The IFN-γ Allele Is Correlated to Moderate-to-Severe Acute Graft-Versus-Host Disease After Allogeneic Stem Cell Transplant

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Abstract

Objectives: **Analysis of nonhistocompatibility leucocyte antigen functional genomics in stem cell transplant can lead to prediction of clinical outcomes in histocompatibility leucocyte antigen-matched sibling-transplant recipients. Some of the cytokine gene polymorphisms might be associated with severe, acute graft-versus-host disease after allogeneic stem cell transplant. We evaluated gene polymorphisms of IL-6 G-174C, TGF-ß T+869C, IL-4 C-590T, and IFN-γ T+874A cytokines in bone marrow transplant patients.**

Materials and Methods: **The Amplification refractory mutation system-polymerase chain reaction ARMS-PCR method was used to characterize IL-6 G-174C, TGF-ß T+869C, and IFN-γ T+874A polymorphisms, and PCR-RFLP, using AvaII restriction enzyme, was done for IL-4 C-590T characterization in 35 bone marrow transplant patients. Acute graft-versus-host disease episodes were diagnosed according to EMBT criteria.**

Results: **Analysis showed that IFN-γ +874T allele (*P* = .027, OR=0.198, 95% CI=0.049-0.801) was correlated to moderate-to-severe graft-versus-host disease. TGF-ß T+869C, IFN-γ T+874A, IL-6 G-174C and IL-4 C-590T frequencies were not significantly different in the 2 graft-versus-host disease severity groups (*P* > .05).**

Conclusion: **According to the results, we concluded that the IFN-γ T+874A gene polymorphism has a predictive value for severity of graft-versus-host disease after bone marrow transplant. High producer genotypes of IFN-γ are genetic risk factors for development of graft-versus-host disease.**

Key words: *Bone marrow transplant, Cytokine polymorphism*

Graft-versus-host disease (GVHD) is the most-common serious complication of allogeneic bone marrow transplant (BMT) and severe (grade 3-4) acute GVHD causes increased mortality (1, 2). Acute GVHD is established by a multistep process. A conditioning regimen damages and/or activates recipient tissues, followed by secretion of cytokines (3). A network of various cytokines including Th1, Th2, or proinflammatory and anti-inflammatory cytokines, and their related receptors and inhibitors have been implicated in several immunologic diseases (4), as well as GVHD after allogeneic bone marrow transplant (5). Th1 and proinflammatory cytokines (eg, IL-1, IL-1R, IL-1Ra, IL-2, IL-6, TNF-α) are important mediators and regulators of GVHD and promote GVHD (6), whereas Th2 and anti-inflammatory and immunomodulatory cytokine (eg, IL-4, IL-10, and TGF-β) are associated with transplant tolerance and decreased GVHD (6, 7). Cytokines are multifunctional, and also in a network, influence the effect of each other. The development of GVHD after allogeneic bone marrow transplant is mediated by alloreactive donor T-lymphocytes infused with the bone marrow inoculums (8). Donor T-cells recognize alloantigen disparities, and then cause activation and clonal expansion of T cells occur. These T cells release cytokines, such as TNF-α, IFN-γ, IL-1, IL-10, IL-4, and

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TGF-β, resulting in activation and recruitment of other effector cells, such as macrophages and natural killer cells. Finally, these effector cells mediate the pathological processes associated with GVHD (3, 6). A variety of risk factors contribute to GVHD development, one of the important factors being a patient's genetics. Analysis of nonhistocompatibility leucocyte antigen functional genomics, together with conventional risk factors in hematopoietic stem cell transplant can lead to prediction of clinical outcomes in histocompatibility leucocyte antigen-matched sibling transplant recipients. Polymorphisms in cytokine and cytokine receptor genes have been shown to be associated with posttransplant outcome in many forms of transplant (9). Cytokine gene single nucleotide polymorphisms can regulate cytokine production or function, and are suggested as a possible factor for graft rejection (10).

