P2X7 receptor polymorphism and clinical outcomes in HLA-matched sibling allogeneic hematopoietic stem cell transplantation

Kyung-Hun Lee, Sung Sup Park, Inho Kim, Jin Hee Kim, Eun Kyung Ra, Sung-Soo Yoon, Yun-Chul Hong, Seonyang Park, Byoung Kook Kim

Background and Objectives
The P2X7 receptor (P2X7R) is a key player in the processing and release of interleukin (IL)-1. To evaluate whether the A1513C polymorphism of the P2X7R gene is related to allogeneic stem cell transplantation outcome, we performed an association analysis between this polymorphism and clinical outcomes in patients treated with an HLA-matched sibling stem cell transplant.

Design and Methods
Patients (n=152) with a malignancy or aplastic anemia underwent allogeneic stem cell transplantation at a single institute. Peripheral blood DNA of these 152 patients and their 152 donors was genotyped. Genotypes of 145 recipients and 150 donors were obtained and analyzed for the polymorphism.

Results
The frequencies of the A and C alleles in all 295 study subjects were 72% and 28%, respectively. The genotypes in patients were AA in 75, AC in 58, and CC in 12; the genotypes in donors were AA in 74, AC in 70, and CC in 6. Overall survival was significantly shorter for recipients with the CC genotype than for those with the AA or AC genotype (92 days for 1513CC vs. 821 days for 1513AA or 1513AC, p=0.012), and for recipients from donors with the CC genotype than for recipients from donors with the AA or AC genotype (63 days for 1513CC vs. 702 days for 1513AA or 1513AC, p=0.024). Multivariate analyses, which included sex, age, transplant method (reduced intensity conditioning vs. conventional conditioning), stem cell source, risk group, and P2X-R recipient and donor genotypes, as parameters, identified high-risk group (hazard ratio 3.25, 95% confidence interval 1.83–5.77) and a donor 1513CC genotype (hazard ratio 2.66, 95% confidence interval 1.02–6.91) as risk factors for a shorter survival. Microbiologically documented bacteremia occurred in 66.7% of recipients with the CC donor genotype and in 17.6% of recipients of transplants of AA or AC genotype (p=0.014).

Interpretation and Conclusions
We conclude that the A1513C polymorphism in the P2X7R gene is related to the occurrence of infections and survival after allogeneic stem cell transplantation. Thus, the determination of this polymorphism may be helpful for the optimal selection of patients and donors.

Key words: P2X7, polymorphism, hematopoietic stem cell transplantation, IL-1, IL-18.

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Hematopoietic stem cell transplantation (HSCT) is a potentially curative treatment for many hematologic malignancies and for aplastic anemia and inherited immune disorders. However, the incidence of transplant-related mortality (TRM) is substantial and it is important that risks be reduced by tailoring and individualizing therapies. Single nucleotide polymorphisms (SNP) are believed to play an important role in determining individual characteristics, and are also being actively investigated in the field of HSCT.\textsuperscript{1-3} Several polymorphisms of cytokine genes, including those for interleukin (IL)-1, IL-2, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and TNF-β, have been investigated in relation to HSCT outcomes, i.e., with respect to graft-versus-host disease (GVHD) and infections.\textsuperscript{4-8} However, cytokine pathways are diverse and complex, and thus polymorphisms of cytokine receptors and their regulatory molecules must be considered in studies on the biological effects of cytokines.

