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Research Article

Cancer Immunology Research

Allelic Polymorphisms of *KIR*s and *HLA*s Predict Favorable Responses to Tyrosine Kinase Inhibitors in CML

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Abstract

Response to tyrosine kinase inhibitors (TKIs) is variable in chronic myeloid leukemia (CML), and elevated natural killer (NK) cells during TKI therapy are positively correlated with superior outcomes. NK cell function involves interactions of their killer immunoglobulin-like receptors (KIRs) and human leukocyte antigen (HLA) class I on target cells, and the avidity of KIR–HLA interactions depends on the combination of *KIR* and *HLA* alleles. We hypothesized that *KIR* and *HLA* polymorphisms may influence response to TKIs. *KIR* and *HLA* allele genotyping was performed by next-generation sequencing for 76 CML cases, and association with clinical outcome was analyzed. Second-generation TKIs as first-line therapy and patients' sex (female) were strongly associated with achievement of complete molecular response (CMR: $MR^{4.0}$) after 2 years ($P < 0.001$ and $P = 0.002$, respectively). After adjustment for these two characteristics, several *KIR* alleles remained associated with achievement of $MR^{4.0}$: *KIR2DL4*005/011* or **008* (HR = 1.797, $P = 0.032$); *KIR2DS4*003* or **007/010* (HR = 3.348, $P < 0.001$); *KIR3DL1*005* (HR = 2.746, $P = 0.003$); and *KIR3DL2*009* or **010* [HR = 1.980 (1.109–3.524), $P = 0.021$]. Strong linkage among these alleles exists, implying that they comprise favorable KIR allele haplotypes. Allelic polymorphisms of *KIR3DL1* and HLA-B determine their differential avidity into strong/weak or no interaction. Patients carrying noninteracting *KIR3DL1* and *HLA-B* allele pairs achieved better outcomes than those with strongly interacting pairs, and *KIR3DL1*005* associated with a positive outcome among patients with weak-interacting pairs. Thus, *KIR3DL1*005* and its associated haplotypes associated with superior TKI therapeutic effects. The combinations of these *KIR* and *HLA* alleles may correlate with potent NK cell immunity against CML. *Cancer Immunol Res; 6(6); 745–54. ©2018 AACR.*

Introduction

Tyrosine kinase inhibitors (TKIs) can suppress chronic myeloid leukemia (CML) cells that harbor *BCR–ABL1* mutations (Philadelphia chromosome) in an ATP-competitive manner, and have dramatically improved long-term outcomes of CML patients (1). However, it remains unclear whether TKIs enable treatment-free remission (TFR) in CML. Because TKIs do not kill leukemia stem cells (2), TKI treatment was considered to be required for all through life, even after achievement of deep molecular remission (3- to 4-log reduction in *BCR–ABL1* transcripts; ref. 3). However, long-term TKI therapy can cause late-onset adverse events, including renal insufficiency, pleural effusion, cardiovascular events, and can also result in high costs (4, 5). Hence, several clinical trials have investigated the feasibility of discontinuing TKIs (6–9). Overall, these trials have demonstrated that approximately a half of patients who achieve deep molecular remission experience TFR.

Given that TKIs do not directly kill leukemia stem cells (2) but achieve TFR, TKIs may trigger cytotoxicity against CML cells indirectly. Several lines of evidence demonstrate that TKIs enhance antitumor immunity by suppressing regulatory T cells (10, 11). Treatment with interferons, allogeneic hematopoietic stem cell transplantation (allo-HSCT), and donor lymphocyte infusion can suppress CML and have definite positive clinical impacts (12). Antitumor immunity can be carried out by T cells, B cells, and natural killer (NK) cells and, interestingly, increased numbers of NK cells are positively associated with TFR in several TKI discontinuation trials (7, 13, 14). Collectively, TKIs may not only directly suppress the activity of BCR–ABL1 but also enhance antitumor NK cell immunity against CML. However, responses to TKIs vary among patients, and not all cases achieve TFR. The factors that determine the effects of TKIs in individual patients have yet to be fully elucidated.