In bone marrow transplant patients, we evaluated the polymorphisms for IFN-γ T+874A, IL-4 C-590T, IL-6 G-174C, and TGF-β T+869C that previously have been reported to be associated with cytokine production or function (11, 12, 13). In the present study, the effects of functional polymorphisms of Th1, Th2, proinflammatory, and anti-inflammatory cytokine genes in the outcome of bone marrow transplant were investigated. Also, the polymorphisms of IFN-γ T+874A, IL-4 C-590T, IL-6 G-174C, and TGF-β T+869C genes were evaluated.

Materials and Methods

Subjects

In the current study, 35 consecutive patients receiving an allogeneic stem cell transplant in Namazi Hospital between 2006 and 2007 were enrolled. Histocompatibility leucocyte antigen matching was considered in this study. In stem cell transplant recipients, we investigated the graft outcome and GVHD episode(s) in the 12 weeks after transplant. Acute GVHD signs and symptoms were identified by an expert hematologist team based on European group for blood and marrow transplantation criteria. This study was approved by Research Ethics Committee of our institute. Written, informed consent, conforming with the ethical guidelines of the 1975 Helsinki Declaration, was obtained from the patients. There were 35 cases including 20 acute myelogenous leukemia, 10 acute lymphogenous leukemia, 3 chronic myelogenous leukemia, and 2 aplastic anemia patients. The source of hematopoietic stem cells was peripheral blood in all cases except in 2 cases of aplastic anemia. Conditioning chemotherapy regimen included busulfan 16 mg/kg or busulfex IV (80% of oral dose) and cyclophosphamide 120-200 mg/kg in leukemia patients (acute myelogenous leukemia, acute lymphogenous leukemia, chronic myelogenous leukemia) and cyclophosphamide 60-120 mg/kg +ATG 90 kg/mg for severe aplastic anemia and Fanconi's anemia. Graft-versus-host disease prophylaxis consisted of cyclosporine and methotrexate. Prophylactic antibiotic, antifungal, and antiviral drugs were prescribed for all patients. All blood products were irradiated with gamma rays to prevent posttransfusion GVHD.

DNA Extraction

Harvested buffy coats from the whole blood of bone marrow transplant patients were available in the sample bank of Shiraz Transplant Research Center. Genomic DNA was extracted from buffy coat using a DNGplus extractor WB kit (Cinagen, Iran) according to the manufacturer's instructions.

Determination of Cytokine Genotype

Cytokine gene polymorphisms were evaluated by polymerase chain reaction (PCR) using a thermal cycler (Techne, Genius, UK). Polymerase chain reaction conditions, PCR cycles, and primers are summarized in Tables 1 and 2. Amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method was carried out for IL-6 G-174C (11), TGF-β T+869C (12), and IFN-γ T+874A (13) was genotyped in 10 μL reaction mixtures. A beta globin gene primer was used as an internal control. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in a final volume of 25 μL was used to determine the IL-4 C-590T gene polymorphism (13). After PCR, products were digested by AvaII restriction enzyme, and the amplified products were monitored by agarose gel electrophoresis and ethidium bromide staining.

Statistical analyses

Allele and genotype frequencies were calculated in patient and control subjects by direct gene counting. Statistical analyses were performed with SPSS software for Windows (Statistical Product and Service

Table 1. PCR materials and cycles.

Locus	PCR conditions
IL-6 G-174C	10 cycles: 94°C 30s, 61°C 50s, 72°C 40s 20 cycles: 94°C 20s, 56°C 50s, 72°C 40s 50 ng DNA, 200 μmol dNTPs, 0.7 mM $MgCl_2$
TGF-ß T+869C	10 cycles: 94°C 30s, 64°C 50s, 72°C 40s 20 cycles: 94°C 20s, 59°C 50s, 72°C 40s 50 ng DNA, 200 μmol dNTPs, 3 mM $MgCl_2$
IL-4 C-590T	35 cycles: 94°C 20s, 53°C 50s, 72°C 50s 50 ng DNA, 200 μmol dNTPs, 3 mM $MgCl_2$
IFN-γ T+874A	10 cycles: 94°C 30s, 62°C 50s, 72°C 40s 20 cycles: 94°C 20s, 56°C 50s, 72°C 40s 50 ng DNA, 200 μmol dNTPs, 0.7 mM $MgCl_2$

Table 2. Cytokine and internal control primers.