The P2X\textsuperscript{7} receptor (P2X-R), a plasma membrane receptor for extracellular ATP, is known to be a key player in the processing and release of IL-1 cytokines.\textsuperscript{9-11} The IL-1 family presently contains nine members, including IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra) and IL-18. These cytokines are released from a variety of cells including activated monocytes, macrophages, and microglia, and are known to play an important role in immune responses. The loss-of-function SNP at nucleotide position 1513 of the human P2XR gene (1513A→C) changes a glutamic acid to alanine at amino acid 496. The presence of this polymorphism impairs ATP-induced Ca\textsuperscript{2+} influx, ATP-induced Rb\textsuperscript{-} efflux, ATP-induced release of IL-1β and IL-18, and the killing of intracellular bacteria.\textsuperscript{9-11}

Several studies have concluded that IL-1 is related to HSCT outcome, i.e., GVHD and mortality, and that polymorphisms of the gene for IL-1Ra affect the incidence and severity of acute GVHD.\textsuperscript{4} Moreover, because IL-1 expression is regulated by P2X-R, it is possible that P2X-R status may also affect HSCT outcome. Therefore, we investigated the role of the P2X-R polymorphism in HSCT. Association analysis was performed to determine the relations between this polymorphism and the clinical outcomes of patients treated with HLA matched sibling stem cell transplants at a single institute.

### Design and Methods

#### Patient population and data collection

Patients who underwent allogeneic HSCT from human leukocyte antigen (HLA)-matched sibling donors at a single institution (Seoul National University Hospital) between 1998 and 2005 for malignancies or aplastic anemia were included in this study. All the patients were 16 years or older at transplantation. Finally, 152 patients were included.

Clinical data were obtained from thorough review of medical records before P2X-R genotyping. The data collected were: patient demographics, transplantation procedure details including the conditioning regimens, time to recovery of platelet and granulocyte counts, liver and renal function as reflected by total bilirubin, aspartic transaminase (AST), alanine transaminase (ALT), and creatinine levels, transplantation complications such as acute GVHD and hepatic veno-occlusive disease (VOD), infections documented by blood culture and febrile episodes not confirmed microbiologically, survival status and cause of death. Times to platelet and granulocyte recovery were defined as the time to the first of 3 consecutive days of counts exceeding 500/µL, and to the first of 7 consecutive days of counts exceeding 20,000/µL without transfusion, respectively. Acute GVHD was graded from 0 to IV using conventional criteria.\textsuperscript{4} VOD of the liver was defined as an increase in bilirubin of more than 2 mg/dL with at least two of the following: hepatomegaly, ascites, and a body weight gain of greater than 5%.\textsuperscript{17}

The patients’ characteristics, including underlying diseases and conditioning regimens are shown in Table 1. Acute myeloid leukemia (AML) was the most common underlying diagnosis (51 acute myeloid leukemia; 21 chronic myeloid leukemia; 21 acute lymphocytic leukemia; 21 severe aplastic anemia; 12 myelodysplastic syndrome; 10 non-Hodgkin's lymphoma). Low-risk disease was defined as follows: acute leukemia in first remission, chronic myeloid leukemia in first chronic phase, and severe aplastic anemia. Other diseases were

### Table 1. Patients’ characteristics (n=152).

<table>
<thead>
<tr>
<th>Age (median, range), years</th>
<th>40 (16-70)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, n (%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>86 (56.6%)</td>
</tr>
<tr>
<td>Female</td>
<td>66 (43.4%)</td>
</tr>
<tr>
<td>Transplant method, n (%)</td>
<td></td>
</tr>
<tr>
<td>Reduced intensity conditioning</td>
<td>70 (46.1%)</td>
</tr>
<tr>
<td>Conventional conditioning</td>
<td>82 (53.9%)</td>
</tr>
<tr>
<td>Stem cell source</td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>64 (42.1%)</td>
</tr>
<tr>
<td>Peripheral blood stem cells</td>
<td>80 (52.6%)</td>
</tr>
<tr>
<td>Bone marrow and peripheral blood stem cells</td>
<td>8 (5.3%)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>51</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>21</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>21</td>
</tr>
<tr>
<td>Severe aplastic anemia</td>
<td>19</td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>12</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
<td>10</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>5</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>5</td>
</tr>
<tr>
<td>Others</td>
<td>8</td>
</tr>
<tr>
<td>High risk disease*</td>
<td>79/152 (46%)</td>
</tr>
<tr>
<td>Granulocyte recovery</td>
<td></td>
</tr>
<tr>
<td>Median days to reach 500/µL</td>
<td>15 (4-38)</td>
</tr>
<tr>
<td>Platelet recovery</td>
<td></td>
</tr>
<tr>
<td>Median days to reach 20,000/µL</td>
<td>16 (5-73)</td>
</tr>
</tbody>
</table>

