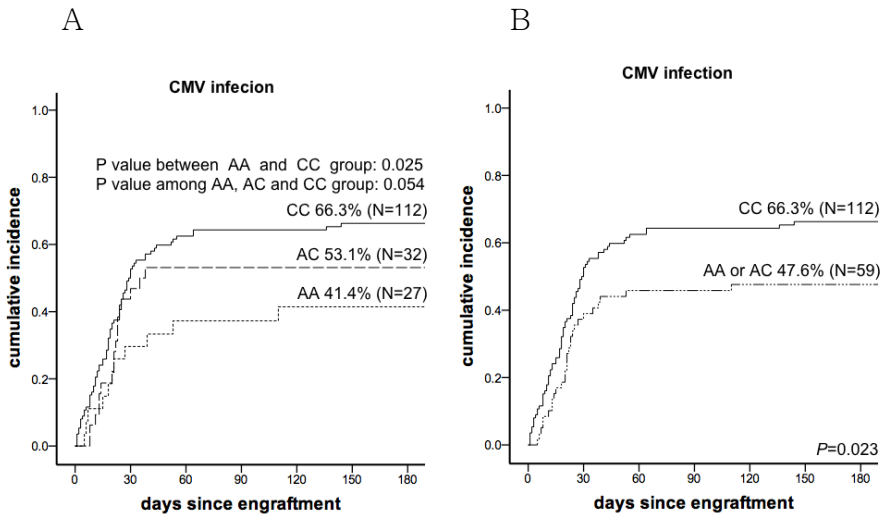

Figure 2. The CI of CMV infection by AA, AC and CC genotype groups and the CI of CMV infection were 41.4%, 53.1% and 66.3%, respectively (A). There was no statistically significant difference among the three genotypes groups, however, CC genotype group had higher incidence of CMV infection than AA genotype group; AA and AC genotype groups were grouped together and compared to the CC genotype group (B), AA or AC genotype group showed lower incidence of CMV infection than the CC genotype group. Patients were monitored up to 180 days after neutrophil engraftment. CI, cumulative incidence.

