lentivirus particles were delivered at a multiplicity of infection (MOI) of 50, corresponding to the transducing units of $50\times10^6$/mL. The effective over-expression of miR-1245 was confirmed by quantitative reverse transcriptase (RT)-PCR.

### Construction of a short hairpin RNA microRNA-1245 lentivirus delivery system

A lentivirus system for delivering a target sequence of the mature human miR-1245 was constructed using the pENTR/U6 BLOCK-iT entry vector and BLOCK-iT Lentiviral RNAi Expression System (Invitrogen) and was used to establish miR-1245-deficient NK cells. Further details are given in the *Online Supplementary Design and Methods*.

### Transfection with a synthetic inhibitor of microRNA-1245

A synthetic miR-1245 inhibitor, anti-has-miR-1245 (catalog # MIN0005897), and a miScript Inhibitor Negative Control (Catalog # 1027271) anti-microRNA-NC, designated hereafter as antago-miR-1245 and antago-NC, respectively, were obtained from Qiagen. These synthetic antagonists were used to transfect cultured NK cells.

### Measurement of cytokines and granzyme B secretion

The levels of tumor necrosis factor-α, interferon-γ and granzyme B in the culture supernatants secreted by primary NK cells or from NK cell lines in response to NKG2D receptor stimulation were measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Mabtech Nacka Strand Sweden), as described in more detail in the *Online Supplementary Design and Methods*.

### Induction of NKG2D ligands on leukemia cells

To induce the expression of NKG2D ligands, OUN-1 leukemia cells were cultured for 24 h in the presence or absence of 200 μg/mL valproic acid, as described in a previous report.[9] The effective induction of the expression of the NKG2D ligands, MICA/B and ULBP-2, in this cell line was determined by flow cytometry.

### Cytotoxicity assay

The cytotoxic activity of NK cells was measured with the LDH Non-Radioactive Cytotoxicity Assay (Roche Applied Science) using OUN-1 leukemia cells as target cells. Further details are given in the *Online Supplementary Design and Methods*.

### Statistical analysis

All data are reported as means ± standard errors. When comparisons were made between two different groups, statistical significance was determined using Student's t-test. The statistical significance of multiple comparisons was determined using a one-way analysis of variance. The data were considered statistically significant when the $P$ value was $\leq 0.05$. All statistical analyses were performed using the GraphPad Prism software package (San Diego, USA).

## Results

### Identification of microRNA-1245 as a potential negative regulator of NKG2D in natural killer cells

Estimated to regulate more than 30% of all human genes, microRNA have emerged as important regulators of gene expression.[12] Since NKG2D is a powerful receptor modulated by numerous factors, many of which are known to induce changes in microRNA expression,[13] we hypothesized that the 3'UTR of the *NKG2D* gene may contain target sites for microRNA. To test this hypothesis, we first screened potential conserved microRNA targeting the 3'UTR of the *NKG2D* gene by using computational prediction tools. Three different online algorithms, TargetScan,[7] miTarget[14] and MicroSniper,[15] were used to demonstrate that the *NKG2D* 3'UTR contains a conserved region which represents a putative target site for miR-1245 (Figure 1A). The RNA hybrid software program, which is a computer alignment model based on the prediction of the minimal free energy of hybridization,[16] further predicted the interaction of *NKG2D* 3'UTR with miR-1245 (Figure 1B). Next, the endogenous expression of miR-1245 was assessed in NK cells to determine the functional significance of the predicted miR-1245 binding site in the 3'UTR of the *NKG2D* gene. Quantitative RT-PCR revealed that miR-1245 is expressed in fresh NK cells and was also detected in cultured NK cells (Figure 1C). Variable levels of mature miR-1245 were also detected in the four NK cell lines tested (Figure 1D). Since exosomes from serum samples have been reported to contain functional microRNA,[11,17] the expression of miR-1245 was examined in the exosomes from the plasma of patients with hematologic malignancies and from healthy donors. While miR-1245 was undetectable in the plasma exosomes of seven healthy individuals, the exosomes of three out of seven patients with non-Hodgkin's lymphoma and two out of three patients with acute myelogenous leukemia/myelodysplastic syndrome contained abundant levels of miR-1245 (Figure 1E), thus substantiating the biological relevance of this novel microRNA.

### Interaction of microRNA-1245 with the 3'-untranslated region of the NKG2D gene

A luciferase reporter assay was used to directly assess whether the 3'UTR region of *NKG2D* interacts with miR-1245. A fragment of the 3'UTR region of *NKG2D* was inserted downstream of the luciferase open reading frame of the luciferase reporter vector pGL3-TK-Luc and the resultant construct was transfected into either stable miR-1245-overexpressing NKL cells or their wild-type counterparts. The presence of a *NKG2D* 3'UTR fragment in the reporter vector suppressed the luciferase activity in NKL cells but this effect was significantly greater in cells overexpressing miR-1245 (80% luciferase repression) than in the wild-type cells (44% luciferase repression) (Figure 2A). The forced expression of miR-1245 in HEK cells resulted in greater repression of luciferase activity induced by the *NKG2D* 3'UTR construct as well (Figure 2B). Remarkably, when the cells were transfected with a mutant reporter plasmid, which included a deletion of the microRNA-1245 target sequence region, there was a significant decrease in the repression of luciferase activity induced by the *NKG2D* 3'UTR fragment, which completely abolished the increased repression induced by ectopic miR-1245 expression. As expected, there were no differences in the luciferase activity in either the miR-1245-overexpressing cells, or in the wild-type cells transfected with the pGL3-TK-Luc vector (without *NKG2D* 3'UTR fragments). Taken together, these results indicate that the microRNA-1245 binding motif in the *NKG2D* 3'UTR is targeted by miR-1245.

### Inducible over-expression of microRNA-1245 in natural killer cells

To investigate the potential modulation of endogenous miR-1245 expression in NK cells, we first examined the expression of the primary gene transcript of miR-1245,

pri-miR-1245, which is the starting point in the biogenesis of mature miR-1245. Freshly isolated or activated NK cells (Figure 3A) and NK cell lines (Figure 3B) invariably expressed high levels of pri-miR-1245; these levels appeared to be higher than those of mature miR-1245, suggesting that mature miR-1245 may be markedly inducible. TGF-β1 was thought to be a candidate capable of modulating miR-1245 since it is a negative regulator of NKG2D expression[18,19] and TGF-β1 signals up-regulate other microRNA.[10,20] Primary NK cells or NK cell lines were treated with TGF-β1 and miR-1245 expression was examined. A quantitative RT-PCR analysis revealed that TGF-β1 treatment of primary NK cells induced a consistent and dose-dependent up-regulation of the level of mature miR-1245 (Figure 3C). Of note, TGF-β1 treatment did not increase the expression of pri-miR-1245. NK cell lines treated with TGF-β1 also had up-regulated mature miR-1245 levels (Figure 3D). To investigate whether the TGF-β1-mediated inducible over-expression of miR-1245 occurs at the transcriptional or post-transcriptional level, the time-based kinetic accumulation of pri-miR-1245 and mature miR-1245 in response to TGF-β1 treatment was examined in fresh NK cells derived from seven individuals. As shown in Figure 3E, the mature miR-1245 levels started increasing at 8 h and peaked at 16 h after starting TGF-β1 treatment. Conversely, no significant changes in the expression of pri-miR-1245 were detected. These results suggest that the TGF-β1-induced miR-1245 over-expression occurs at the level of post-transcriptional processing. The effect of other cytokines on the expression of miR-1245 was investigated. While the treatment of NK cells with interleukin-2 or interferon-γ had no significant effects on mature or pri-miR1245 levels, interleukin-15, which is a potent inducer of NKG2D expression, decreased the expression of mature miR1245 by 20-fold in NK cells (Figure 3F). Dose-dependent inhibition of mature miR-1245 by interleukin-15 further confirmed this finding (*Online Supplementary Figure S1*).

### Effect of exogenous microRNA-1245 on NKG2D expression in natural killer cells

To investigate the effect of miR-1245 on the expression of NKG2D receptor on NK cells, fresh NK cells were transduced with a miR-1245 precursor vector (miR-1245-vector) or a negative control vector (NC-vector) using a lentiviral delivery system and the NKG2D receptor levels were

