

Cytokine polymorphisms

Polymorphisms of transforming growth factor- β 1 and transforming growth factor- β 1 type II receptor genes are associated with acute graft-versus-host disease in children with HLA-matched sibling bone marrow transplantation

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Summary:

The aim of this study was to determine whether the gene polymorphisms of Th1/Th2 and immunoregulatory cytokines were associated with aGVHD in Japanese children receiving allogeneic bone marrow transplantation (allo BMT). We investigated polymorphisms of genes encoding interleukin (IL)-4, IL-4 receptor (IL-4 R), IL-10, transforming growth factor (TGF)- β 1, TGF- β 1 type II receptor (TGF- β 1 RII), interferon (IFN)- γ , IFN- γ type 2 receptor (IFN- γ R2), and IFN regulatory factor (IRF)-1. Sixty-seven patients were treated with alloBMT from HLA-identical siblings, and aGVHD was observed in 38. TGF- β 1 codon 10 leucine (Leu) /proline (Pro) polymorphism in donors was associated with the development of aGVHD. Patients having donors with the Pro allele had aGVHD more frequently than those without Pro allele (30/45 vs 8/20, odds ratio = 3.00; $P = 0.04$). TGF- β 1 RII 1167 C/T polymorphism in recipients was also associated with the development of aGVHD. The incidence was significantly higher in recipients with T allele than in those without T allele (21/27 vs 16/35, odds ratio = 4.16; $P = 0.01$). In conclusion, genetic backgrounds of TGF- β 1 and TGF- β 1 RII may be involved in the development of aGVHD in HLA-matched sibling BMT in Japanese children.

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Graft-versus-host disease (GVHD) is a major complication of allogeneic bone marrow transplantation (alloBMT). Although the incidence of acute GVHD (aGVHD) in children is known to be lower than that in adults, approximately 45% of pediatric patients suffer from aGVHD after alloBMT from an HLA-identical sibling.¹ Elucidation of the mechanisms of onset and progression of aGVHD should lead to better prediction and prophylaxis for aGVHD.

Acute GVHD is established by a multistep process. A conditioning regimen damages and/or activates recipient tissues, followed by the secretion of the inflammatory cytokines.² This reaction facilitates donor T cells to recognize alloantigen disparities, then causing activation and clonal expansion of the T cells occur. These T cells release cytokines such as tumor necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-1, IL-10, IL-4 and transforming growth factor (TGF)- β 1,³ resulting in the activation and recruitment of other effector cells, such as macrophages and NK cells. Finally, these effector cells mediate the pathological processes associated with aGVHD.⁴ Thus, donor T cells play a critical role in the induction of aGVHD, and the interactions of several cytokines are involved in its progression. The imbalance between Th1 and Th2 cytokines has been suggested to be responsible for the development of severe GVHD by the analysis of cytokine mRNA expression in patients with aGVHD.⁵ It is proposed that Th1 cytokines such as IFN- γ , IL-1, IFN regulatory factor (IRF)-1 are critical for inducing aGVHD, while Th2 cytokines such as IL-4, IL-10 and the immunoregulatory cytokine, TGF- β , have a suppressive effect.^{6,7} However, contradictory data also exist, indicating that Th1 cytokines such as IL-12 and IFN- γ could be protective.⁸ The absence of a Th1 cytokine can be deleterious in aGVHD, whereas the lack of a Th2 cytokine can be protective in knockout mice.⁹ The contribution of Th1 and Th2 cytokines to aGVHD may be more complicated than was previously considered. Thus, an investigation into the immunogenetics of cytokines is needed in order to clarify more fully their potential role in the development of aGVHD in man.

Recently, several gene polymorphisms of cytokines such as TNF- α , IFN- γ , IL-6, IL-1 and IL-10, involved in the inflammatory response, have been reported to be associated with the development of aGVHD in HLA-matched related BMT in adults.^{10–13} However, the comprehensive examination of the influence of gene polymorphism of the Th1 and Th2 cytokines on the development of aGVHD has not been performed. The aim of this study is to determine whether the gene polymorphisms of Th1/Th2 and immunoregulatory cytokines are associated with the development of aGVHD. The results suggested the significant role of TGF- β 1 and its receptor polymorphisms in aGVHD.