NK cells are a facet of innate immunity and can damage tumor cells and pathogens via secretion of cytotoxic granules or initiating antibody-dependent cellular cytotoxicity (15). The balance of activating and inhibitory signals received through NK cell receptors determines their cytotoxic activity (16). In particular, killer immunoglobulin-like receptors (KIRs) play pivotal roles in the

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recognition of human leukocyte antigen (HLA) class I molecules on target cells (17). KIRs are classified into activating and inhibitory KIRs depending on their transducing intracellular signals (18). Humans typically carry 9 to 15 activating or inhibitory *KIR* genes, combinations of which are classified into haplotypes A and B. Currently, haplotype B is defined by the presence of one or more of the following genes: *KIR2DS1/2/3/5*, *KIR3DS1*, and *KIR2DL2/5*. Haplotype A is defined by the absence of all these genes (19). Interaction between inhibitory KIRs and their cognate HLA is also crucial in the mediation of NK cell responsiveness during differentiation, referred to as "licensing" or "education" (20). KIRs also exhibit extensive allelic polymorphisms, which can confer functional diversity (21).

Polymorphic *KIR* genotypes have clinical impacts in viral infections, autoimmune disorders, and malignancies (17, 22). Regarding hematologic malignancies, antibodies targeting inhibitory KIRs enhance antilymphoma activity (23). Several reports have investigated the clinical impacts of specific KIRs. Although *KIR2DS1* positivity is associated with a better outcome in allo-HSCT recipients (24, 25), TKI-treated CML patients lacking *KIR2DS1* achieve superior outcomes (26, 27). Assuming that each KIR has cognate immunologic functions, it may be expected that investigations of different diseases would reach similar conclusions. However, studies investigating the associations between disease outcome and KIR molecules have reached inconsistent conclusions regarding the immunomodulatory impacts of KIRs, possibly because of insufficient knowledge of KIR functions.

Next-generation sequencing can fully define *KIR* allelic polymorphisms, which have been associated with infections, autoimmune disorders, and several cancers (28). In particular, *KIR3DL1* exhibits abundant allelic polymorphisms and is associated with three differential expression levels: high (*KIR3DL1*-h), low (*KIR3DL1*-l), and null (*KIR3DL1*-n; refs. 29, 30). Among HLA-Bw4 epitopes, an amino acid dimorphism at position 80 (isoleucine (80I) and threonine (80T)) defines differential inhibition of KIR3DL1 activity (31). Therefore, the combination of *KIR3DL1* alleles and HLA-B epitopes may determine the avidity of each specific interaction and, consequently, the magnitude of NK cell immune responses (21, 32–34). For example, KIR3DL1-h alleles appear to be suppressive (33), and alleles encoding HLA-Bw4-80I epitopes result in stronger ligands for KIR3DL1 than HLA-Bw4-80T epitope–encoding alleles (31, 35). Although allelic combinations of KIR and HLA with high avidity correlate with protection against HIV infection (36), such pairs are also associated with worse outcome in neuroblastoma patients treated with monoclonal anti-GD2 (37) and higher leukemia relapse rates after allo-HSCT (38). Strong interactions between inhibitory KIRs and their ligands, the allelic combinations of KIR and HLA with high avidity, may be associated with vigorous inhibition of NK cell immunity, whereas weak or no interactions are related to elevated NK cell function (37, 38). In the meantime, strong interactions may also be associated with high levels of licensing of NK cell immunity.

We hypothesized that polymorphisms of *KIR* and *HLA* genes correlated with the therapeutic effects of TKIs in CML. In particular, we focused on the avidity of interactions between KIR3DL1 and HLA-B epitopes that may define the magnitude of NK cell cytotoxicity. We performed allelic genotyping of activating and inhibitory *KIR*s and *HLA*s in 76 CML patients by next-generation sequencing. Activating *KIRs* (*KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, and *KIR3DS1*) and inhibitory *KIRs* (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR3DL1*, *KIR3DL2*, and *KIR2DL5*) were genotyped. In particular, three activating *KIRs (KIR2DS1*, *KIR2DS4*, and *KIR3DS1)* and six inhibitory *KIRs (KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL4*, *KIR3DL1*, and *KIR3DL2)* with known ligands were genotyped. We then analyzed associations of genotypes, as well as their combinations, with patient clinical outcomes.