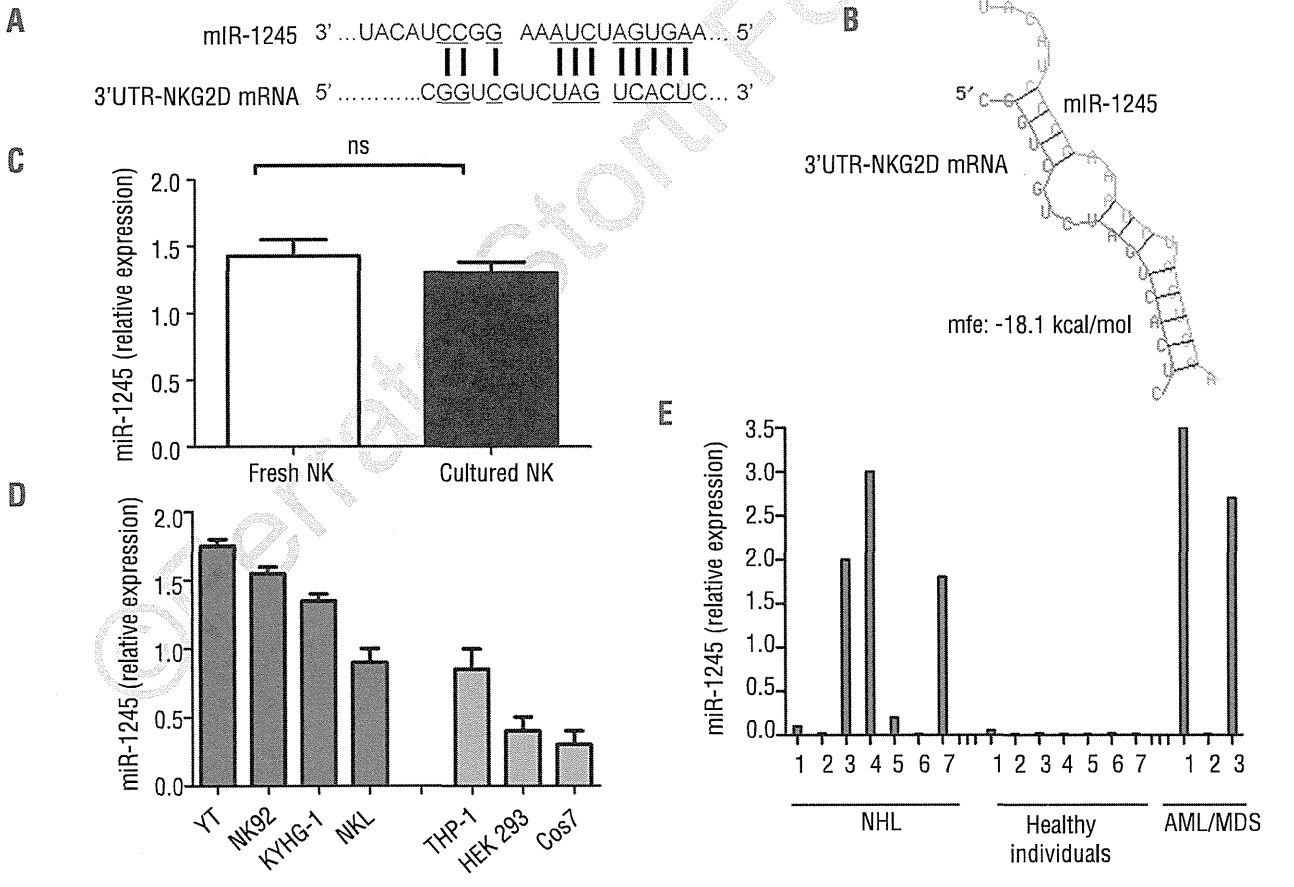


Figure 1. **Identification of miR-1245 as a potential negative regulator of NKG2D in NK cells.** (A) A schematic representation of the interaction between miR-1245 and its target site in the 3'UTR region of *NKG2D* mRNA. (B) Computational modeling showed the hybridization of miR-1245 and the 3'UTR region of *NKG2D* mRNA; mfe represents the calculated minimal free energy. (C) Mature miR-1245 expression in fresh or activated primary NK cells was measured by quantitative RT-PCR and normalized to U6B RNA. The data are the means ± S.E.M. (n= 13). (D) The expression of mature miR-1245 normalized to that of U6B RNA in NK cell lines YT, KYHG-1, NK92 and NKL. (E) The expression of mature miR-1245 in exosomes isolated from plasma samples from patients with non-Hodgkin's lymhoma (NHL) (n=7), acute myelogenous leukemia/myelodysplastic syndrome (AML/MDS) (n=3) and from seven healthy donors.

examined after transduction using flow cytometry. Effective over-expression of mature miR-1245 levels, which were dependent on the amount of lentivirus particles transduced, was achieved in primary NK cells, KHYG-1 cells and HEK cells as confirmed by quantitative RT-PCR (*Online Supplementary Figure S2*). The mature miR-1245 levels in NK cells transduced with a MOI of 50 were equivalent to those induced by treatment with 10 ng/mL of TGF-β1 for 16 h, and was achieved in NK cells transduced with miR-1245-vector. The over-expression of mature miR-1245 in NK cells resulted in a consistent down-regulation of the NKG2D receptor. (Figure 4A and 4B).

The over-expression of miR-1245 in primary NK cells did not reduce the expression of other activator receptors, including NKp30, NKp44 and NKp46, in primary NK cells indicating the specificity of miR-1245 in the NKG2D down-regulation (*Online Supplementary Figure S3*). Next, quantitative real-time PCR was used to measure NKG2D mRNA levels in NK cells transduced with miR-1245 in order to identify the suppressive mechanism used by this particular microRNA, since microRNA can suppress gene expression by inhibiting translation or by inducing mRNA degradation. There were significantly lower levels of NKG2D transcripts in NK cells transduced with the miR-1245-vector in comparison with the untreated cells or the NC-vector transduced cells ($P<0.05$) (Figure 4C)

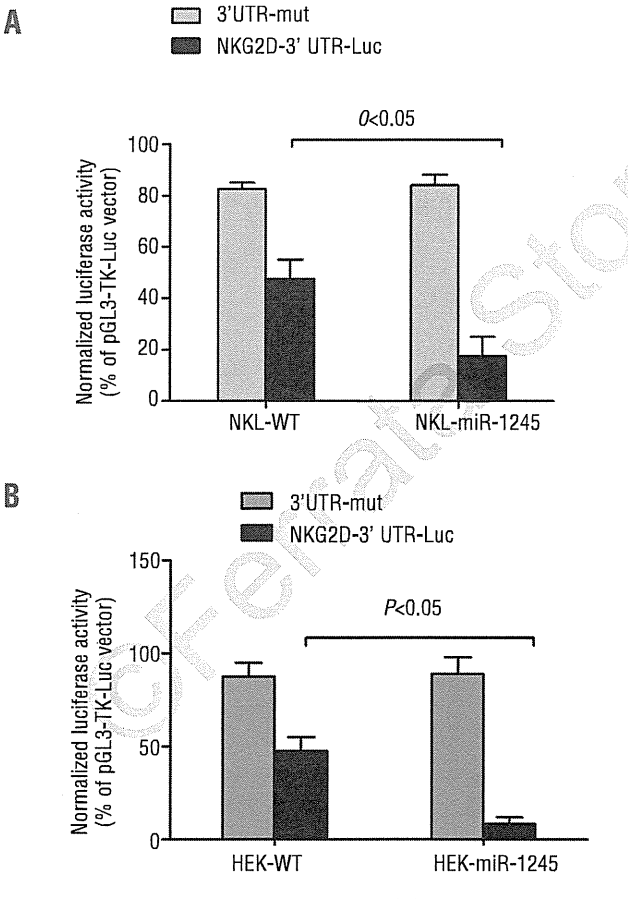


Figure 2. **The interaction of miR-1245 with the *NKG2D* gene 3'UTR. (A) NKL cells or (B) HEK cells overexpressing miR-1245, or their wild type (WT) counterparts, were transfected with a luciferase expression vector (pGL3-TK-Luc), or with constructs including the *NKG2D* 3'UTR (NKG2D-3' UTR-Luc) or with a luciferase expression vector that included the *NKG2D* 3'UTR with a 16-bp deletion of the miR-1245 targeting site (3' UTR-mut). The firefly luciferase activities measured 48 h after transfection were normalized to the Renilla luciferase expression and the mean activities ± S.E.M. from three independent experiments are shown.**

To substantiate whether the down-regulation of NKG2D by miR-1245 in NK cells has functional significance, the NKG2D-mediated effector functions were assessed in NK cells over-expressing miR-1245. Previous studies demonstrated that *in vitro* engagement of the NKG2D receptor by recombinant NKG2D ligands is sufficient to deliver activator signals leading to cytokine release from the NK cells.[21] To examine whether the NKG2D down-regulation induced by miR-1245 has functional implications, primary NK cells from seven healthy donors transduced with the miR-1245-vector, and their counterparts transduced with the NC-vector, were cultured in plates coated with a mixture of recombinant NKG2D ligands or on plates coated with mouse IgG, and the levels of tumor necrosis factor-α in the cell culture supernatants were measured by an ELISA. The NK cells over-expressing miR-1245 secreted significantly lower levels of tumor necrosis factor-α in response to NKG2D engagement with recombinant NKG2D ligands compared to the NK cells transduced with the NC-vector (Figure 4D). Consistent with these results of the cytokine secretion assay, the miR-1245 over-expressing NK cells displayed significantly lower cytotoxicity against the myeloid leukemia OUN-1 cells treated with valproic acid, an inducer of NKG2D ligands in this cell line (*Online Supplementary Figure S4*), compared with the NK cells transduced with NC-vector (Figure 4E). There was no difference in the killing effect between NK cells and miR-1245-transduced NK cells when the target cells were pretreated with vehicle alone, or when the effector NK cells were pretreated with anti-NKG2D monoclonal antibodies, thus suggesting that this effect was ligand-dependent. Taken together, these results suggest that the NKG2D expression regulated by microRNA-1245 in NK cells impairs the cytotoxic activity of NK cells.

### Natural killer cell lines over-expressing microRNA-1245 down-regulate NKG2D expression

NK cell sub-lines derived from NK-92 and KYHG-1 cells lines were established by lentivirus delivery of the miR-1245-vector. The effective over-expression of miR-1245 in NK cell lines resulted in a significant decrease in cell surface NKG2D expression in KHYG-1 and NK-92 cells (Figure 5A and 5B) and reduced NKG2D transcripts (Figure 5C), indicating that miR-1245 is a negative regulator of NKG2D expression. The over-expression of miR-1245 in these cell lines was not associated with a reduction in the expression of other activator receptors (*Online Supplementary Figure S5*). Notably, the KHYG-1 and NK-92 cells over-expressing miR-1245 secreted significantly lower levels of interferon-γ and granzyme B in response to NKG2D receptor engagement by NKG2D ligands (*Online Supplementary Figure S6*).