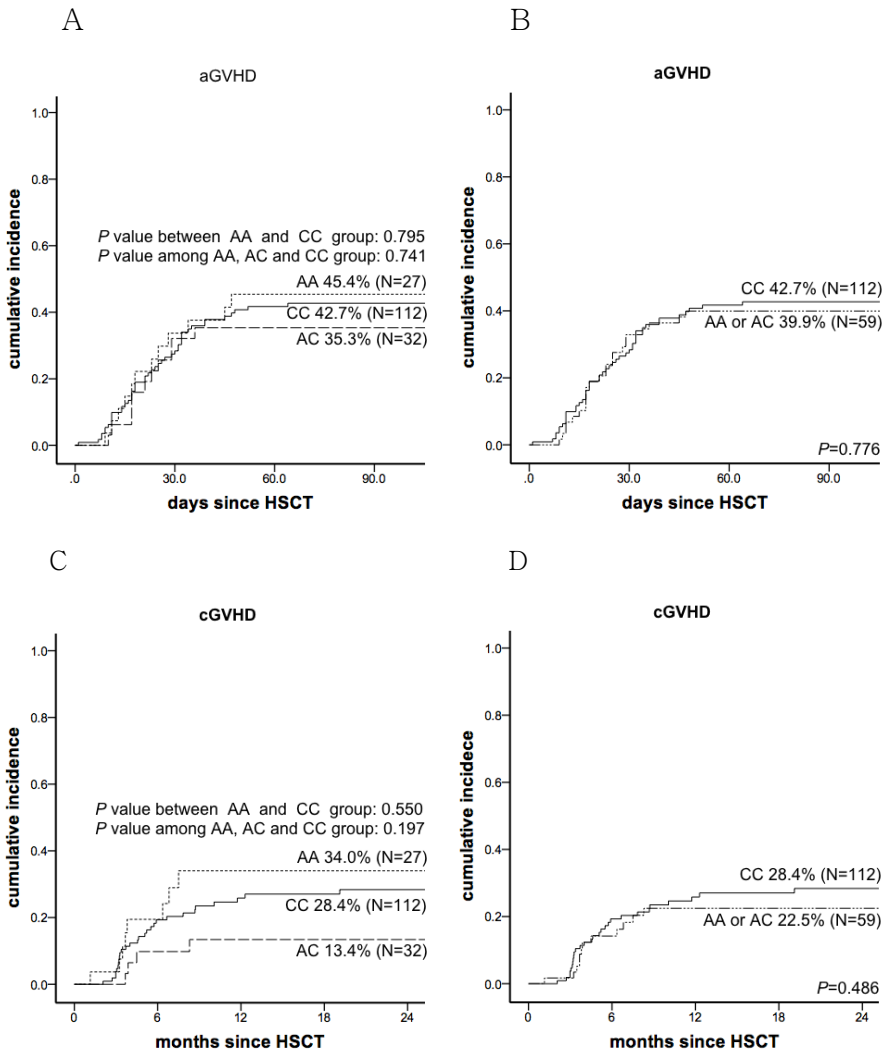

Figure 3. The CI of GVHD were classified by AA, AC and CC genotype groups and the CI of aGVHD were 45.4%, 35.3% and 42.7%, respectively (A); the CI of cGVHD were 34.0%, 13.4% and 28.4%, respectively (C) (In the analysis of aGVHD, grade II-IV are classified as positive indication of aGVHD); AA and AC genotype groups together considered a group compared to CC genotype group (B, D). Statistically significant difference was not observed in any of the four diagrams. CI, cumulative incidence; aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease.