Methods and Materials

Patients

Patients with Philadelphia chromosome–positive CML in chronic phase (CML-CP; $n = 76$) treated at Saga University Hospital between April 2002 and September 2016 with TKIs (imatinib, dasatinib, nilotinib, or bosutinib) as first-line therapy were investigated for distributions of *KIR* gene frequencies, *KIR* and *HLA* genotypes (including allele typing), and KIR–HLA combinations. Diagnosis of CML was according to the World Health Organization classification of myeloid neoplasms and acute leukemia (39). Baseline patient characteristics were obtained from the hospital records and included general characteristics (age, sex), hematologic data (complete blood count, blast count, percentage of eosinophils, and basophils), molecular diagnosis (*BCR–ABL* transcript level), and spleen size. All the patients were treated with only TKIs, and none received allo-HSCT. Written informed consent was obtained from all the patients before registration, in accordance with the Declaration of Helsinki. The study was approved by the institutional review board of Saga University, enabling gene-panel sequencing and data sharing with qualified investigators. The study was registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID: R000020356).

Real-time quantitative PCR

The therapeutic effects of TKIs were evaluated based on peripheral blood *BCR–ABL* mRNA expression. Patients' RNA was purified from their whole blood by QIAamp RNA Blood Mini Kit (QIAGEN). *BCR–ABL* mRNA expression was measured by real-time quantitative (RQ)-PCR adjusted to the international reporting scale (IS) by ODK-1201 kit (Otsuka Pharmaceutical Co.) or by the transcription-mediated amplification (TMA) method using DNA probe FR Amp-CML kit (Fujirebio Inc.). Major molecular response (MMR) was defined as a 3-log reduction ($MR^{3.0}$) of RQ-PCR (IS) or *BCR–ABL* transcript expression of <50 copies/0.5 μg RNA using the TMA method. We also defined complete molecular response (CMR) as a 4-log reduction ($MR^{4.0}$) of RQ-PCR (IS), which was equivalent to undetectable *BCR–ABL* transcript expression using the TMA method.

Genotyping of KIR and HLA

Patients' DNA was purified from their whole blood with QIAamp DNA Blood Midi kit (QIAGEN) and stored at −20°C. Allelic genotyping of *HLA* and *KIR* genes was performed using DNA samples by next-generation sequencing (NGS) using Illumina MiSeq technology. For genotyping, commercially available typing kits, HLA version 3.0 IGS kit (Scisco Genetics Inc.) for HLA genotyping and KIR IGS kit version 1.0 (Scisco Genetics Inc.) for KIR genotyping were used according to the manufacturer's instructions (40). Briefly, DNA samples were subjected to amplicon PCR and pooling, enzymatic cleanup, barcoding, barcode

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pooling, magnetic bead–based size selection, and finally quantification at MiSeq platform. Regions targeted for multiplex short-range PCR were exons 1 to 7 of *HLA-A, B*, and *C*; exons 1 to 4 of *HLA-DRB1*, *-DRB3*, *-DRB4*, *-DRB5*, *-DQA1*, *-DQB1*, *-DPA1*, and *-DPB1*; and exons 3, 4, 5, and 9 of *KIR* genes. Gene copy numbers of one or two were categorized as "positive," while samples with a copy number of zero were classified as "blank." *KIR2DL4–3DL1/3DS1–2DS4–3DL2* haplotypes were estimated at the allele level, and their frequencies were determined using Haplo.stats software (version 1.7.7) operated in R.

Antibodies and flow cytometry

Patients' peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using lymphocyte separation medium (MP Biomedical) and were subjected to flow cytometric analyses. Cells (1×10^6) suspended in phosphate-buffered saline with 5% fetal bovine serum (FBS; Sigma-Aldrich), 2 mmol/L EDTA, and 0.05% NaN_3 were stained with the following monoclonal antibodies for 20 minutes at room temperature in dark: anti-CD3–FITC (OKT3; TONBO Biosciences), anti-CD56–PE-Cy7 (HCD56; Biolegend), anti-CD107a–Pacific Blue (H4A3; BioLegend), anti-CD16–APC-Cy7 (3G8; BD Biosciences), and anti-CD158e1–APC (DX9; BD Biosciences). For staining IFNγ, surface antigen-stained cells were fixed for 30 minutes and then permeabilized and also intracellularly stained for anti-IFNγ–V450 (B27; BD Biosciences) for 30 minutes with respective buffers (Thermo Fisher Scientific). Stained samples were assessed using a FACSVerse cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Assessment of NK cell activity