Patients and methods

Patient characteristics

Sixty-seven Japanese patients were treated with alloBMT from HLA-identical siblings between 1987 and 1999 at National Kyushu Cancer Center and Osaka Medical Center and Research Institute for Maternal and Child Health. All patients were examined for HLA-A, B, C and DR by conventional serological methods. Table 1 shows the patient characteristics. The diagnoses included acute lymphoblastic leukemia (ALL) in 16, acute myelogenous leukemia (AML) in 25, acute unclassified leukemia (AUL) in two, myelodysplastic syndrome (MDS) in 10, severe aplastic anemia (SAA) in five, neuroblastoma (NB) in three, non Hodgkin's lymphoma (NHL) in two and others in four. Thirty-two patients were treated with the regimens including total body irradiation (TBI) and other 35 without it (non-TBI). The TBI regimen was used for 13 ALL patients in combination with melphalan and busulfan or thio-TEPA. In AML, TBI regimen was used only for four patients, while 17 received a combination of busulfan and melphalan. All SAA patients received cyclophosphamide in combination with TBI in three and anti-thymocyte globulin in two. Other patients received several types of conditioning regimens. GVHD prophylaxis regimen included methotrexate (MTX) in 25, cyclosporine (CsA) in 29, CsA with short-term MTX in 11 and tacrolimus in two. T cell depletion was not performed for all grafts.

Patient samples

Peripheral blood or bone marrow samples were obtained from 67 pairs of donors and patients. Peripheral blood samples were also obtained from 100 healthy childhood as controls. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation and were cryopreserved until preparation of genomic DNA. All guardians of patients and controls gave informed consent for the use of their samples. Genomic DNA was extracted using standard techniques with a QIAmp Blood kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Detection of polymorphism in cytokine genes

Table 2 shows the polymorphic sites of each cytokine gene and their primers for polymerase chain reaction (PCR).

Table 1 Patient characteristics

Total (male/female)	67 (37/30)
Age (year) median (range)	9 (1–28)
Diagnosis	
ALL	16
AML	25
AUL	2
MDS	10
SAA	5
NB	3
NHL	2
Others	4
GVHD prophylaxis	
MTX	25
CsA	29
CsA+ MTX	11
Tacrolimus	2
Conditioning regimen	
TBI	32
Non TBI	35
Status at BMT	
1CR or 2CR	34
\geq 3CR or relapse	18
Others	15
Acute GVHD	
Yes	38
Grade I	21
II	10
III	6
IV	1
No	27
Not evaluated ^a	2
Chronic GVHD	
Yes	10
Limited	3
Extensive	7
No	49
Not evaluated	8
Early death	5
Not reached ^b	3
Outcome	
Alive	54
Dead	13

^aEngraftment was not obtained.

^bNot reached day 100 post transplant during the observation period. CR = complete remission.

IFN- γ gene polymorphism (intron 1 CA repeat) and IRF-1 (intron 7 GT repeat) gene polymorphism were examined using genotyping with ABI PRISM 310 genetic analyzer (Perkin-Elmer, Foster City, CA, USA). IFN- γ type 2 receptor (IFN- γ R2) gene polymorphism (64 Arg/Gln) was tested with PCR-single stranded conformation polymorphism (SSCP).¹⁴ IL-4 gene polymorphism (-590C/T) was examined using PCR-restriction fragment length polymorphism (RFLP) with *Ava*II (TakaraShuzo, Ohtsu, Japan). IL-4 receptor (IL-4 R) gene polymorphism (50 Ile/Val) was examined using allele-specific PCR. IL-4 R gene polymorphism (551 Gln/Arg) was examined using PCR-SSCP according to the previous report.¹⁵ IL-10 gene polymorphism (-571C/A) was tested using PCR-SSCP. TGF- β 1 gene polymorphism (-509C/T) was detected using PCR-RFLP with *Eco*81I (TakaraShuzo). TGF- β 1 gene polymorphism (10 Leu/Pro) was examined using PCR-SSCP. TGF- β 1 type II receptor (TGF- β 1 RII) gene polymorphism (1167 C/T (codon 389 AAC/AAT)) was tested with PCR-SSCP according to the previous report.¹⁶