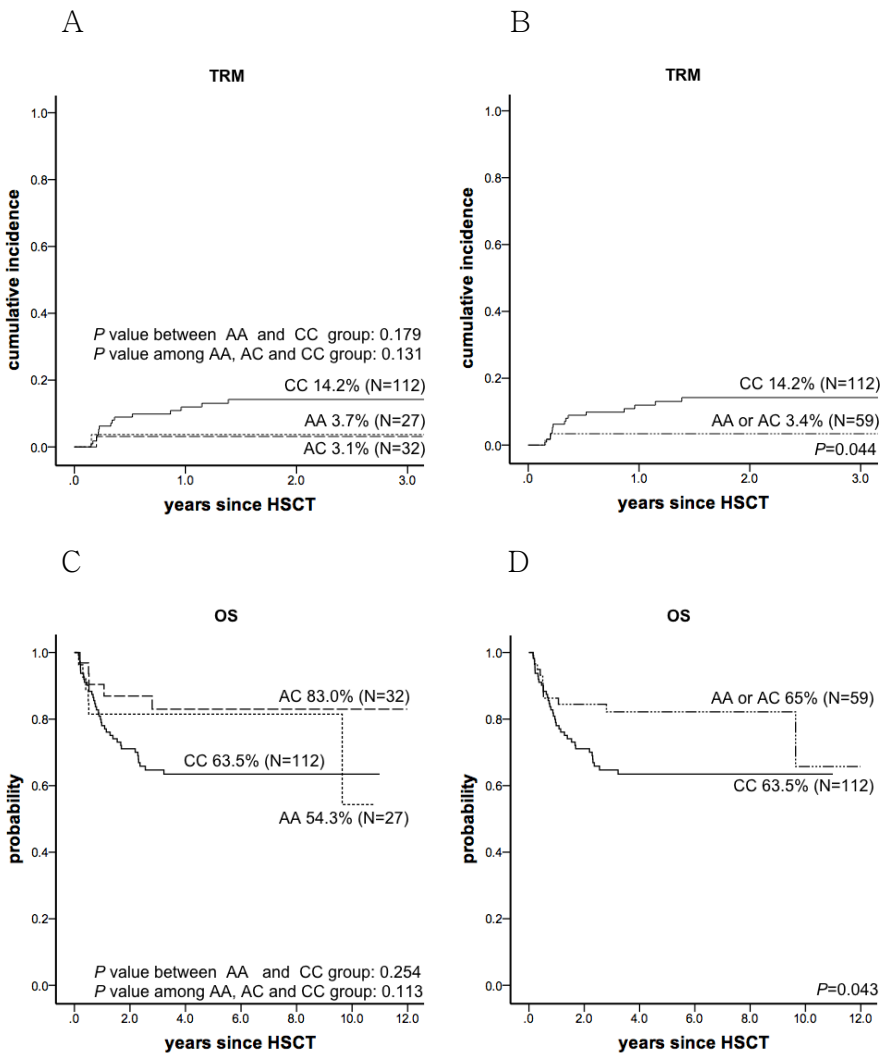

Figure 4. The CI of TRM by AA, AC and CC genotype group and the CI of TRM were 3.7%, 3.1% and 14.2%, respectively (A), CC genotype group had higher incidence of TRM trend; AA and AC genotype groups together considered a group compared to CC genotype group (B), AA or AC genotype group had lower transplant-related mortality rate than that of CC genotype group; Kaplan-Meier Analysis of OS by AA, AC and CC genotype group and OS were 54.3%, 83.0% and 63.5%, respectively (C); AA and AC genotype were grouped together and compared to the CC genotype group (D), the AA or AC genotype group showed higher OS rate than CC genotype group. CI, cumulative incidence; TRM, treatment-related mortality; OS, overall survival.