### Knocking down microRNA-1245 in natural killer cells resulted in higher NKG2D expression and relative resistance to the effect of transforming growth factor-β1

miR-1245-deficient NK cell lines were established by lentivirus delivery of the anti-miR-1245 to substantiate the role of miR-1245 in the regulation of NKG2D expression in NK cells. A KYHG-1 clone was identified, in which effective miR-1245 knockdown was consistently achieved as demonstrated by quantitative RT-PCR. In contrast to their wild-type counterpart or in clones transduced with shRNA-NC, these cells did not express miR-1245 even in the pres-

ence of TGF-β1 treatment (*Online Supplementary Figure S7*). Interestingly miR-1245 5KO cells expressed higher levels of NKG2D receptor at both protein (Figure 6A) and transcript levels (Figure 6B). In addition, although TGF-β1 treatment also resulted in the down-regulation of NKG2D receptor in miR-1245 5KO cells, this effect was less notable than in wild-type cells (Figure 6C) or in those cells transduced with shRNA-NC (*data not shown*). Moreover, miR-1245 5KO cells secreted higher levels of interferon-γ in response to NKG2D receptor engagement by NKG2D ligands compared to KYHG-1 cells transduced with shRNA-NC or with their wild-type counterpart (*Online Supplementary Figure S8*). These results suggest that NKG2D down-regulation induced by TGF-β1 in NK cells involves the induction of miR-1245.

To address whether the effects of knocking down miR-1245 are also observed in primary NK cells, cultured NK cells from four healthy individuals were transiently transfected with a synthetic antagonist of miR-1245 or a negative control antago-NC. The antago-miR-1245- transfected cells up-regulated NKG2D receptor (Figure 6D) and were less sensitive to the effects of TGF-β1 treatment on the down-regulation of NKG2D expression compared to their antago-NC-transfected counterparts (Figure 6E).

## Discussion

Normal cells require a protective mechanism against NKG2D receptor overactivation to prevent self-attack, since NKG2D is implicated in certain autoimmune conditions.[22] This study has identified a novel mechanism for the modulation of *NKG2D* gene expression in NK cells. Several *in vitro* approaches demonstrated that miR-1245 interacts with the 3'UTR region of *NKG2D*, and that this interaction resulted in the down-regulation of NKG2D expression in NK cells, thus leading to an impairment in NKG2D-mediated immune functions, including less effective cytotoxicity and lower cytokine secretion.

One question remains with regard to how miR-1245 contributes to regulating the expression of NKG2D *in vivo*. The constitutive expression of miR-1245 in NK cells suggests that it may represent an endogenous autoregulatory mechanism to maintain NKG2D expression at physiological levels. NK cells are a pivotal source of endogenous TGF-β1, and this immunosuppressive cytokine secreted from NK cells represses NK cell functions, including the down-regulation of the NKG2D expression, in a paracrine/autocrine manner,[18,23,24] thus indicating that TGF-β1 plays an important role as negative feedback to prevent overstimulation of NK cell functions. The current findings suggest that miR-1245 induc-

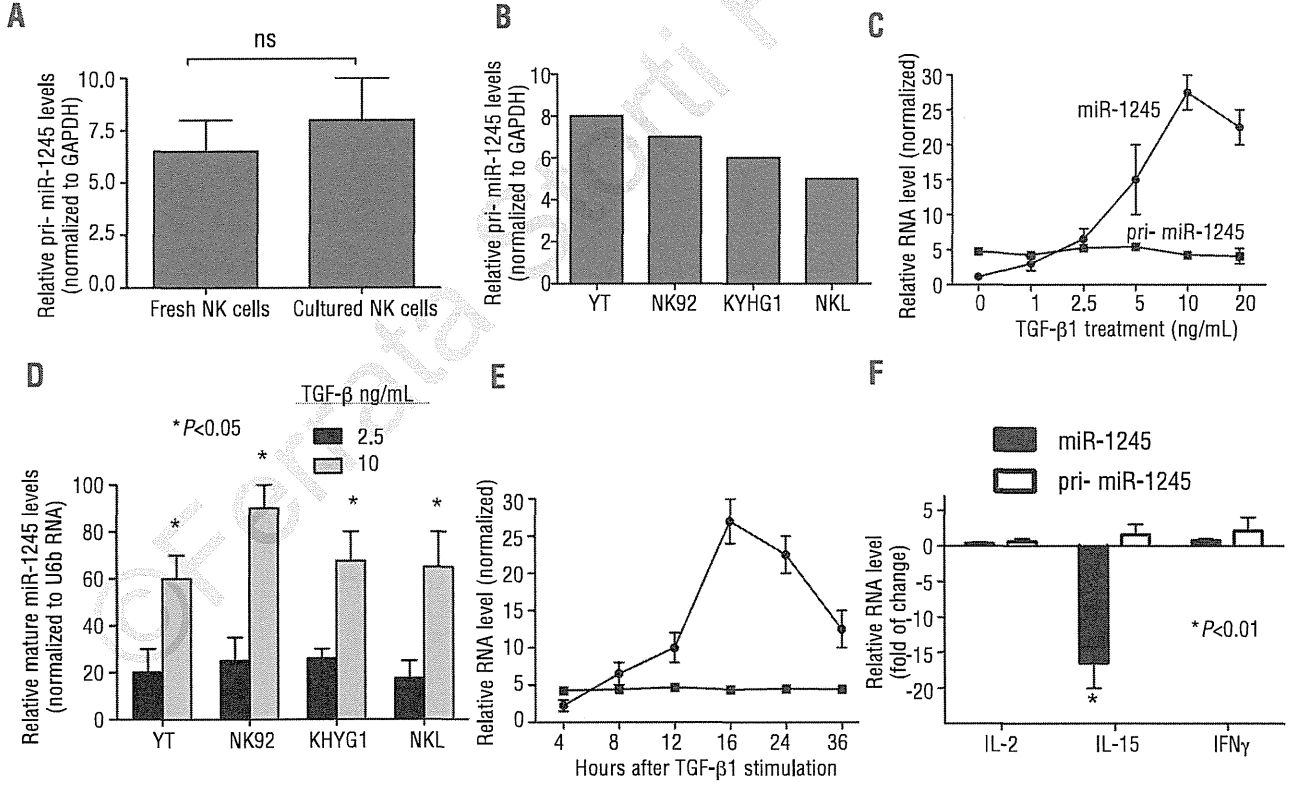


Figure 3. **Post-transcriptional regulation of mature miR-1245 biogenesis in NK cells by TGF-β1.** (A) The expression of the primary gene transcripts (pri-miR-1245) in fresh and cultured NK cells normalized to GAPDH. The data are the mean values ± S.E.M. from measurements in seven donors. (B) The expression of pri-miR-1245 normalized to GAPDH in the NK cell lines YT, NK92, KYHG-1, and NKL. (C) The expression of pri-miR-1245 and mature miR-1245 in primary NK cells treated with various concentrations of TGF-β1 for 24 h (n=7). (D) The expression of mature miR-1245 in NK cell lines after treatment with TGF-β1 (2.5 or 10 ng/mL) for 24 h. The error bars in (C) and (D) show the ± S.E.M. from three independent experiments, each measured in duplicate. (E) The time course of pri-miR-1245 and mature miR-1245 expression in primary NK cells after treatment with TGF-β1 (10 ng/mL) (n=7). The data are the means ± S.E.M. from three independent experiments, each measured in duplicate. (F) The expression of pri-miR-1245 (open bar) and mature miR-1245 (closed bar) in primary NK cells treated with interleukin (IL-2) (150 U/mL), interleukin-15 (IL-15) (50 ng/mL) or interferon-γ (IFN-γ) for 24 h. The error bars show the ± S.E.M. from three independent experiments, each measured in duplicate.

tion may, therefore, represent one of the downstream mechanisms by which TGF-β1 exerts such negative feedback to control NK cell activation.

NKG2D receptor plays a relevant role in tumor immunosurveillance and cancer cells use several mechanisms to escape NKG2D-mediated cytotoxicity including the down-regulation of NKG2D ligands on their surface, the release of soluble NKG2D ligands, to induce NKG2D silencing and the secretion of immunosuppressive cytokines. Indeed various cancer cells produce TGF-β1, which impairs NK cell functions in cancer patients, leading to stimulation of tumor growth[18,19,25] and TGF-β1 released by tumor-arising cells has been suggested to influence immunosurveillance; the tumor microenvironment represents an important source of TGF-β1 impairing NKG2D-mediated functions.[26] The present study suggests the involvement of miR-1245 in the mecha-