The activation status of NK cells was evaluated by staining for CD107a degranulation and intracellular IFNγ secretion of $CD3^-CD16^+CD56^+$ NK cells using flow cytometry. PBMCs (5×10^5) from patients were cocultured with K562 cells, which lack HLA class I expression. The effector–target ratio was adjusted to 10 to 1 in RPMI 1640 medium supplemented with 10% FBS (41). Anti-CD107a–Pacific Blue was added, followed by a 6-hour incubation, during which brefeldin A (GolgiPlug; BioLegend) and monensin (GolgiStop; BioLegend) were added after 1 hour. Prior to staining for intracellular IFNγ, the cells were fixed and permeabilized with the described buffers (Thermo Fisher Scientific) after staining surface antigens.

The K562 cell was purchased from RIKEN, and short tandem repeat DNA profiling analyses using PCR authenticated that it was the same as those registered in the ATCC (CCL-243, K-562), the Japanese Collection of Research Biosources (JCRB) Cell Bank (JCRB0019, K-562; JCRB1002, K562/ADM; and JCRB1402, K562-Luc), the RIKEN BRC Cell Bank (RCB0027, K562; RCB1197, P2UR/K-562; RCB0474, K562/MTX-2; RCB1898, K562/Adr; and RCB2111, K562/Vin), and in Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; ACC-10, K-562), by comparison with the JCRB database. K562 was used within 2 months after thawing or 25 passages, and *mycoplasma* infection was regularly checked.

Statistical analyses

Cumulative incidence probabilities were calculated using the Kaplan–Meier method. All curve comparisons were completed using a log-rank test for time to achievement of $MR^{4.0}$. Crude and covariate adjusted hazard ratios (HRs) and their 95% confidence intervals (95% CIs) were computed using the Cox proportional hazards model. In these estimates, sex and first-line treatment were selected as clinical covariates. Two-tailed P values < 0.05 were considered statistically significant. All the statistical analyses were performed using R Version 3.3.1 downloaded from http://cran.ism.ac.jp.

Results

Characteristics and *KIR* alleles of CML patients

A total of 76 patients with CML-CP who received TKI treatment were investigated in this study. The median follow-up time was 4.9 years (range, 0.7–14.6 years), and the median age of the total cohort was 62.5 years (range, 21–88 years). Forty-seven patients were male and 29 were female, and 39, 24, 10, and 3 patients had low, intermediate, high, and unknown Sokal scores, respectively. All patients received the first-generation TKI imatinib ($n = 30$) or the second-generation TKIs dasatinib or nilotinib ($n = 46$) as first-line treatment. After two years of treatment, $MR^{4.0}$ was achieved by 48% of patients (Fig. 1A). Second-line treatment with second-generation TKIs was required for 36 patients, due to adverse events

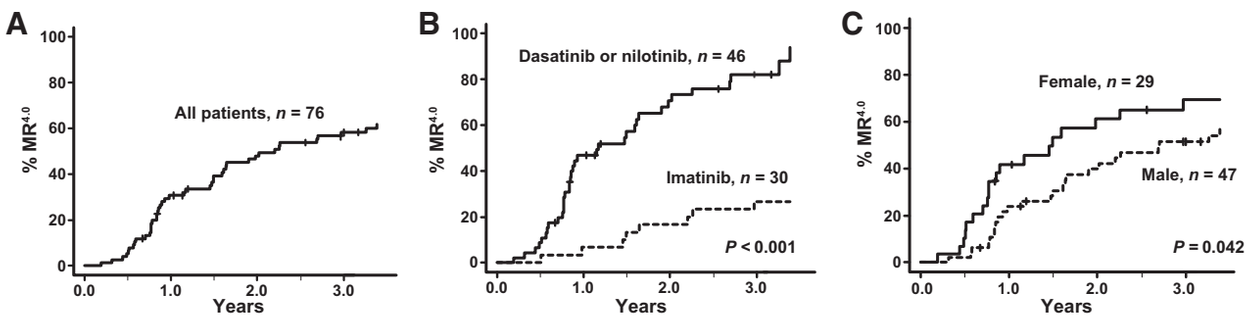

Figure 1.