*Includes all other states than acute leukemia in first complete remission, chronic myeloid leukemia in first chronic phase and severe aplastic anemia.
P2XR A1513C genotyping

Genomic DNA was prepared from peripheral blood samples using a Puregene® DNA purification kit (Gentra, Minneapolis, MN, USA). Peripheral blood DNA of the 152 patients was genotyped. Genotypes of 145 recipients and 150 donors were obtained and analyzed for the polymorphism. SNP genotyping was performed using SNP-IT™ assays and the SNPstream 25K System (Orchid Biosciences, Princeton, NJ, USA). Briefly, the genomic DNA region spanning the polymorphic site was amplified using one phosphothiolated primer and one regular polymerase chain reaction (PCR) primer (sense: AAGCTGCCTCCATCTCA, antisense: AACAGCTCTGAGGTGGTGAT). Amplified PCR products were then digested with exonuclease (Amersham Biosciences, Uppsala, Sweden). 5′ phosphothiolates were used in this study to protect one strand of the PCR-product from exonuclease digestion. The single-strand PCR template generated by exonuclease digestion was overlaid onto a 384-well plate precoated covalently with the extension primer (SNP-IT™ primer with the GAGAGC-CACAGGTGCCTGGAGG sequence). These SNP-IT™ primers were designed to hybridize immediately adjacent to the polymorphic site. After hybridization of template strands, SNP-IT™ primers were extended by a single base using DNA polymerase at the polymorphic site of interest. Extension mixtures contained two labeled terminating nucleotides (one fluorescein isothiocyanate, one biotin) and two unlabeled terminating nucleotides. The final single base incorporated was identified by serial colorimetric reactions using anti-fluorescein-AP (Roche, Basel, Switzerland) and streptavidin-horse radish peroxidase (Fierce, Rockford, IL, USA), respectively. Respective blue and/or yellow color developments were analyzed using an enzyme linked immunosorbent assay (ELISA) reader and the final genotyping calls were made using the QC Review™ program.

Statistical data analysis

Statistical analyses of categorical variables were performed using the Pearson’s χ² test or Fisher’s exact test as appropriate. Continuous data were compared using linear regression. To determine whether the P2XR SNP site is in Hardy-Weinberg equilibrium, the distributions of observed genotype frequencies and expected genotype frequencies were compared using the χ² test. Mean values of peak total bilirubin, AST, and ALT were calculated for individual genotypes. Engraftment rates and the incidences of complications, such as acute GVHD and hepatic VOD, were compared between genotypes. Treatment-related mortality (TRM) was defined as death from any cause, other than progression of the underlying disease, during the course of treatment. TRM and overall survival (OS) were calculated using the Kaplan-Meier method and comparisons between genotypes were made using the log-rank test. Cox regression analysis was used for multivariate analyses. The variables included in the models were age, gender, stem cell source, conditioning regimen used, and disease status at the time of transplantation (i.e., low risk vs. high risk). Two-sided p values < 0.05 were considered statistically significant.

Ethics

The study protocol was reviewed and approved by the institutional review board of Seoul National University Hospital, and complied with the recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

Results

Frequencies of P2XR A1513C genotypes

The frequencies of the A and C alleles of the P2XR A1513C polymorphism were 72% and 28%, respectively. The genotypes in patients were AA in 75 (51.7%), AC in 58 (40.0%), and CC in 12 (8.3%); the genotypes in donors were AA in 74 (49.3%), AC in 70 (46.7%), and CC in 6 (4.0%). The P2XR SNP site was in Hardy-Weinberg equilibrium (p > 0.05). The baseline characteristics of patients with different genotypes were comparable (Table 2).