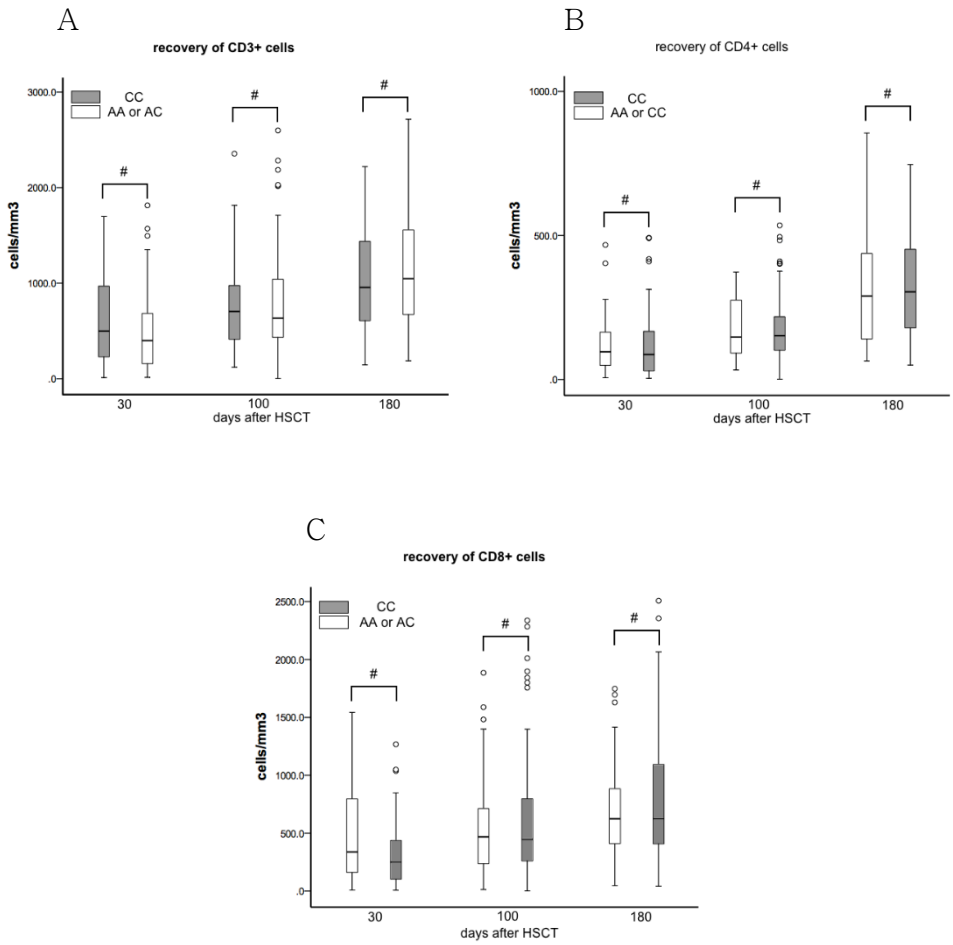

Figure 5. Recovery of T-cell subtypes after HSCT and changes in $CD3^+$ (A), $CD4^+$ (B) and $CD8^+$ (C) cell count 30-, 100- and 180-days after HSCT was monitored. No difference was observed when AA or AC genotype group was compared to the CC genotype group for each T-cell subtypes on the different days. # , P>0.05.

Table 6 Clinical Characteristics of allo-HSCT patients

Characteristics	N=171
Gender, No. (%)	
Male	92 (53.8%)
Female	79 (46.2%)
Median age, yr. (range)	11.1 (0.6~22.5)
Genotype, No. (%)	
CC	112 (65.5%)
AC	32 (18.7%)
AA	27 (15.8%)
Underling disease, No. (%)	
ALL	77 (45.0%)
AML	74 (43.3%)
ABL	20 (11.7%)
Conditioning regimens, No. (%)	
BU-based	132 (77.2%)
TBI-based	37 (21.6%)
Others	2 (1.2%)
Type of donor, No. (%)	
Related donor	43 (25.2%)
Unrelated donor	128 (74.8%)
Type of stem cell source, No. (%)	
Bone marrow	44 (25.7%)
Peripheral blood	85 (49.7%)
Cord blood	42 (24.6%)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ABL, acute biphenotypic leukemia; BU, busulfan; TBI, total body irradiation.

Table 7 Multivariate Analysis For Transplantation Outcomes

		HVOD			CMV			OS			TRM		
	N	HR	95.0% CI	*P*	HR	95.0% CI	*P*	HR	95.0% CI	*P*	HR	95.0% CI	*P*
rs3761548													
AA&AC	59	1			1			1			1		
CC	112	3.97	1.47 −10.74	0.01	1.88	1.19 −2.97	0.01	2.25	1.10 −4.62	0.03	3.56	0.76 −16.74	0.11
Gender													
male	92	1			1			1			1		
female	79	0.68	0.32 −1.46	0.33	1.02	0.68 −1.54	0.91	0.65	0.35 −1.22	0.18	0.53	0.19 −1.50	0.24
Age at HSCT	171	1.03	0.95 −1.12	0.48	1.02	0.97 −1.06	0.43	1.00	0.93 −1.07	0.92	0.98	0.88 −1.10	0.75
Disease													
ALL	77	1			1			1			1		
ABL	20	0.67	0.19 −2.37	0.54	1.08	0.58 −2.00	0.81	0.76	0.28 −2.06	0.59	1.78	0.44 −7.16	0.42
AML	74	0.71	0.31 −1.63	0.41	0.60	0.38 −0.97	0.04	0.96	0.48 −1.93	0.92	1.90	0.43 −8.35	0.40
preSCT status													
1st CR	119	1			1			1			1		
2nd CR	34	0.58	0.19 −1.72	0.32	0.87	0.51 −1.50	0.61	1.08	0.49 −2.41	0.85	1.20	0.32 −4.55	0.79
advanced	18	1.93	0.68 −5.50	0.22	1.37	0.70 −2.68	0.36	4.21	1.89 −9.38	0.00	2.84	0.69 −11.74	0.15
Donor type													
related BM/PB	43	1			1			1			1		
unrelated BM/PB	86	1.70	0.62 −4.71	0.31	1.04	0.62 −1.75	0.88	1.58	0.77 −3.26	0.22	1.78	0.44 −7.16	0.42
cord	42	1.74	0.58 −5.19	0.32	1.20	0.68 −2.13	0.53	0.71	0.27 −1.87	0.48	1.90	0.43 −8.35	0.40
Donor Sex													
male	100	1			1			1			1		
female	71	1.94	0.86 −4.40	0.11	1.14	0.74 −1.74	0.56	1.37	0.72 −2.62	0.34	1.03	0.33 −3.19	0.96
Conditioning Regimen													
BU	134	1.19	0.10 −14.14	0.89	3.76	0.76 −18.60	0.10	1.40	0.23 −8.58	0.72	0.00	0.00 −1.53E+98	0.94
TBI	39	1.42	0.13 −15.95	0.78	3.02	0.64 −14.31	0.16	3.58	0.57 −22.71	0.18	0.00	0.00 −4.03E+98	0.94