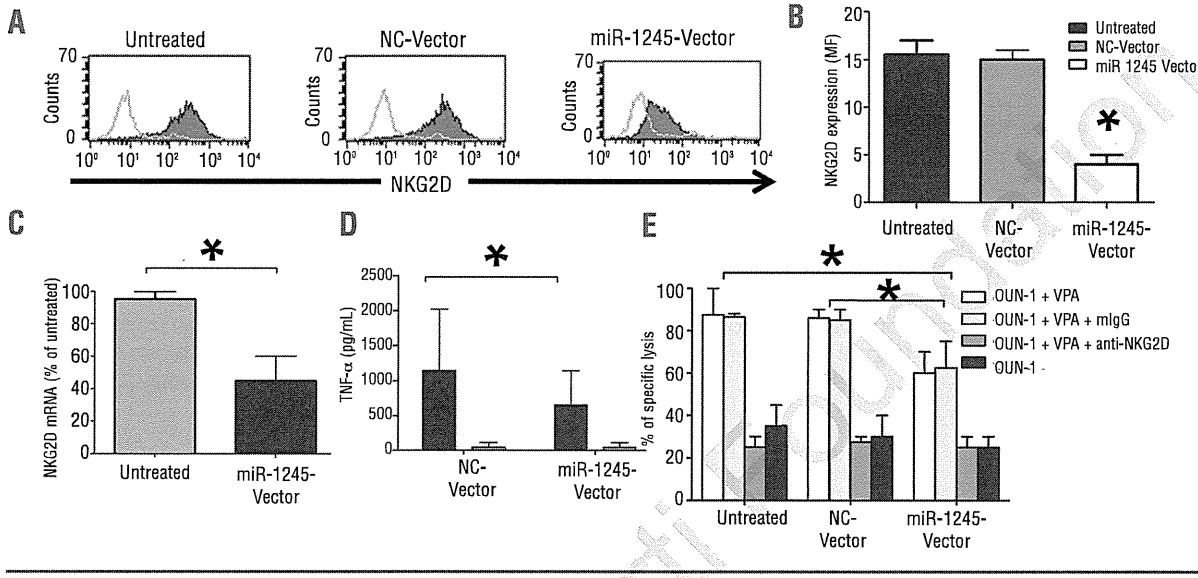


Figure 4. Exogenous miR-1245 decreases NKG2D expression in NK cells. (A) A representative result showing the cell surface expression of NKG2D on miR-1245-transduced primary NK cells. Cells stained with anti-NKG2D (filled histograms) or with the isotype antibody (open histogram). (B) The summarized data from 12 donors are shown. (C) Fresh NK cells were transduced as in the panels A and B, and the NKG2D mRNA levels were measured by quantitative RT-PCR 24 h after transduction. The relative results normalized to U6b RNA are expressed as the percentage of the mRNA compared to that for the control conditions (untreated cells). (D) Fresh NK cells transduced with the miR-1245-vector or with the NC-vector (n=7) and cultured in plates coated with a mixture of three recombinant NKG2D-Ls (MICA, ULBP-1 and ULBP-2) or control IgG. (E) Fresh NK cells treated as in panel D were left untreated (medium) and then assessed for their cytotoxicity against OUN-1 leukemia cells. The summarized data from seven donors (effector: target ratio, 5:1) are shown. The data are the means ± S.E.M. from three independent experiments. *$P<0.05$.

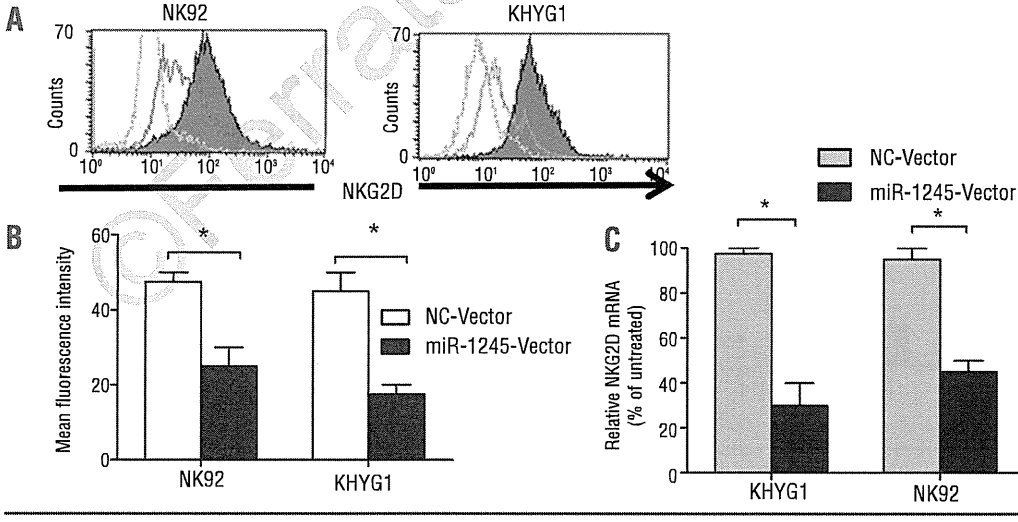


Figure 5. Down-regulation of the NKG2D receptor in NK cell lines stably overexpressing miR-1245. NK cell lines, NK92 andKHYG1 overexpressing miR-1245 were established by lentiviral delivery of a vector containing the miR-1245 precursor. (A) miR-1245 overexpressing cells or those transduced with a negative control vector were examined for NKG2D expression by flow cytometry. The filled histograms represent cells transduced with negative control vector, empty histograms represent cells transduced with the miR-1245-vector, and dashed line histograms represent cells stained with the isotype antibody. A representative figure showing the results of three independent experiments is shown. (B) Summarized results of three independent experiments for each cell line. (C) Total RNA was extracted from the above-described cells, and the normalized levels of the NKG2D transcript were measured by quantitative RT-PCR.

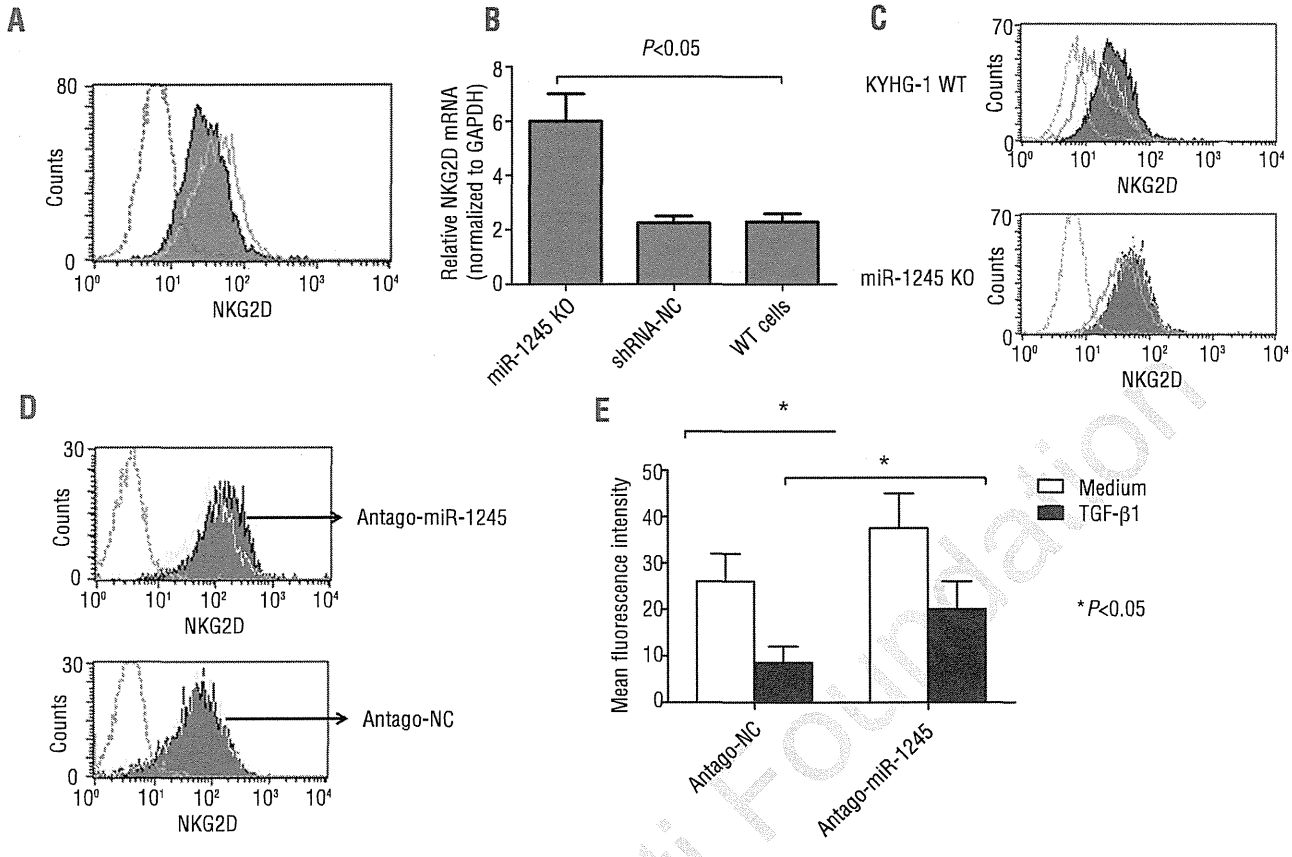


Figure 6. **Knockdown of miR-1245 increases the expression of NKG2D in NK cells, and renders the cells less sensitive to TGF-β1.** (A) KYHG-1 cells were examined by flow cytometry for NKG2D expression. The filled histogram represents shRNA-NC-transduced KHYG-1 cells stained with anti-NKG2D antibody. In the open histogram, the solid line shows the data from the miR-1245 5KO KHYG-1 cells stained with the anti-NKG2D antibody, and in the open histogram, the dotted line indicates cells stained with the isotype antibody. A representative figure from three independent experiments is shown. (B) Total RNA was extracted, and the levels of *NKG2D* mRNA normalized to the levels of *GAPDH* mRNA were measured by RT-PCR. The figure shows the means ± S.E.M. from three independent experiments. (C) miR-1245 5KO KHYG-1 cells or wild-type (WT) KHYG-1 cells were cultured for 48 h in the presence or absence of TGF-β1, and their NKG2D expression levels were examined by flow cytometry. The filled histogram represents untreated cells, the green histograms represent cells treated with TGF-β1 (5 ng/mL), the red histograms represent cells treated with TGF-β1 (10 ng/mL) and the blue histograms with the dotted line represent cells stained with the isotype antibody. A representative figure from the data obtained from three independent experiments is shown. (D) Cultured NK cells were left untreated or were transfected with antago-miR-1245 or antago-NC, and 48 h later, their NKG2D expression levels were examined by flow cytometry. The filled histogram represents cells transfected with antago-miR-1245 or antago-NC, the open histogram with solid lines represents untreated cells, and the open histograms with the dotted line represent cells stained with the isotype antibody. A representative figure from the data obtained from three independent experiments is shown. (E) Primary NK cells were transfected as in the panel (D). Twenty-four hours later, they were treated with TGF-β1 (10 ng/mL) and cultured for another 24 h, and their NKG2D expression levels were examined by flow cytometry. Summarized data for the NK cells from five donors are shown. The data are the means ± S.E.M. from three independent experiments.

nism(s) whereby the TGF-β1 produced in cancer patients may modulate NK cell functions. It is also conceivable that tumor cells might secrete miR-1245 and use this as a way to down-regulate NKG2D expression in order to escape NK cell-mediated killing. Although it is highly speculative, the release of microRNA through exosomes could provide a route by which tumor cells may deliver functional miR-1245 to impair NKG2D expression. In fact, tumor-derived exosomes have been reported to down-regulate NKG2D expression in NK cells.[19] Our finding that miR-1245 was detectable in exosomes of some patients with non-Hodgkin's lymphoma as well as in patients with acute myelogenous leukemia adds support to this hypothesis, although we were unable to investigate the possible correlation between miR-1245 expression in exosomes and NKG2D levels, since no NK cells from those patients were available for this study. Thus, further studies on the functional relevance of exosome derived miR-1245 are warranted.