Treatment-related mortality, disease-free survival, and overall survival analysis according to P2XR A1513C genotype

Treatment-related mortality and survival analyses were performed in donors and recipients separately. Overall survival was significantly shorter for recipients with the CC genotype than for those with the AA or AC genotype (92 days for 1513CC vs. 821 days for 1513AA or 1513AC, p = 0.0121), and for recipients from
donors with the CC genotype than for recipients from donors with the AA or AC genotype (63 days for 1513CC vs. 702 days for 1513AA or 1513AC, \( p = 0.0241 \); Figure 1). Multivariate analyses including sex, age, transplant method (reduced intensity conditioning vs. conventional conditioning), stem cell source, risk group, and P2X7R genotype as variables identified high-risk group (hazard ratio 3.25, 95% confidence interval 1.83–5.77) and donor 1513CC genotype (hazard ratio 2.66, 95% confidence interval 1.02–6.91) as risk factors for shorter survival (Table 3).

Median disease-free survival was shorter for recipients with the CC genotype than for those with the AA or AC genotype (76 days for 1513CC vs. not reached for 1513AA or 1513AC, \( p = 0.0283 \)), and for recipients from donors with the CC genotype (163 days for 1513CC vs. not reached for 1513AA or 1513AC, \( p = 0.054 \)). However, more high-risk patients were of the CC genotype, but the CC genotype in patients was not found to be an independent predictor of shorter disease-free survival (\( p = 0.372 \)) by Cox regression analysis using genotype and risk group as covariates. The 1-year TRM rate after transplantation appeared to be higher for recipients from donors with the CC genotype (33.3% for donors with 1513CC vs. 22.2% for 1513AA or 1513AC, \( p = 0.3325 \)), although this was not statistically significant.

**Complications after HSCT and the A1513C polymorphism**

Microbiologically documented infections with a positive blood culture were significantly more frequent in recipients of grafts from donors with a CC genotype (66.7% for donors with 1513CC genotype vs. 17.6% for those with a 1513AA or 1513AC donor, \( p = 0.014 \)). However, no difference was observed according to recipient genotype (27.3% for recipients with 1513CC vs. 22.2% for recipients with 1513AA or 1513AC, \( p = 0.712 \)). In addition, the occurrence of TRM was found to be related to a microbiologically documented infection (TRM rate, 71.4% in patients with a positive blood culture vs. 12.2% in patients without a positive blood culture, \( p < 0.001 \)). The identified pathogens were Gram-

**Table 2. Comparison of patients’ characteristics according to the P2X7R genotypes of donors and patients.**

<table>
<thead>
<tr>
<th>Patient P2X7R A1513 genotype</th>
<th>Donor P2X7R A1513 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (n=75)</td>
<td>AA (n=74)</td>
</tr>
<tr>
<td>AC (n=58)</td>
<td>AC (n=70)</td>
</tr>
<tr>
<td>CC (n=12)</td>
<td>CC (n=6)</td>
</tr>
<tr>
<td>p value(^a)</td>
<td>p value(^a)</td>
</tr>
<tr>
<td>Sex (male), n (%)</td>
<td>45 (60%)</td>
</tr>
<tr>
<td>Age [mean±SD(years)]</td>
<td>41.3±12.6</td>
</tr>
<tr>
<td>Transplant method</td>
<td>40 (53.3%)</td>
</tr>
<tr>
<td>(reduced intensity conditioning), n (%)</td>
<td>32 (55.2%)</td>
</tr>
<tr>
<td>Stem cell source</td>
<td>33 (44.0%)</td>
</tr>
<tr>
<td>(BM), n(%)</td>
<td>24 (41.4%)</td>
</tr>
<tr>
<td>High-risk disease, n(%)</td>
<td>43 (57.3%)</td>
</tr>
</tbody>
</table>

Excluding patients whose laboratory tests or genotyping results were unavailable; p-values were calculated using the \( \chi^2 \) test or ANOVA.