Table 2 Gene polymorphic site of each cytokine and its primers for PCR

Cytokine	Polymorphism site	Primers	
		Forward	Reverse
TGF- β 1	-509C/T	CAGTTGGCACGGGCTTTC	ACCGTCTCATCTCGCGT
TGF- β 1	10 Leu/Pro	CGTGGGATACTGAGACACCC	CCAGTCCATGTGCGATAG
TGF- β 1 RII	1167 C/T (codon 389 AAC/AAT)	CAGTTGGCACGGGCTTTC	ACGCGAGATGAGGACGGT
IL-10	-571C/A	GCCTGGAACACATCCTGTGACCCCGCCAGT	GGGTGGGCTAAATATCCTCA
IL-4	-590C/T	TAAACTTGGGAGAACATGGT	TGGGAAAGATAGAGTAATA
IL-4 R	50 Ile/Val	GAAGCCCACACGTGTA (Ile) GAAGCCCACACGTGTG (Val)	TCGCTGGGCTTGAAGGACT
IL-4 R	551 Gln/Arg	GCCCCAACCTGAGCCAGAAA	ATGTGAGCTCTGCGGACTGC
IFN γ	intron 1 CA repeat	GCTGTCATAATAATATTCAGAC	CGAGCTTTAAAAGATAGTTCC
IFN γ R2	64 Arg/Gln	CCCCGCCAGACCCTCTTTCC	ACTGTCGGTGTATTTAACTGCACT
IRF-1	intron 7 GT repeat	GGTTTGAGAGGCTGAGTCACT	TCACTGAGAAACGGTCACTTC

Each PCR amplification was performed in a total reaction volume of 25 μ l containing 10 mM Tris (pH 9.0), 50 mM KCl, 2 mM MgCl₂, 0.1% Triton X-100, 0.2 mM each of dNTPs, 0.5 μ M of each primer, 0.625 units of Promega Taq polymerase (Promega, Madison, WI, USA) and 20 ng of template genomic DNA. Amplified PCR products were electrophoresed on agarose gels and stained with ethidium bromide or on polyacrylamide gels with silver staining. SSCP was performed as follows: 3 μ l of PCR products were mixed with 3 μ l deionized formamide, denatured for 5 min at 95°C and analyzed by electrophoresis on 12.5% polyacrylamide gels.

Statistical analysis

A χ^2 test with 2 \times 2 contingency tables was used to evaluate differences between phenotype or allele frequencies of two groups. A whole allele distribution of microsatellite polymorphism was analyzed by χ^2 test with 2 \times 6 or 2 \times 7 contingency tables. When expected values of the cells were not more than 5, Fisher's exact test was applied to obtain χ^2 values. A *P* value less than 0.05 was considered to be statistically significant.

Results

Acute GVHD

Acute GVHD was diagnosed and graded according to previously published criteria.¹⁷ Of 67 patients who received BMT, 27 showed no evidence of aGVHD and two did not achieve engraftment. Acute GVHD was observed in 38 patients; grade I in 21, grade II in 10, grade III in six and grade IV in one. The incidence of aGVHD (grade 0 vs grades I–IV) was not associated with age, pre-conditioning regimen (TBI or non-TBI), GVHD prophylaxis, disease status at BMT and diagnosis (Table 3).