Locus	Primers	Method
IL-6 G-174C	Common: 5'- GAGCTTCTCTTTCGTTCC -3' C allele: 5'- CCCTAGTTGTGTCTTGCC -3' G allele: 5'- CCCTAGTTGTGTCTTGCG -3'	ARMS-PCR
TGF-ß T+869C	Common: 5'- TCCGTGGGATACTGAGACACC -3' C allele: 5'- GCAGCGGTAGCAGCAGCG -3' T allele: 5'- AGCAGCGGTAGCAGCAGCA -3'	ARMS-PCR
IL-4 C-590T	Forward primer 5'- TAAACTTGGGAGAACATGGT -3' Reverse primer 5' - TGGGGAAAGATAGAGTAATA -3'	Ava II based RFLP
IFN-γ T+874A	Common: 5'- TCAACAAAGCTGATACTCCA -3' A allele: 5'- TTCTTACAACACAAAATCAAATCA -3' T allele: 5'- TTCTTACAACACAAAATCAAATCT -3'	ARMS-PCR
Beta globin	Forward: 5'- ACACAACTGTGTTCACTAGC -3' Reverse: 5'- CAACTTCATCCACGTTCACC -3'	Internal control

Solutions, version 15.0, SSPS Inc, Chicago, IL, USA). Frequencies of alleles/genotypes were compared in cases and controls by the chi-square test and the Fisher exact test. Odds ratios and 95% confidence intervals for relative risks were calculated. A probability value of $P < .05$ was considered statistically significant, and all reported P values were 2-tailed.

Results

Frequencies of cytokine genes in transplanted patients are shown in Table 3. Allele frequencies of IFN-γ variants were significantly different between rejected patients. The frequency of IFN-γ 874T allele was significantly higher than that of IFN-γ 874A allele ($P = .027$, OR=0.198, 95% CI=0.049-0.801) in acute rejection patients. With regard to IL-6, G-174C proinflammatory cytokine, IL-4 C-590T Th2 cytokine marker, and TGF-β T+869C suppressive cytokine gene polymorphisms, the differences between the 2 groups of patients was not significant ($P > .05$).

Discussion

Hematopoietic stem cell transplant can be lifesaving for patients with otherwise fatal diseases. However, it is associated with detrimental outcomes, such as GVHD, a potentially fatal complication (14). Several risk factors revealed for acute GVHD include histoincompatibility, age, sex mismatch, viral status, and prophylaxis (15). Cytokines and other regulators of the immune response may have an important role in the pathogenesis of GVHD (6). Inflammatory and anti-inflammatory mediators affect graft microenvironment, and transplant conditioning regimens can lead to the release of cytokines before transplant in the patient, which may depend on several factors in particular genetic polymorphisms. Genetic variations in transplanted patients could determine the outcome of bone marrow transplant as severity of GVHD (16).

The importance of nonhistocompatibility leucocyte antigen genetic variation in alloresponses is evidenced by the observation that some degree of GVHD and rejection still occur after bone marrow and solid-organ transplant between histocompatibility leucocyte antigen-identical siblings. Three general areas of genetic polymorphism have been studied in relation to stem cell and solid-organ transplant: natural killer receptor genes, minor histocompatibility antigens, and cytokine gene polymorphism (9). Polymorphisms in cytokine genes may alter the amount of cytokine production or cytokine function; we therefore postulated that polymorphisms in the cytokine genes determine the severity of GVHD.

Previous studies have suggested an association between polymorphisms in the IL-1RA, IL-6, IL-10, TNF-α, and IFN-γ genes, and the outcome of hematopoietic stem cell transplant (1, 17, 18, 19). In the present study, we showed that IFN-γ +874 A allele is associated with more-severe GVHD. It has been postulated that cytokines produced by Th1 cells (IL-2 and IFN-γ), being primarily associated with cell-mediated immune responses, would augment the T-cell responses against the host, thereby leading to greater GVHD in the recipients (20). Conversely, cytokines from Th2 cells (ie, IL-4 and IL-10) were considered immunosuppressive and therefore, capable of inhibiting GVHD (20). Transfer of Th2 cells after allogeneic bone marrow transplant resulted in protection from GVHD (21). But there have been conflicting reports indicating that Th2 cytokines can worsen the outcome of GVHD (22).