The mature miR-1245 over-expression induced by TGF-β1 in NK cells occurs at the post-transcriptional level which is consistent with the mechanism by which TGF-β1 induces mature miR-21 in human vascular smooth muscle cells.[10,20] The regulation of miR-21 biogenesis involves the recruitment of TGF-β1–ligand specific SMAD signal transducer to members of the DROSHA microprocessor complex which facilitate the processing of primary transcript into pre-microRNA.[27]

The over-expression of miR-1245 reduced NKG2D expression at both the protein (Figure 4B) and mRNA (Figure 4C) levels in primary NK cells, thus suggesting that miR-1245 suppresses the expression of NKG2D through mRNA degradation, which is consistent with a recent study showing that decreasing target mRNA is the main mechanism by which mammalian microRNA regulate gene expression.[28]

However, the reduction of the expression of NKG2D appears to be greater at the protein level than at the mRNA level. Accordingly, in addition to mRNA degradation, miR-1245 could cause translational repression of mRNA; this possibility is supported by previous reports that microRNA reduces translation initiation[29] and increases ribosome drop-off.[30]

The present study did not investigate miR-1245 expression in different NK cell subsets such as $CD56^{bright}$ and $CD56^{dim}$. However, NK cells isolated from healthy individuals were more than 95% $CD16^{pos}$, suggesting that the vast majority of the NK cells were $CD56^{dim}$ and responsible for cytolytic activity and target killing.[31] Elucidating the functional roles of miR1245 in $CD56^{bright}$ $CD16^{neg}$ NK cells, which are the main source of cytokine production, will be an interesting challenge.

In conclusion, the current study has identified miR-1245 as a novel regulator of NKG2D expression by targeting the 3'UTR region of the *NKG2D* gene. TGF-β1, which is a potent NKG2D suppressor in NK cells, post-transcriptionally enhanced the expression of mature miR-1245, leading to down-regulation of the NKG2D receptor. There is a large amount of evidence to support the role of NKG2D in tumor immunosurveillance and progression. Although several clinical trials of TGF-β1 antagonists for cancer treatment are underway,[32] the main concern is that TGF-β1 antagonists could enhance the proliferation of tumor cells, which may counterbalance their potential therapeutic benefits on immunomodulation.[33] A recent study has shown that the processing of pri-microRNA could be selectively blocked.[34] miR-1245 could, therefore, be a promising target for tumor immunotherapy to improve NK cell activity.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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# Recipient *PTPN22* −1123 C/C Genotype Predicts Acute Graft-versus-Host Disease after HLA Fully Matched Unrelated Bone Marrow Transplantation for Hematologic Malignancies

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*PTPN22* is a critical negative regulator of T cell responses. Its promoter gene variant (rs2488457, −1123G>C) has been reported to be associated with autoimmune diseases. This study analyzed the impact of the *PTPN22* variant on transplantation outcomes in a cohort of 663 patients who underwent unrelated HLA-matched bone marrow transplantation (BMT) for hematologic malignancies through the Japan Marrow Donor Program. The recipient C/C genotype versus the recipient G/G genotype resulted in a lower incidence of grade II-IV acute graft-versus-host disease (hazard ratio [HR], 0.50; 95% confidence interval [CI], 0.29-0.85; $P = .01$), as well as a higher incidence of relapse (HR, 1.78; 95% CI, 1.10-2.90; $P = .02$), as demonstrated on multivariate analysis. In patients with high-risk disease, the recipient C/C genotype was associated with significantly worse overall survival rates than the recipient G/G genotype (HR, 1.60; 95% CI, 1.02-2.51; $P = .04$), whereas this effect was absent in patients with standard-risk disease. In addition, the donor G/C genotype was associated with a lower incidence of relapse (HR, 0.58; 95% CI, 0.40-0.85), which did not influence survival. Our findings suggest that *PTPN22* genotyping could be useful in predicting prognoses and creating therapeutic strategies for improving the final outcomes of allogeneic BMT.



## INTRODUCTION

The *PTPN22* gene encodes lymphoid specific phosphatase (Lyp), expressed in T and B lymphocytes, monocytes, dendritic cells (DCs), neutrophils, natural killer cells and thymocytes [1]. *PTPN22* is an important negative regulator of T cell activation involved in the dephosphorylation and inactivation of TCR-associated kinases. A single nucleotide variant of the *PTPN22* promoter gene, rs2488457 (−1123G>C), is associated with susceptibility to autoimmune diseases, including type 1 diabetes and rheumatoid arthritis, in Caucasian and Asian populations [2-6].

The role of *PTPN22* in the immune response, as well as the association of the *PTPN22* variant with autoimmunity, prompted us to investigate the impact of donor and recipient −1123G>C variation in the *PTPN22* gene on the clinical outcomes of patients undergoing allogeneic bone marrow transplantation (BMT) using an HLA allele-matched unrelated donor through the Japan Marrow Donor Program (JMDP). Our data show that the recipient C/C genotype is associated with a significantly lower incidence of grade II-IV acute graft-versus-host disease (aGVHD) and a higher incidence of relapse, which predict worse survival outcomes for patients with high-risk disease.

## PATIENTS AND METHODS

### *Patients*

*PTPN22* genotyping was performed on 663 patients with hematologic malignancies and their unrelated donors who underwent BMT through the JMDP with T cell–replete marrow from HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 allele-matched donors between January 1993 and December 2007. This cohort represents 7% (663 of 9229) of all recipients of unrelated BMT in Japan during the study period. All available data and samples for eligible patients and their donors were analyzed. None of the patients had a history of previous transplantation. The study cohort included Asian patients only. The final clinical survey of these patients was completed by November 1, 2008. Diagnoses included acute myelogenous leukemia (AML) in 215 patients (32%), acute lymphoblastic leukemia (ALL) in 164 patients (25%), chronic myelogenous leukemia (CML) in 118 patients (18%), myelodysplastic syndrome (MDS) in 89 patients (13%), malignant lymphoma (ML) in 73 patients (11%), and multiple myeloma in 4 patients (1%) (Tables 1 and 2). The median follow-up duration in the survivors was 2103 days (range, 124-5136 days); 183 recipients (28%) relapsed or progressed, and 322 (49%) died, 16 (2%) before engraftment. Recipients with AML or ALL in first complete remission, CML in any chronic phase, ML in any complete remission, or MDS were classified as having standard-risk disease. All others were classified as

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**Table 1**
Donor and Recipient Characteristics

| Variable | Value |
|---|---|
| Number of cases | 663 |
| Recipient age, years, median (range) | 34 (1-67) |
| Donor age, years, median (range) | 34 (20-57) |
| Year of BMT, median (range) | 2001 (1993-2007) |
| Recipient *PTPN22* genotype, n (%) | |
| G/G | 228 (34) |
| G/C | 331 (50) |
| C/C | 104 (16) |
| Donor *PTPN22* genotype, n (%) | |
| G/G | 219 (33) |
| G/C | 324 (49) |
| C/C | 120 (18) |
| Recipient sex, n (%) | |
| Male | 395 (60) |
| Female | 268 (40) |
| Donor sex, n (%) | |
| Male | 420 (63) |
| Female | 243 (37) |
| Donor/recipient sex match, n (%) | |
| Sex-matched | 426 (64) |
| Female/male | 106 (16) |
| Male/female | 131 (20) |

having high-risk disease. Myeloid malignancies included AML, CML, and MDS, and lymphoid malignancies included ALL, ML, and multiple myeloma. All patients received cyclosporine- or tacrolimus-based therapy for GVHD prophylaxis; none received anti–T cell therapy, such as antithymocyte globulin or ex vivo T cell depletion. All patients and donors provided written informed consent to participate in molecular studies of this nature at the time of transplantation, in accordance with the Declaration of Helsinki. This project was approved by the Institutional Review Board of Kanazawa University Graduate School of Medicine and the JMDP.

### *PTPN22 Genotyping*

Genotyping of *PTPN22* was performed using the TaqMan-Allelic discrimination method as described previously [7]. The genotyping assay was conducted in 96-well PCR plates using specific TaqMan probes for the *PTPN22* gene single nucleotide polymorphism rs2488457 (catalog C_16027865_10) in a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA).

**Table 2**
Pretransplant Characteristics

| Variable | Value |
|---|---|
| Disease, n (%) | |
| AML | 215 (32) |
| ALL | 164 (25) |
| MDS | 89 (13) |
| ML | 73 (11) |
| CML | 118 (18) |
| Multiple myeloma | 4 (1) |
| Disease stage, n (%) | |
| Standard risk | 406 (61) |
| High risk | 257 (39) |
| ABO matching, n (%) | |
| Major or/and minor mismatch | 255 (38) |
| Major mismatch | 145 (22) |
| Minor mismatch | 129 (19) |
| Bidirectional | 19 (3) |
| Missing | 9 (1) |
| Conditioning regimen, n (%) | |
| Myeloablative | 583 (88) |
| Reduced intensity | 80 (12) |
| With total body irradiation | 525 (79) |
| Pretransplantation CMV serostatus, n (%) | |
| CMV-positive recipient | 420 (72) |
| Missing | 80 (12) |
| GVHD prophylaxis, n (%) | |
| With cyclosporine | 376 (57) |
| With tacrolimus | 285 (43) |
| Missing | 2 (0) |
| TNC, $\times 10^8$/kg, median (range) | 5.0 (0.1-316.8) |

### *Data Management and Statistical Analysis*

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days and 1 year post-transplantation, and annually thereafter. Pretransplantation cytomegalovirus (CMV) serostatus was routinely tested in recipients only, not in donors. Engraftment was confirmed by an absolute neutrophil count of $>0.5 \times 10^9$/L for at least 3 consecutive days. Outcome classification, including GVHD, did not change over time.