Chronic GVHD

Of 65 patients who achieved engraftment, 10 patients showed chronic GVHD (cGVHD) and 49 did not (Table 1). Five patients died before 100 days post BMT and three

Table 3 Risk factors of acute GVHD

Risk factor	Acute GVHD		No.	<i>P</i> value
	(+)	(-)		
Age (years)				
\geq 9	20	12	32	
<9	18	15	33	0.52
Conditioning regimen				
TBI-included	22	10	32	
Non-TBI	16	17	33	0.10
GVHD prophylaxis				
MTX	11	13	24	
CsA-included	25	14	39	
Tacrolimus	2	0	2	0.20
Status at BMT				
1CR or 2CR	20	13	33	
Others	18	14	32	0.72
Diagnosis				
ALL	9	7	16	
AML	14	10	24	
MDS	6	4	10	
SAA	2	3	5	
Others	7	3	10	0.89
Total	38	27	65	

Association between risk factor and aGVHD was analyzed by the χ^2 test (2 \times 2 table) or the two-sided Fisher's exact test (2 \times 3 or 2 \times 5 table).

patients did not reach day 100 post transplant during the observation period.

Clinical outcome

Fifty-four patients survived, and the other 13 died. The causes of death were relapse in six, interstitial pneumonia in two, severe infection in two, regimen-related toxicity, hemophagocytic syndrome and sudden death in one each. Acute GVHD-related death was observed in two patients.

Cytokine gene polymorphisms and aGVHD

When the association of gene polymorphisms in donors with aGVHD was examined, TGF- β 1 polymorphism (10 Leu/Pro) was significantly associated with the development

of aGVHD (Tables 4 and 5). Patients having donors with Pro (Leu/Pro+Pro/Pro) allele developed aGVHD more frequently than those without Pro (Leu/Leu) allele (30/45 vs 8/20, odds ratio = 3.00; $P = 0.04$). The polymorphisms of TGF-β1 RII (1167 C/T), TGF-β1 (-509) and IL-10 (-571) tended to be associated with the development of aGVHD, although they were not statistically significant (Table 5).

When the association was examined regarding recipients' genotypes, TGF-β1 RII polymorphism (1167 C/T) was significantly associated with the development of aGVHD (Tables 4 and 5). The incidence was significantly higher in recipients with T (C/T + T/T) allele than those without T (C/C) allele (21/27 vs 16/35, odds ratio = 4.16; $P = 0.01$). IL-10 (-571) and IFN-γ intron 1 CA repeat polymorphisms seemed to be associated with aGVHD, although there were not significant (Tables 5 and 6).

The gene polymorphisms in IL-4, IL-4 R, IRF-1 did not show any association with development of aGVHD (Tables 4, 5 and 6). In addition, no associations between cGVHD and cytokine gene polymorphisms were observed. The frequency distribution of each genotype of the genes in both

donors and recipients showed no differences from those observed with normal healthy controls. No significant deviation from Hardy-Weinberg expected frequencies of these genotypes was observed for either donor or recipient (data not shown).

Discussion

TGF-β1 has been shown to enhance the Th2 type response and inhibit the Th1 type response.¹⁸ TGF-β1 in the presence of IL-10 showed induction of alloantigen-specific tolerance *ex vivo*, resulting in protection from GVHD.¹⁹ TGF-β1, therefore, is considered to be a negative regulator of aGVHD because of its immunosuppressive and anti-inflammatory effects. Expression of TGF-β1 mRNA in peripheral blood mononuclear cells decreases during aGVHD.²⁰ Thus, low TGF-β1 production in the early phase of BMT has been suggested to be insufficient to suppress BMT-associated immune activation, which leads to the induction and progression of aGVHD.²¹

The leucine (Leu)/proline (Pro) polymorphism at codon 10 of TGF-β1 in donors was associated with the development of aGVHD in our present study. Associations of this polymorphism with several diseases have been reported: graft vascular disease after heart transplantation;²² pulmonary fibrosis after lung transplantation;²³ myocardial infarction²⁴ and alteration of bone mineral density.²⁵ On the contrary, the development of inflammatory bowel disease²⁶ and moyamoya disease¹⁶ did not correlate with the polymorphism. Our results demonstrate that patients receiving bone marrow from donors with the Pro allele developed aGVHD more frequently than those with the Leu allele, suggesting that the Pro allele is a risk factor of aGVHD, whereas the Leu allele is protective for aGVHD. The differences in the biological activity of TGF-β1 based on the gene polymorphism may influence the development of aGVHD.