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ABL, acute biphenotypic leukemia; advanced disease include 3rd or subsequent CR, primary refractory of disease, 1st relapse and refractory, 2nd or subsequent relapse and refractory and other; BM, bone marrow; PB, peripheral blood; BU, busulfan; TBI, total body irradiation.

Discussion

In this study, we assessed the clinical impact of *FOXP3* rs3761548 polymorphism in donor on the outcomes of allogeneic HSCT. Patients who received HSCT from a donor with CC genotype showed higher incidence of HVOD, CMV infection, and lower overall survival in both univariate as well as multivariate analysis.

Previous studies have shown rs3761548 to be associated with the development of cancers and autoimmune diseases, but not much is known about the mechanism. It is well established that function of Tregs is positively correlated with expression of FOXP3[16, 34]. Polymorphisms of the *FOXP3* gene may result in decreased FOXP3 function or quantity, thus leading to a lack of functional Tregs. From the TRANSFAC® Factor database, the number of potential transcription factors which can bind to the promoter of *FOXP3* is lower when the allele C is changed to A (The search was performed in November, 2015). Oda *et al.*[35] indicted that rs3761548 AA genotype leads to a loss in binding with *E47* and *c-Myb*, leading to defective transcription of FOXP3. Qiu *et al.*[36] proved that patients with AA genotype were more prone to allograft rejection in renal transplantation and the function of Treg in patients with AA genotype is weaker than that of CC genotype.

Hepatic sinusoidal obstruction syndrome (HSOS) is the other name given to HVOD, which classically presents with tender hepatomegaly, hyperbilirubinaemia and ascites after HSCT[37]. The definitive incidence of HVOD is difficult to estimate though one large study showed an overall rate of about 5%[38]. Severe HVOD that evolves into multi-organ failure has a mortality rate higher than 80%[39]. The pathogenesis of HVOD is complicated. The initial pathogenic event may be damage to the sinusoidal endothelium and to hepatic venules[40] caused by conditioning regimen. Subsequently, infiltration of inflammatory cells, fibrin deposition and platelet aggregation contribute to the pathogenesis of HVOD[41]. In animal studies, more platelet aggregation, higher incidence of HVOD and mortality were observed in BU/CY group compared to TBI[41]. There is a lack of published data on the contribution of Tregs to the incidence of HVOD. Some studies have shown an association of Tregs with thrombus formation in stroke[42] and hepatocellular carcinoma[43]. Kleinschnitz *et al.*[44] demonstrates that Tregs induce micro-vascular dysfunction and thrombosis in acute ischemic stroke. In view of these studies as well as our data, we believe that patients with CC genotype have stronger Treg function than individuals with AA or AC genotype and this leads to easier development of HVOD in patients with CC genotype. In our results,

patients with CC genotype had 4-fold higher incidence of HVOD than AA or AC group. Although, further research is both necessary and important to confirm our findings, but we propose that rs3761548 may be a suitable biomarker for predicting the occurrence of HVOD.

CMV disease can damage several organs and is one of the most common causes of early death after HSCT[45]. To prevent CMV infection, prophylactic and preemptive therapies are usually administered, but the antiviral agents such as ganciclovir are associated with myelotoxicity, particularly neutropenia, which could contribute to a fatal bacterial or fungal infection[46]. Therefore, selecting the high-risk population is important in prevention and treatment of CMV infection. In our data, patients who received HSCT from donors with rs3761548 CC genotype had higher incidence of CMV infection in the post-transplantation phase. This was supported by other research showing that CMV infection is dependent on T cell response of the host. Jost *et al.*[47] showed that natural Tregs as well as induced Tregs interfere with an effective anti-mouse CMV (mCMV) immune response. Depletion of $FOXP3^{+}$Tregs resulted in enhanced T-cell activation and was associated with reduced viral titers in the organ where mCMV

mainly persists. This implies that SNP rs3761548 of *FOXP3* could affect the incidence of CMV infection through the influence of T cell response. In this study, patients with CMV infection had higher incidence of TRM, and one of the major cause of TRM was CMV infection in this population. These results suggest that selection of donors with rs3761548 AA or AC genotype could positively affect the outcome of allo-HSCT.