**Figure 1.** Kaplan-Meier plots of overall survival according to (A) patient and (B) donor P2X7R A1513C genotype.
positive bacteria in 21 patients, Gram-negative bacteria in nine patients, and Candida species in three patients (four patients were infected by more than one pathogen). Peak bilirubin, AST, ALT, and creatinine levels during the initial 30-day post-transplantation period were not associated with the A1513C polymorphism in either donors or recipients, and neither was platelet or granulocyte recovery.

Thirty-seven patients (24.3%) developed grade II-IV acute GVHD; 16 patients grade II acute GVHD, 10 patients grade III, and 11 patients grade IV. The occurrence of acute GVHD was not related to the P2X7R A1513C polymorphisms. Hepatic VOD developed in 19 patients (12.5%), but showed no association with A1513C genotype (Table 4).

**Discussion**

The present study describes the importance of the A1513C P2XR gene polymorphism in HSCT, with regard to infections and survival after transplantation. Patients who received stem cells from matched sibling donors and who were homozygous for the minor C allele were found to be vulnerable to infections (as proven by blood culture) and had a shorter survival. However, no association was found between the polymorphism and acute GVHD.

IL-1α, IL-1β and IL-18 belong to the IL-1 family, and mediate inflammation and a wide range of other biological effects associated with infection, inflammation, and autoimmune processes. IL-1 is pyrogenic, induces hepatic acute-phase proteins, activates lymphocytes, and promotes prostanoid synthesis. It also augments antimicrobial defenses and enhances immunologic responses; on the other hand it causes hypotension and has a deleterious effect in the pathogenesis of sepsis syndrome. With regard to tumors and chemotherapy, IL-1 has a direct antiproliferative effect on human tumors and can accelerate hematopoietic recovery after myelosuppressive therapies. Moreover, IL-1Ra binds competitively to IL-1α and IL-1β.

P2XR plays an important role in secreting IL-1 family cytokines in response to bacterial lipopolysaccharides. Pro-IL-1β and pro-IL-1α, which accumulate in the cytosol in the presence of lipopolysaccharides, are released after stimulation of the P2XR by extracellular ATP. IL-1Ra is also released by blood cells such as monocytes, but not by endothelial cells after P2XR activation. P2XR consists of 13 exons, of which exons 12 and 13 code for the C-terminus. The P2XR gene is highly polymorphic and is known to harbor more than 260 SNP. The A to C polymorphism at position 1513 of the coding region of the P2XR gene causes the substitution of Glu-496 by Ala in the intracellular C-terminal tail. This polymorphism was
found not to affect the surface expression of P2X-R, though the C allele was non-functional when expressed at low density but regained normal function at high density in terms of monocyte differentiation to macrophages and the apoptosis of lymphocytes. In addition, the A1513C polymorphism has been linked to reduced release of IL-1β and IL-18 from human macrophages. With regard to infections, the Glu-496 to Ala polymorphism has been shown to impair ATP-mediated immune responses, such as the killing of mycobacteria by human macrophages, and it is also believed to contribute to the pathogenesis of chronic lymphocytic leukemia. Moreover, because P2X-R-dependent apoptosis is impaired in the presence of the C allele, its expression could result in the accumulation of neoplastic B cells.

The shorter survival of recipients whose donors were homozygous for the C allele could be explained by a higher incidence of bacteremia. IL-1 family cytokines are secreted mainly by immune cells (e.g. monocytes, macrophages, and dendritic cells), and recipients of hematopoietic stem cells of the P2X-R CC genotype might show impaired IL-1 cytokine release, which would contribute to the observed high incidence of bacteremia. In addition, evidence associates a reduced IL-1 level and increased susceptibility to infection. Serious infections tended to increase after the administration of anakinra, a recombinant human IL-1Ra, in patients with rheumatoid arthritis. Moreover, a low dose of recombinant IL-1 protected granulocytopenic mice from lethal Gram-negative infection.