The current study showed that the incidence of aGVHD was higher in patients with T allele of TGF-β1 RII 1167 C/T polymorphism than without it. This polymorphism was reported in the squamous cell carcinoma cell line (CE-48).²⁷ In addition, the frequency of T allele at 1167 polymorphism was significantly higher in early-onset colorectal cancer patients than in nonmalignant control group.²⁸ These results raise the possibility that the 1167 C/T polymorphism influences the biological activity of TGF β1 RII, which may lead to the alteration of the efficiency of TGF-β1 signaling.

Several reports demonstrated the association of the gene polymorphism of IL-10 with the development of aGVHD.^{10,11,13} Our study also showed the marginal association of IL-10 gene polymorphism with the development of aGVHD (Table 5). Several polymorphisms in the 5' flanking region of IL-10 gene have been known to constitute haplotypes. Studies in asthma patients demonstrated the association of low IL-10 producing haplotypes with the severe disease.²⁹ Although both the IL-10⁻¹⁰⁸² A allele and the IL-10⁻¹⁰⁶⁴ longer allele were linked in BMT patients, significant association with aGVHD was detected only in the IL-10⁻¹⁰⁶⁴ longer allele, but not in the IL-10⁻¹⁰⁸² A allele.¹⁰ The association of IL-10 haplotypes with the devel-

Table 4 Association between cytokine polymorphisms and acute GVHD

Genotype	Donor type			Recipient type		
	Acute GVHD		P value	Acute GVHD		P value
	(+)	(-)		(+)	(-)	
TGF-β1 (-509)						
C/C	7	10	0.24	6	7	0.55
C/T	18	11		20	12	
T/T	13	6		10	6	
TGF-β1 (10)						
Leu/Leu	8	12	0.12	10	9	0.68
Leu/Pro	17	10		17	9	
Pro/Pro	13	5		10	7	
TGF-β1 RII (1167)						
C/C	17	18	0.16	16	19	0.03
C/T	20	9		18	5	
T/T	1	0		3	1	
IL-10 (-571)						
C/C	6	1	0.37	7	1	0.18
C/A	16	13		15	14	
A/A	16	13		16	11	
IL-4 (-590)						
C/C	4	5	0.58	6	5	1.00
C/T	17	9		11	7	
T/T	17	13		20	13	
IL-4 R (50)						
Ile/Ile	5	4	0.83	5	3	0.93
Ile/Val	23	14		20	13	
Val/Val	10	9		11	9	
IL-4 R (551)						
Gln/Gln	26	22	0.42	27	19	1.00
Gln/Arg	10	5		9	6	
Arg/Arg	2	0		1	0	
IFN γR2 (64)						
Gln /Gln	7	9	0.12	8	9	0.15
Arg/Gln	21	8		23	9	
Arg/Arg	10	10		6	7	

The number of donors or recipients possessing each cytokine genotype is shown together with aGVHD (+) or (-). The two-sided Fisher's exact test was used to analyze each of genotypes (2 × 3 table).