Absence of a Th1 cytokine can be deleterious in acute GVHD, whereas lack of a Th2 cytokine can be protective in knockout mice (23). Our results indicate

Table 3. Distribution of cytokine polymorphism in 2 GVHD severity groups.

GENE	Genotype/Allele	Production	Grade 1 (No or mild) GVHD % (n)	Grade 2 to 4 (Moderate to severe) GVHD % (n)	*P* value	OR	95% CI
	Genotype						
	CC-CG§	Intermediate-Low	27.3 (3)	55.6 (5)	.362	0.300	0.046-1.943
	GG	High	72.7 (8)	44.4 (4)			
IL-6	Allele						
	C allele$^{\Omega}$	Low	13.6 (3)	27.8 (5)	.430	0.411	0.083-2.025
	G allele	High	86.4 (19)	72.2 (13)			
	Genotype						
	CC-CT$^{<}$	Intermediate-Low	63.6 (7)	44.4 (4)	.653	2.188	0.362-13.226
	TT	High	36.4 (55.6)	55.6 (5)			
TGF-ß	Allele						
	C allele$^{\infty}$	Low	40.9 (9)	22.2 (4)	.312	2.423	0.598-9.816
	T allele	High	59.1 (13)	77.8 (14)			
	Genotype						
	CC$^{\varnothing}$	Low	72.7 (8)	77.8 (7)	.795	0.762	0.097-5.958
	CT-TT	Intermediate-High	27.3 (3)	22.2 (2)			
IL-4	Allele						
	C allele$^{\#}$	Low	86.4 (19)	88.9 (16)	.810	0.792	0.117-5.340
	T allele	High	13.6 (3)	11.1 (2)			
	Genotype						
	TT†	High	18.2 (2)	55.6 (5)	160	0.178	0.024-1.339
	AT-AA	Intermediate-Low	81.8 (9)	44.4 (4)			
IFN-γ	Allele						
	T allele$^{\Delta}$	High	40.9 (9)	77.8 (14)	.027*	0.198	0.049-0.801
	A allele	Intermediate-Low	59.1 (13)	22. 2(4)			

Abbreviations: CI, confidence interval; N, absolute number; OR, odds ratio.
*statistically significant
§CC and CG vs GG genotype; $^{\Omega}$C vs G allele.
$^{<}$CC and CT vs TT genotype; $^{\infty}$C vs T allele.
$^{\varnothing}$CC vs CT and TT genotype; $^{\#}$C vs T allele.
†TT vs AT and AA genotype; $^{\Delta}$T vs A allele.

that IFN-γ +874 T allele is associated with higher production of IFN-γ, and Th1 cytokines promote GVHD incidence. So, this polymorphism will be a risk factor for GVHD. Also, in another study, the IFN-γ intron1 (CA)n microsatellite was significantly associated with more-severe acute GVHD (1). In the case of Th2 cytokine, it has been shown that anti-IL-4 antibody prevents GVHD in mice after BMT (24), and the polymorphism is promoter of IL-10 associated with acute GVHD. However, in our results, there was no significant association between IL-4 -590 SNP and severity of GVHD. Transforming growth factor-β C-509T polymorphism was not associated with development of acute GVHD (3), and the results indicate that another important SNP of TGF-β, TGF-β C +869T does not affect the severity of GVHD. For regulatory cytokine IL-6, an increased level of IL-6 in the serum of acute GVHD patients has been seen (25), and its serum level has been correlated with GVHD severity (26).

Cavet and associates have shown a correlation of IL-6 G-176C variants with acute GVHD severity (1). In our study, there was no significant association between IL-6 polymorphism and severity of GVHD. The results of different studies have been inconsistent, probably because of the heterogeneity among the patients and genetic composition of different ethnicities, or study subjects who have taken various immunosuppressive therapies or relatively small sample size. In this study, we showed that a candidate polymorphism in IFN-γ gene was correlated with acute GVHD severity. For confirmation of our results and other aspects of cytokine genetic effect on bone marrow transplant outcome, further studies, with a larger sample size, are recommended.

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