After data collection, aGVHD and chronic GVHD (cGVHD) were diagnosed and graded based on classically defined criteria [8,9]; namely, aGVHD was defined as GVHD developing within the first 100 days post-transplantation, and cGVHD was defined as GVHD occurring after day 100. Data using the updated criteria for assessment of GVHD [10,11] were not available for our cohort. The overall survival (OS) rate was defined as the number of days from transplantation to death from any cause. Disease relapse was defined as the number of days from transplantation to disease relapse. Transplantation-related mortality (TRM) was defined as death without relapse. Any patients alive at the last follow-up date were censored. Data on infectious organisms, postmortem changes in causes of death, and supportive care, including prophylaxis for infections and therapy for GVHD given on an institutional basis, were not available for this cohort.

All statistical analyses were performed with the EZR software package (Saitama Medical Center, Jichi Medical University), a graphical user interface for R version 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria) [12], as described previously [13]. The probability of OS was calculated using the Kaplan-Meier method and compared using the log-rank test. The probabilities of TRM, disease relapse, aGVHD, cGVHD, and engraftment were compared using the Gray test [14] and analyzed using cumulative incidence analysis [15], considering relapse, death without disease relapse, death without aGVHD, death without cGVHD, and death without engraftment as respective competing risks. Variables included recipient age at the time of BMT, sex, pretransplantation CMV serostatus, disease characteristics (ie, disease type, disease lineage, and disease risk at transplantation), donor characteristics (ie, age, sex, sex compatibility, and ABO compatibility), transplant characteristics (ie, conventional or reduced-intensity conditioning [16], total body irradiation–containing regimens, tacrolimus versus cyclosporine, and total nucleated cell count harvested per recipient weight), and year of transplantation. The median was used as the cutoff point for continuous variables. The $\chi^2$ test and Mann-Whitney $U$ test were used to compare data between 2 groups. The Hardy-Weinberg equilibrium for the *PTPN22* gene variant was determined using the Haploview program [17].

Multivariate Cox models were used to evaluate the hazard ratio (HR) associated with the *PTPN22* variation. Covariates found to be significant in the univariate analyses ($P \leq .10$) were used to adjust the HR. For both the univariate and multivariate analyses, $P$ values were 2-sided, and $P \leq .05$ was considered to indicate statistical significance.

## RESULTS

### *Frequencies of PTPN22 Genotypes*

The rs2488457 single nucleotide polymorphism in the *PTPN22* gene was genotyped in 663 unrelated BMT donor–recipient pairs (Table 1). The genotype frequencies of G/G, G/C, and C/C were 34%, 50%, and 16% in recipients and 33%, 49%, and 18% in donors, respectively. These results are in accordance with the Hardy-Weinberg equilibrium ($P = .49$) and similar to HapMap data reported in the Japanese population [5]. Donor and recipient *PTPN22* genotype did not significantly influence the cumulative incidence of engraftment (data not shown).

### *Effects of Recipient PTPN22 Genotype on Transplantation Outcomes*

Transplantation outcomes according to *PTPN22* genotype are summarized in Table 3. Recipient C/C genotype was significantly associated with a lower incidence of grade II-IV aGVHD (18%) compared with recipient G/G (33%; $P = .009$) and G/C (35%; $P = .02$) genotypes (Figure 1A), suggesting the homozygous recessive effects of the C allele. We randomly split the study cohort into 2 subcohorts to test the validity of these associations. Subcohort 1 included 116 (35%) recipient C/C, 164 (49%) recipient G/C, and 52 (16%) recipient G/G genotypes, and subcohort 2 comprised 116 (35%) recipient G/G, 167 (50%) recipient G/C, and 52 (16%) recipient C/C

**Table 3**
Univariate Analysis of Associations between *PTPN22* Variations and Clinical Outcomes after BMT

| Variable | Number | 5-Year OS, % | P | 5-Year TRM, % | P | 5-Year Relapse, % | P | Grade II-IV aGVHD, % | P | Grade III-IV aGVHD, % | P | Chronic GVHD, % | P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Recipient *PTPN22* genotype | | | | | | | | | | | | | |
| G/G | 228 | 48 | | 25 | | 28 | | 33 | | 11 | | 43 | |
| G/C | 331 | 50 | .73 | 28 | .67 | 27 | .75 | 35 | .69 | 15 | .26 | 47 | .36 |
| C/C | 104 | 48 | .64 | 19 | .43 | 40 | .06 | **18** | **.009** | 6 | .18 | 42 | .79 |
| Donor *PTPN22* genotype | | | | | | | | | | | | | |
| G/G | 219 | 48 | | 22 | | 34 | | 32 | | 13 | | 42 | |
| G/C | 324 | 48 | .59 | 30 | .08 | **27** | **.04** | 31 | .73 | 11 | .57 | 45 | .42 |
| C/C | 120 | 53 | .38 | 21 | .62 | 29 | .35 | 33 | .85 | 14 | .79 | 49 | .24 |

Significant values ($P \leq .05$) are in bold.

genotypes, leading to an estimated statistical power of 57% to detect the difference between the recipient C/C genotype and recipient G/C or G/G genotype in both subcohort analyses. The association between recipient C/C genotype and a lower incidence of grade II-IV aGVHD remained positive in the analyses of subcohort 1 ($P = .04$) and subcohort 2 ($P = .03$) (Supplemental Figure 1).

In addition, the recipient C/C genotype was associated with a higher incidence of relapse (40%) compared with that seen in the recipient G/G (28%; $P = .06$) and G/C (27%; $P = .02$) genotypes (Figure 1B). This difference had no significant influence on OS or TRM, however.

In a comparison of the impact of the *PTPN22* genotype in recipients with standard-risk disease and those with high-risk disease to investigate the significant effect of recipient genotype on relapse rate, the effect of recipient genotype on the incidence of grade II-IV aGVHD appeared unchanged. In patients with high-risk disease, the incidence of grade II-IV aGVHD was 33% in those with the recipient G/G genotype, 38% in those with the G/C genotype, and 17% in those with the C/C genotype ($P = .10$). In patients with standard risk disease, these values were 33%, 34%, and 18% ($P = .09$), respectively. In patients with high-risk disease, the 5-year cumulative incidence of relapse associated with the recipient C/C genotype was as high as 50%, which was not significantly different from that in those with the recipient G/G (39%; $P = .28$) and G/C (35%; $P = .14$) genotypes; however, this likely contributed to a significantly lower 5-year OS rate associated with the recipient C/C genotype (20%) compared with the recipient G/C (37%; $P = .02$) and G/G genotypes (32%; $P = .05$) (Figure 2A). In patients with standard-risk disease, the 5-year cumulative incidence of relapse was 32% in those with the recipient C/C genotype, 22% in those with the G/G phenotype ($P = .23$), and 32% in those with the G/C genotype ($P = .17$), and there were no significant differences in OS rate (Figure 2B).

After adjusting for clinical factors in the multivariate model, recipient C/C genotype remained statistically significant compared with the recipient G/G genotype with respect to the development of grade II-IV aGVHD (HR, 0.50; 95% confidence interval [CI], 0.29-0.85; $P = .01$; Table 4) and relapse (HR, 1.78; 95% CI, 1.10-2.90; $P = .02$; Table 5). Although analysis of the entire cohort revealed no considerable effects of the *PTPN22* genotype on OS rates (Table 5), compared with recipient G/G genotype. recipient C/C genotype was associated with significantly lower OS in patients with high-risk disease (HR, 1.60; 95% CI, 1.02-2.51; $P = .04$; Table 6) and with a significantly higher incidence of relapse in patients with standard-risk disease (HR, 2.02; 95% CI, 1.02-4.00; $P = .04$). No effects of recipient C/C genotype on OS rates were seen in patients with standard-risk disease.

The increased risk of relapse associated with recipient C/C genotype could be outweighed by the decreased risk of grade II-IV aGVHD, given that the absence of grade II-IV aGVHD was closely linked to the higher incidence of relapse (31% versus 19% at 5 years; $P = .01$) in the landmark analysis completed at day 60, in agreement with a previous report [18]. Consequently, we analyzed the impact of recipient *PTPN22* genotype on relapse according to the development of grade II-IV aGVHD. The landmark time for aGVHD analysis was chosen as day 60 post-BMT, as in a previous study [18], because more than 90% of patients who develop grade II-IV aGVHD do so within 60 days after transplantation [19]. In patients who developed grade II-IV aGVHD before day 60, the cumulative incidence of relapse was higher in those with the recipient C/C genotype (47% at 5 years) compared with

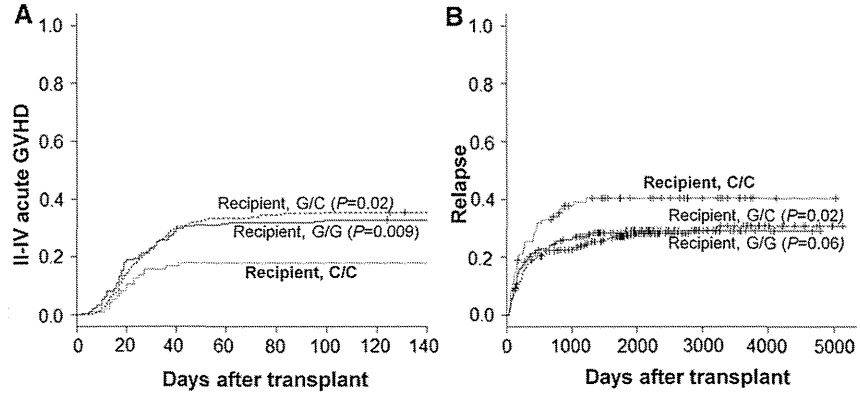


**Figure 1.** Estimated cumulative incidence curves of grade II-IV aGVHD (A) and relapse (B) according to recipient *PTPN22* genotype. Solid lines represent the recipient G/G genotype; dashed lines, the recipient G/C genotype; and dotted lines, the recipient C/C genotype.