Table 5 Association between cytokine polymorphisms and acute GVHD

Genotype	Donor type			Recipient type				
	Acute GVHD		Odds ratio	P value	Acute GVHD		Odds ratio	P value
	(+)	(-)			(+)	(-)		
TGF- β 1 (-509)								
C/T + T/T	31	17	2.61	0.08	30	18	1.94	0.23
C/C	7	10			6	7		
TGF- β 1 (10)								
Leu/Pro + Pro/Pro	30	15	3.00	0.04	30	16	1.69	0.25
Leu/Leu	8	12			10	9		
TGF- β 1 RII (1167)								
C/T + T/T	21	9	2.47	0.07	21	6	4.16	0.01
C/C	17	18			16	19		
IL-10 (-571)								
C/C	6	1	4.88	0.13	7	1	5.65	0.09
C/A + A/A	32	26			31	25		

χ^2 test or Fisher's exact test was used to analyze each of genotypes (2×2 table).

Table 6 Association of allele frequencies between IFN γ and IRF-1 microsatellite polymorphisms and acute GVHD

Acute GVHD polymorphism	Donor type		Recipient type		P value
	Acute GVHD		Acute GVHD		
	(+)	(-)	(+)	(-)	
IFN γ intron 1 CA repeat					
12	5	5	10	2	0.12
13	45	30	42	35	
14	2	0	0	0	
15	21	18	21	13	
16	1	0	3	0	
17	0	0	0	0	
18	2	1	0	0	
Whole distribution					
IRF-1 intron 7 GT repeat					
11	27	12	24	17	0.24
12	21	20	23	14	
13	0	0	0	0	
14	0	0	0	0	
15	1	0	3	0	
16	14	17	17	16	
17	9	5	6	3	
18	1	0	0	0	
19	1	0	1	0	
Whole distribution					

Whole distributions between acute GVHD (+) and (-) were evaluated by the two-sided Fisher's exact test for the 2×6 or 2×7 table.

opment of aGVHD has not been clear in BMT patients, and further investigation is needed to elucidate the role of IL-10 haplotypes in aGVHD.

IL-1 receptor antagonist (IL-1Ra) has been reported as the first recognized donor gene polymorphism associated with aGVHD.¹² Our study also revealed that a potential association between TGF- β 1 gene polymorphism in donors and the development of aGVHD. These results suggest that a donor-recipient interaction in genetic predisposition plays a role in the development of aGVHD and that donor cyto-

kine gene polymorphisms can be applied for the selection of the suitable donor.

A previous study on IFN- γ microsatellite polymorphism in the first intron, detected by the difference in number of CA repeats, showed the association of the polymorphism with pulmonary fibrosis after lung transplantation.³⁰ IFN- γ production *in vitro* showed a significant correlation with the presence of allele 2, which corresponds to 12 CA repeats.³¹ In the present study, the allele frequency of 12 CA repeats was higher in patients with aGVHD than the

other alleles, although it was not statistically significant. The reason why the current results did not show significant correlation of IFN- γ polymorphism with aGVHD might be due to the age-dependent maturation and expansion of the Th1 cell population.³²

The low incidence of severe GVHD in this study probably resulted from the relative genetic homogeneity in the Japanese population. Japanese patients who received BMT from HLA-compatible siblings showed a relatively low incidence of aGVHD compared with that in the patients of the United States, even though T cell depletion was not performed.³³ In Japan, only 50 000 donors are enough to supply at least one HLA-identical donor for 80% of patients, whereas 1 000 000 and 400 000, may be needed in Europe and North America, respectively.³⁴ The population in this study comprises only Japanese people, excluding minor distinct ethnic groups and immigrants from overseas. This genetic homogeneity may explain the low incidence of severe aGVHD in this study, which results in the lack of statistical correlation between the gene polymorphism of cytokines and the severity of aGVHD. This association may become more apparent if studied in higher risk groups for GVHD, such as in unrelated or mismatched donor transplantation.

In conclusion, the current study revealed that the polymorphisms of TGF- β 1 in donors and TGF- β 1 RII in recipients were associated with the development of aGVHD after HLA-identical BMT in Japanese children. Genetic backgrounds of TGF- β 1 and TGF- β 1 RII may influence the development of aGVHD. Further investigation is needed to clarify the effect of the polymorphisms of TGF- β 1 and TGF- β 1 RII genes on the donor T cell activation after alloBMT.

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