Several studies have shown that Tregs are associated with the occurrence of infection, GVHD and relapse. Of these studies, many implicate a negative correlation of Tregs with the occurrence of infection[48], GVHD[49, 50] and relapse[51]. However, a study by Perz *et al.*[52] showed that there was no relation between Tregs and cGVHD. In concordance with Perz *et al.*, we did not see an association of rs3761548 polymorphism with occurrence of BSI, GVHD or relapse. The occurrence of BSI, GVHD and relapse requires the involvement of multiple factors and just the presence of the rs3761548 polymorphism might not be a sufficient trigger for development of BSI, GVHD or relapse.

Conclusion

We present the first association between *FOXP3* rs3761548 SNP and the occurrence of HVOD and CMV infection after allo-HSCT in pediatric patients. Based on our results, *FOXP3* rs3761548 SNP can be considered a candidate marker for predicting the development of HVOD. It also affects CMV infection susceptibility, which influences TRM and survival after allo-HSCT. Further studies are needed to shed more light on the mechanisms involved in this effect.

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국문초록

동종 조혈모세포이식은 고위험군 급성 백혈병환자에게 치유를 가져다 줄 수 있는 치료기법이지만 또한 엄중한 합병증을 초래하기도 한다. 이러한 합병증의 발생에서 면역기능의 항상성이 중요한 역할을 하고 조절 T세포가 면역기능의 항상성의 유지에서 관건적인 작용을 한다고 알려져 있다. Forkhead BOX P3 (FOXP3)의 발현은 조절 T세포의 발생, 발달 과정에서 필수적이다. 하여 본 연구에서는 공여자의 *FOXP3* rs3761548 다형성이 동종 조혈모세포이식후 이식결과에 미치는 영향에 대하여 연구하였다. 총 171명의 환자가 본 연구에 등록 되였고 공여자의 유전자형을 확인하기 위하여 이식 후 완전히 공여자의 조혈세포로 치환된 결과를 보이는 환자 군의 샘플을 선택하였다. PCR기법을 통하여 유전자를 증폭시킨 후 직접염기서열결정법으로 공여자의 유전자형을 측정하였다. 결과적으로 간정맥폐색성질환에서는 공여자의 유전자형 CC 그룹에서 AA 혹은 AC 유전자형 그룹보다 더 높은 발생률을 나타내었다. 사이토메갈로바이러스감염에서도 CC유전자형인 경우 더 높은 감염발생률을 나타내었고 이식 관련 사망률도 CC 유전자형 환자 군에서 더 높아 이로 하여 CC유전자형 환자 군에서 전체 생존율은 AA 혹은AC 유전자형 환자 군보다 훨씬 낮게 나타내었다. 그러나 이식대숙주병, 재발, 혈류 감염에서는 유전자형에 의한 차이를 보이지 않았다. 다변량분석을 진행하여 보면 CC유전자형은 간정맥폐색성질환, 사이토메갈로바이러스 감염과 전체 생

존율에서 위험인자로 나타나고 있다. 이 연구를 통해 처음으로 *FOXP3* rs3761548 단일 염기 변이와 동종 조혈모세포이식후 이식결과에 대한 연관성을 제시하였고 *FOXP3* rs3761548 다형성은 동종 조혈모세포 이식 후 간정맥폐색성질환 및 CMV 감염의 발생을 예측할 수 있는 후보 마커로 간주 될 수 있다고 생각한다.

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주요어: FOXP3, rs3761548, 동종 조혈모세포이식, 이식결과

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