Cumulative incidences of $MR^{4.0}$ at 2 years according to first-line therapy and sex. **A,** Cumulative incidence of $MR^{4.0}$ (%$MR^{4.0}$) achievement is shown for all patients. Forty-eight percent of patients achieved $MR^{4.0}$ at 2 years. **B** and **C,** Cumulative incidence of $MR^{4.0}$ achievement according to first-line treatment (**B**) and sex (**C**) are presented. **B,** Solid line, patients treated with second-generation TKIs as first-line therapy; dashed line, patients treated with imatinib ($P < 0.001$). **C,** Solid line, female patients; dashed line, male patients ($P < 0.039$). Number of patient indicted in **A–C**. All curve comparisons were completed using log-rank tests for time to achievement of $MR^{4.0}$, and $P < 0.05$ were considered statistically significant.

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Table 1. Patient characteristics

Sex (%)	
Male	47 (61.8)
Female	29 (38.2)
Age, mean years (range)	62.5 (21–88)
Age ≥65 years, number (%)	22 (28.9)
Age at diagnosis, mean years (range)	55.5 (17–83)
Complete blood count at diagnosis, mean value (range)	
White cell count $\times 10^9$/L	24 (8.2–290)
Hemoglobin g/L	13.6 (5.6–16.8)
Platelet count $\times 10^9$/L	495 (106–2032)
Sokal risk group, number (%)	
Low	39 (51.3)
Intermediate	24 (31.6)
High	10 (13.2)
Unknown	3 (3.9)
First-line TKI, number (%)	
Imatinib	30 (39.5)
Dasatinib	35 (46.1)
Nilotinib	11 (14.5)
Second-line TKI, number (%)	
Dasatinib	24 (31.6)
Nilotinib	9 (11.8)
Bosutinib	3 (3.9)
Third-line TKI, number (%)	
Dasatinib	5 (6.6)
Nilotinib	2 (2.6)
Median follow-up, years (range)	4.9 (0.7–14.6)

(AEs) or suboptimal responses. Seven patients needed third-line TKI treatment. The clinical characteristics of patients are summarized in Table 1.

NGS of DNA extracted from patient peripheral blood samples was performed to determine their *KIR* alleles (Table 2). *KIR2DL4*, *KIR2DS4*, *KIR3DL1/3DS1*, and *KIR3DL2* exhibited diverse polymorphisms, consistent with a previous report of *KIR* alleles in the Japanese population (33).

First-line treatment and female sex are associated with achievement of $MR^{4.0}$

First-line treatment with second-generation TKIs and the patients' sex (female) were associated with achievement of $MR^{4.0}$ at 2 years (second-generation TKIs: HR = 7.305; 95% CI = 3.377–15.803, $P < 0.001$; female sex: HR = 1.709; 95% CI = 1.028–2.842, $P = 0.039$; Table 3, Fig. 1B and C). Because only these two characteristics were statistically significant in multivariate analysis adjustment for covariates, we analyzed other potentially prognostic factors after adjustment for these variables. Sokal scores segregated patients into low ($n = 39$), intermediate ($n = 24$), and high ($n = 10$) groups. Scores were not associated with achievement of $MR^{4.0}$ in univariate analyses. However, adjustment for sex (female) and first-line treatment revealed that high Sokal scores were associated with achievement of $MR^{4.0}$, compared with low or intermediate scores (low vs. high: HR = 0.462; 95% CI = 0.218–0.982, $P = 0.045$; intermediate vs. high: HR = 0.384; 95% CI = 0.168–0.881, $P = 0.024$; low vs. intermediate: HR = 1.033; 95% CI = 0.582–1.833, $P = 0.913$; Table 3).