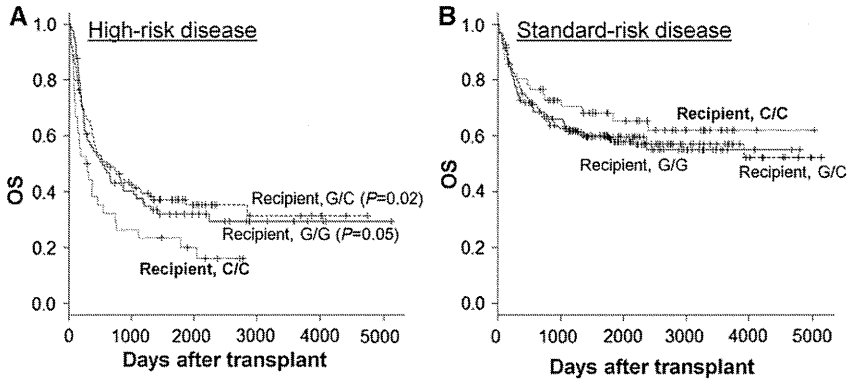


**Figure 2.** Kaplan-Meier analysis of OS after BMT according to the recipient *PTPN22* genotype in patients with high-risk disease (A) and those with standard-risk disease (B). Solid lines represent the recipient G/G genotype; dashed lines, the recipient G/C genotype; and dotted lines, the recipient C/C genotype.

those with the G/G (22%; $P = .04$) or G/C (20%; $P = .03$) genotype. The increased incidence of relapse associated with the recipient C/C genotype was confirmed on multivariate analysis, with an HR for relapse for the recipient C/C genotype versus G/G genotype as high as 4.5 (95% CI, 1.56-12.78; $P = .005$). In patients who survived more than 60 days without developing grade II-IV aGVHD, the 5-year cumulative incidence of relapse was higher in those with the recipient C/C genotype (39%) than in those with the recipient G/G (30%; $P = .22$), G/C (28%; $P = .24$), and G/G or G/C genotypes (30%; $P = .21$). After adjustment of covariates using the multivariate model, the increased incidence of relapse associated with the recipient C/C genotype was close to being significant compared with recipient G/G (HR, 1.79; 95% CI, 0.98-3.26; $P = .06$) and G/G or G/C (HR, 1.64; 95% CI, 0.99-2.71; $P = .06$) genotypes. Accordingly, the effects of recipient C/C genotype in increasing the incidence of relapse are considered independently significant irrespective of the development of grade II-IV aGVHD.

### *Effects of Donor PTPN22 Genotype on Transplantation Outcomes*

Compared with donor G/G genotype, donor G/C genotype was correlated with a significantly lower incidence of relapse (27% versus 34%; $P = .04$) and with a trend toward increased TRM (30% versus 22%; $P = .08$). The effects of the lower relapse rate associated with the donor G/C genotype were also evident in the multivariate analysis (HR, 0.58; 95% CI, 0.40-0.85; $P = .005$; Table 5). The effects of donor G/C genotype on relapse and TRM had no significant impact on OS; this also held true in the analysis performed according to disease risk (data not shown).

## DISCUSSION

In our study cohort, the recipient C/C genotype at the rs2488457 (−1123G>C) variant of the *PTPN22* promoter gene was associated with a lower incidence of grade II-IV aGVHD and a higher incidence of relapse after unrelated HLA-matched BMT performed through the JMDP. The recipient C/C genotype negatively affected OS in patients with high-risk disease, but not in those with standard-risk disease. In addition, the donor G/C genotype predicted a lower incidence of relapse, but had no significant impact on OS irrespective of disease risk.

Previous studies have identified 4 variations in the *PTPN22* gene associated with susceptibility to autoimmune diseases. The +1858C>T variation (rs2476601) is in near-perfect disequilibrium with rs6679677 [20] and is closely linked to the −1123G>C variation (rs2488457) analyzed in the present study [2,5,21-23]. The +1858C>T variation was first identified as associated with type 1 diabetes using a candidate gene approach [24]. Subsequent studies have confirmed this finding, as well as the variation's association with other autoimmune diseases, including Crohn's disease, ulcerative colitis, rheumatoid arthritis, Graves disease, autoimmune thyroid disease, vitiligo, alopecia, systemic lupus erythematosus, and acute allograft rejection [25]. The +1858C>T variation is not polymorphic in the Asian population [5]; instead, the −1123G>C variation is associated with type 1 diabetes and rheumatoid arthritis [2]. In addition, the −1123G>C variation is more closely associated with type 1 diabetes than the +1858C>T variation in the European population [5]. The remaining variation, +788G>A (Lyp-R263Q, rs33996649), is associated with ulcerative colitis, rheumatoid arthritis, and systemic lupus erythematosus [26].

**Table 4**
Multivariate Analysis of the Association between *PTPN22* Variations and GVHD after BMT

| Variable | Grade II-IV aGVHD | | | Grade III-IV aGVHD | | | cGVHD | | |
|---|---|---|---|---|---|---|---|---|---|
| | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* |
| Recipient *PTPN22* genotype, G/C (331) versus G/G (n = 228) | 1.64 | 0.79-1.44 | .68 | 1.32 | 0.80-2.18 | .28 | 1.08 | 0.81-1.44 | .59 |
| Recipient *PTPN22* genotype, C/C (104) versus G/G (n = 228) | **0.50** | **0.29-0.85** | **.01** | 0.54 | 0.22-1.34 | .18 | 0.89 | 0.58-1.34 | .59 |
| Donor *PTPN22* genotype, G/C (324) versus G/G (n = 219) | 0.95 | 0.70-1.30 | .76 | 0.81 | 0.48-1.36 | .42 | 1.13 | 0.84-1.53 | .42 |
| Donor *PTPN22* genotype, C/C (120) versus G/G (n = 219) | 1.08 | 0.72-1.61 | .72 | 1.10 | 0.59-2.07 | .76 | 1.33 | 0.93-1.90 | .11 |
| Recipient age ≥34 years | | | | | | | **1.31** | **1.00-1.72** | **.05** |
| Total body irradiation–containing conditioning regimen | | | | | | | **1.44** | **1.01-2.06** | **.05** |
| High-risk disease | | | | | | | **0.75** | **0.56-0.99** | **.05** |
| Year of BMT 2001 or later | | | | 0.69 | 0.42-1.11 | **.12** | | | |

Covariates identified as significant in the univariate analyses ($P \leq .10$) were used to adjust the HR for the *PTPN22* genotype. Significant results ($P \leq .05$) are in bold.

**Table 5**
Multivariate Analysis of the Association between *PTPN22* Variations and Prognostic Outcomes after Transplantation

| Variable | OS | | | TRM | | | Relapse | | |
|---|---|---|---|---|---|---|---|---|---|
| | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* |
| Recipient *PTPN22* genotype, G/C (331) versus G/G (n = 228) | 0.94 | 0.71-1.25 | .69 | 0.84 | 0.55-1.28 | .84 | 1.08 | 0.73-1.64 | .71 |
| Recipient *PTPN22* genotype, C/C (104) versus G/G (n = 228) | 1.03 | 0.68-1.56 | .87 | 0.67 | 0.33-1.35 | .27 | **1.78** | **1.10-2.90** | **.02** |
| Donor *PTPN22* genotype, G/C (324) versus G/G (n = 219) | 0.91 | 0.68-1.21 | .51 | 1.24 | 0.78-1.97 | .37 | **0.58** | **0.40-0.85** | **.005** |
| Donor *PTPN22* genotype, C/C (120) versus G/G (n = 219) | 0.78 | 0.53-1.15 | .21 | 1.08 | 0.60-1.97 | .79 | 0.64 | 0.40-1.04 | .07 |
| Minor ABO incompatibility | | | | **1.74** | **1.10-2.77** | **.002** | | | |
| Recipient age ≥34 years | **1.61** | **1.23-2.10** | **.001** | **2.21** | **1.45-3.37** | **<.001** | | | |
| CMV-positive recipient | | | | **2.15** | **1.13-4.08** | **.002** | 1.49 | 0.95-2.34 | .08 |
| *Conventional conditioning regimen* | | | | 1.33 | 0.64-2.78 | .45 | | | |
| Total body irradiation–containing conditioning regimen | | | | 0.95 | 0.60-1.52 | .84 | | | |
| High-risk disease | 2.08 | **1.60-2.69** | **<.001** | **1.75** | **1.14-2.70** | **.01** | **1.76** | **1.22-2.53** | **.003** |
| Female donor/male recipient | | | | | | | 0.67 | 0.40-1.11 | .12 |
| TNC ≥ 5.0 × $10^8$/kg | | | | 0.92 | 0.63-1.36 | .69 | | | |
| Year of BMT 2001 or later | 0.98 | 0.74-1.31 | .90 | | | | | | |

Covariates identified as significant in the univariate analyses ($P \leq .10$) were used to adjust the HR for the *PTPN22* genotype. Significant results ($P \leq .05$) are in bold.

Recent genome-wide association studies and meta-analyses have validated the association of these variations with type 1 diabetes, inflammatory bowel disease, Graves disease, rheumatoid arthritis, and systemic lupus erythematosus [27].