In the present study, patients with the CC genotype also showed shorter survival. Recurrences of pre-transplant disease were more frequent in these patients, mainly because patients with the CC genotypes had more progressed disease which is reflected by the higher percentages of high-risk patients in the CC genotypes. In addition, the relation between IL-1 family cytokines and tumors should also be considered. IL-1 is thought to be a growth factor for acute and chronic leukemia and to act as a stimulator of colony-stimulating growth factors and other cytokines, such as IL-1β, whereas IL-1 and IL-18 are known to have antitumor effects in experimental models. The direct injection of IL-18 recombinant adenoviral vector into a murine fibrosarcoma model completely eradicated tumors in all animals and concomitantly induced protective systemic immunity. The intratumoral co-administration of IL-18-expressing adenoviral vector and dendritic cells resulted in the complete regression of injected tumors. IL-1 has also been tested in phase I and II clinical trials. The above findings collectively suggest the possibility of a relation between tumor recurrence and P2X-R polymorphisms, but unfortunately this could not be examined during the present retrospective study. Further prospective studies on patients with a homogenous disease status are needed to investigate this issue.

Previous research on the effects of IL-1 and IL-18 in HSCT have focused on the occurrence of acute and chronic GVHD. In general, it has been reported that increased serum levels of IL-18 after engraftment correlate with acute GVHD in allogeneic HSCT. However, we found no correlation between the P2X-R polymorphism and GVHD. IL-1 and IL-1Ra might be affected by P2X-R status, but the P2X-R polymorphism alone was not found to be associated with the occurrence of GVHD in the present study. Heterogeneity of study populations and treatment regimens should be considered and prospective studies on IL-1 family cytokines and GVHD are warranted in a homogenous group of patients.

The effects of IL-1 cytokines on hematopoiesis have been previously described. IL-1 was found to be a myeloprotective agent when administered before myelotoxic chemotherapy. However, we did not find any differences between the recovery times of peripheral blood leukocytes or platelets and P2X-R genotype. One possible explanation for this is that previous studies used exogenous IL-1 species, whereas we examined the effects of endogenous IL-1. Moreover, impaired cytokine secretion, despite similar neutrophil counts, might have influenced leukocyte inflammatory and immunological responses against micro-organisms. As far as we are aware, the present study is the first to find that the A1513C P2X-R gene polymorphism is associated with clinical outcome in HSCT. Several researchers have shown associations between SNP and infections after HSCT, but no data regarding the P2X-R gene have been reported.

Future prospective research on the A1513C P2X-R polymorphism in a larger group of patients should aid our understanding of the role of this polymorphism in HSCT. Moreover, other SNP in this gene, including gain-of-function polymorphisms, should also be investigated to comprehensively define their roles. Ethnicity should also be considered, because the allelic frequency of A1513C determined in the present Korean cohort appears to be higher than frequencies previously reported for Caucasians, which ranged from 8% to 24%.

We conclude that the A1513C polymorphism of the P2X-R gene is related to the occurrence of infections and survival following allogeneic stem cell transplantation, and that the determination of this polymorphism may be helpful for the optimal selection of patients and donors.

**Authors’ Contributions**

K-HL and SSP contributed equally to this work. K-HL collected clinical data and wrote this paper. SSP designed experiments and did genotypings. IK was the principal investigator of this study and was responsible for the conception and supervision of the study and of the paper. JHK and Y-CH performed statistical analysis. EKR collected and prepared samples. IK, SSY, SP and BKK was involved in diagnosis and management of patients.

**Conflict of Interest**

The authors reported no potential conflicts of interest.
References