Experimental evidence has demonstrated that +1858C>T (Lyp-R620W, rs2476601) and +788G>A (Lyp-R263Q, rs33996649) are functional [28,29]. Lyp-Trp620 (+1858T) is associated mainly with an increased risk of autoimmune diseases and impaired constitutive biding of Lyp with c-Src tyrosine kinase (Csk) [30]. The inability of Lyp-Trp620 to bind Csk results in a less efficient inhibition of TCR signaling, because Lyp and Csk concertedly down-regulate TCR signaling [28]. Previous studies in cell lines and primary human cells have shown conflicting results, however [28]. Lyp-Trp620–positive primary human T cells were found to produce less IL-2 on TCR signaling, and Lyp-Trp620 more potently reduced TCR signaling in a dose-dependent manner, suggesting a gain-of-function mutation [30]. Conversely, the

**Table 6**
Impact of Recipient *PTPN22* Genotype on OS and TRM According to Disease Risk in the Multivariate Analysis

| Variable | OS | | | TRM | | | Relapse | | |
|---|---|---|---|---|---|---|---|---|---|
| | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* | Adjusted HR | 95% CI | *P* |
| Patients with high-risk disease | | | | | | | | | |
| Recipient *PTPN22* genotype, G/C (128) versus G/G (n = 89) | 0.95 | 0.57-1.35 | .78 | 0.89 | 0.47-1.69 | .73 | 1.05 | 0.60-1.84 | .87 |
| Recipient *PTPN22* genotype, C/C (40) versus G/G (n = 89) | **1.60** | **1.02-2.51** | **.04** | 0.92 | 0.36-2.34 | .85 | 1.51 | 0.75-3.05 | .25 |
| Donor *PTPN22* genotype, G/C (186) versus G/G (n = 142) | 0.90 | 0.63-1.28 | .55 | 1.29 | 0.60-2.80 | .51 | **0.53** | **0.31-0.91** | **.02** |
| Donor *PTPN22* genotype, C/C (69) versus G/G (n = 142) | 0.81 | 0.51-1.29 | .37 | 1.54 | 0.64-3.75 | .34 | 0.56 | 0.29-1.11 | .10 |
| Minor ABO incompatibility | | | | **2.32** | **1.14-4.73** | **.02** | | | |
| Recipient age ≥34 years | **1.76** | **1.28-2.43** | **.001** | **2.43** | **1.28-4.59** | **.006** | | | |
| CMV-positive recipient | | | | 1.33 | 0.58-3.06 | .50 | 1.26 | 0.66-2.41 | .49 |
| Conventional conditioning regimen | | | | 1.33 | 0.35-5.14 | .68 | | | |
| Total body irradiation–containing conditioning regimen | | | | 1.80 | 0.53-6.15 | .35 | | | |
| Female donor/male recipient | | | | | | | 0.85 | 0.41-1.78 | .67 |
| TNC ≥ 5.0 × $10^8$/kg | | | | 1.11 | 0.61-2.03 | .74 | | | |
| Year of BMT 2001 or later | 0.93 | 0.67-1.29 | .67 | | | | | | |
| Patients with standard-risk disease | | | | | | | | | |
| Recipient *PTPN22* genotype, G/C (199) versus G/G (n = 138) | 0.96 | 0.67-1.37 | .81 | 0.78 | 0.46-1.34 | .37 | 1.12 | 0.63-2.00 | .70 |
| Recipient *PTPN22* genotype, C/C (60) versus G/G (n = 138) | 0.84 | 0.49-1.43 | .52 | 0.51 | 0.18-1.41 | .19 | **2.02** | **1.02-4.00** | **.04** |
| Donor *PTPN22* genotype, G/C (186) versus G/G (n = 142) | 1.17 | 0.82-1.69 | .39 | 1.23 | 0.67-2.24 | .51 | 0.65 | 0.39-1.10 | .11 |
| Donor *PTPN22* genotype, C/C (69) versus G/G (n = 142) | 0.83 | 0.50-1.38 | .48 | 0.81 | 0.35-1.86 | .62 | 0.74 | 0.38-1.45 | .39 |
| Minor ABO incompatibility | | | | 1.39 | 0.72-2.71 | .33 | | | |
| Recipient age ≥34 years | **1.68** | **1.20-2.36** | **.003** | **2.04** | **1.16-3.59** | **.01** | | | |
| CMV-positive recipient | | | | **3.45** | **1.19-9.96** | **.02** | 1.74 | 0.90-3.39 | .10 |
| Conventional conditioning regimen | | | | 1.10 | 0.46-2.64 | .83 | | | |
| Total body irradiation–containing conditioning regimen | | | | 0.79 | 0.45-1.36 | .39 | | | |
| Female donor/male recipient | | | | | | | 0.51 | 0.25-1.07 | .08 |
| TNC ≥ 5.0 × $10^8$/kg | | | | 0.84 | 0.50-1.39 | .50 | | | |
| Year of BMT 2001 or later | 1.24 | 0.88-1.74 | .23 | | | | | | |

Covariates identified as significant in the univariate analyses ($P \leq .10$) were used to adjust the HR for the *PTPN22* genotype. Significant values ($P \leq .05$) are in bold.

Lyp-Gln263 mutation, which is associated with a reduced risk of autoimmune diseases, reportedly results in loss of function [29].

The mechanisms through which the recipient −1123C allele of the *PTPN22* gene affects the incidence of aGVHD and disease relapse remain unclear. Previous reports of the number of regulatory T cells (Tregs) increasing inversely with the level of *PTPN22* in the thymus [31] and of thymus-derived Tregs operating to prevent aGVHD and promote disease relapse [32] suggest the hypothesis that in transplant recipients, the *PTPN22* −1123G>C variant influences the production of Tregs from the thymus. This hypothesis may be supported by the fact that the *PTPN22* gene has a functional variant, +1858C>T, that is closely linked to the −1123G>C variant [2,5,21-23], and that the minor +1858T allele functionally inhibits TCR signaling more potently than the major +1858C allele [30]. Hyporesponsive TCR signaling might lead to increased Treg production by the thymus, given that decreased TCR signaling can promote the development of intrathymic Tregs [33]. Thus, an increased number of Tregs in relation to the recipient −1123C/C genotype might prevent aGVHD at the expense of decreased graft-versus-tumor effects. These hypotheses must be considered speculative, however, given the lack of functional data on the −1123G>C variant. Elucidating the role of the *PTPN22* −1123G>C variant in Treg production will provide useful information in this regard.

A second possible mechanism includes the involvement of host DCs, which are critical for the initiation of aGVHD [34]. This possibility may be supported by a recent report indicating that the *PTPN22*+1858C>T variant plays key roles in antigen receptor signaling of DCs [28].

Why the *PTPN22* −1123G>C genotype displays different behaviors in the donor and recipient genotypes is obscure. Of note, the donor heterozygous −1123G/C genotype was associated with a reduced incidence of relapse, which could be attributed to increased graft-versus-tumor effects owing to donor G/C genotype. The effects of the heterozygous −1123G/C genotype on autoimmunity may be related to the association between this genotype and increased risk of developing autoimmune diseases, including type 1 diabetes and rheumatoid arthritis, in Asian populations [2,3,5]. However the present study showed no gene dose responses, and whether this phenomenon reflects a molecular heterosis is unclear [3,5,35].

The lack of considerable survival advantage in relation to donor *PTPN22* genotype may suggest that the beneficial effects of *PTPN22* genotyping are limited. However, determination of the recipient *PTPN22* genotype before transplantation might provide a recipient harboring the *PTPN22* G/C or G/G genotype an opportunity to avoid the risk of aGVHD by favoring a bone marrow or cord blood HLA-matched graft over a peripheral blood stem cell (PBSC) or HLA-mismatched graft. Conversely, a PBSC or HLA-mismatched graft, along with minimal aGVHD prophylaxis, could be acceptable for a recipient harboring the *PTPN22* C/C genotype. In addition, a recipient with the −1123G/G or G/C genotype may require a bone marrow or cord blood graft to avoid aGVHD. This may apply especially to recipients with a benign disease, such as severe aplastic anemia or primary immunodeficiency, in whom relapse does not matter.

A previous study investigated the impact of the *PTPN22* +1858C>T variant on transplantation outcomes in a cohort of European patients who underwent hematopoietic stem cell transplantation for hematologic malignancies [36]. Although a relatively small number of patients were included in that analysis, the authors found that the donor +1858C/C genotype was consistently linked with severe bacterial infections [36]. Another study [37] showed that recipient–donor pairs carrying 2 or more PTPN22 −1858T alleles were at increased risk for grade III-IV aGVHD, but not for grade II-IV aGVHD. Although determining whether such associations are also present in Japanese patients is not possible, because the +1858C>T variant is not polymorphic in Asian populations [2,3,5], these results might support involvement of the *PTPN22* gene in the pathophysiology of aGVHD, as suggested in the present study.

In conclusion, our data suggest that the specific *PTPN22* variant affects prognosis after unrelated donor BMT. Thus, *PTPN22* genotyping in transplant donors and recipients can be a useful tool for evaluating pretransplantation risk and, in combination with other known risk factors, can form the basis for tailoring individual treatment strategies. Nonetheless, care should be taken when drawing conclusions from our data; experimental evidence is needed to verify the effects of *PTPN22* variations. Moreover, the present study did not include adjustment for multiple testing, because the analyses were conducted in an exploratory context, and thus the interpretation of analyses in the subgroups should be taken into account. Finally, transplantation outcomes, including aGVHD and relapse are multifactorial, and single polymorphisms in one cytokine gene are unlikely to determine the majority of outcomes. Further studies are needed to ascertain whether the findings of this study can be extended to other stem cell sources or to HLA-mismatched transplantation, and to validate these data in other ethnic groups.

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*Authorship statement:* J. Luis Espinoza and Akiyoshi Takami designed research and wrote the manuscript. J. Luis Espinoza performed experiments. Akiyoshi Takami analyzed data and performed statistical analysis. All authors contributed to data and sample collection and reviewed the manuscript.

## SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2012.09.014.

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