No single *KIR* gene was associated with achievement of $MR^{4.0}$

We next analyzed associations between each *KIR* gene and achievement of $MR^{4.0}$. Because almost all patients carried at least one of *KIR2DL1*, *KIR2DL3*, *KIR2DS4*, or *KIR3DL1* genes, patients were segregated into two subgroups: (i) two allelic types of these genes and (ii) zero or one allele. Due to almost all patients having two alleles for *KIR2DL4*, *KIR3DL2*, and *KIR3DL3* genes, these *KIR*s were excluded from further analyses. Consequently, we analyzed associations with alleles for *KIR2DL1*, *KIR2DL2*, *KIR2DL3*, *KIR2DL5*, *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS4*, *KIR2DS5*, and *KIR3DL1*. None of these *KIR*s were associated with achievement of $MR^{4.0}$ (Table 3).

Table 2. Genotyped KIR alleles in CML patients; duplicated allele were not observed

	Number	Frequency
KIR2DL2/3		
3*001	136	89.5%
2*003	11	7.2%
3*023	2	1.3%
2*001	1	0.7%
3*009	1	0.7%
3*019	1	0.7%
KIR2DL1		
*003	128	84.2%
*002	12	7.9%
*004	1	0.7%
Blank	11	7.2%
KIR2DL4		
*001	76	50.0%
*005/011	42	27.6%
*006	24	15.8%
*008	9	5.9%
Blank	1	0.7%
KIR3DL1/S1		
*015	69	45.4%
*007	24	15.8%
3DS1*013	23	15.1%
*005	15	9.9%
*001	9	5.9%
*020	5	3.3%
3DS1*085	3	2.0%
*029	1	0.7%
*038	1	0.7%
3DS1*078	1	0.7%
Blank	1	0.7%
KIR2DS1		
*002	26	17.1%
*002/003	2	1.3%
Blank	124	81.6%
KIR2DS4		
*001	69	45.4%
*004	15	9.9%
*004-variant	9	5.9%
*003	14	9.2%
*007/010	9	5.9%
*014/016	6	3.9%
*015	2	1.3%
Blank	28	18.4%
KIR3DL2		
*002	70	46.1%
*007	23	15.1%
*008	20	13.2%
*010	18	11.8%
*009	9	5.9%
*001/*049	3	2.0%
*015	3	2.0%
*016	2	1.3%
*021	2	1.3%
*024	1	0.7%
*027	1	0.7%

NOTE: As KIR2DL2/DL3 and KIR3DL1/DS1 alleles are shared within the same gene, they are shown in the same category KIR2DL2/3 and KIR3DL1/S1, respectively. KIR2DS4*004 variant is the allele that changed aspartic acid (D) to valine (V) at codon 57 in *004.

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Table 3. Statistical analyses of achievement of $MR^{4.0}$

	Univariate analysis			First-line therapy and sex adjusted	
	***n* (%)**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
KIR2DL1 (2 vs. 1)	65 (85.5)	0.859 (0.405–1.821)	0.692	1.268 (0.585–2.748)	0.548
KIR2DL2 (1 vs. 0)	12 (15.8)	1.243 (0.606–2.546)	0.553	1.115 (0.534–2.330)	0.772
KIR2DL3 (2 vs. 1)	64 (84.2)	0.805 (0.393–1.648)	0.553	1.115 (0.534–2.330)	0.772
KIR2DL5 (1, 2 vs. 0)	29 (37.8)	1.026 (0.983–1.071)	0.236	1.02 (0.976–1.066)	0.38
KIR2DS1 (1, 2 vs. 0)	27 (35.5)	1.023 (0.980–1.068)	0.295	1.015 (0.971–1.061)	0.517
KIR2DS2 (1 vs. 0)	12 (15.8)	1.243 (0.607–2.546)	0.553	0.897 (0.429–1.874)	0.772
KIR2DS3 (1, 2 vs. 0)	8 (10.5)	1.003 (0.939–1.071)	0.93	1.024 (0.958–1.095)	0.478
KIR2DS4 (2 vs. 0, 1)	49 (64.5)	0.966 (0.906–1.031)	0.295	0.978 (0.916–1.045)	0.517
KIR2DS5 (1 vs. 0)	20 (26.3)	1.430 (0.823–2.483)	0.205	1.151 (0.652–2.034)	0.627
KIR3DL1 (2 vs. 0, 1)	49 (64.5)	0.966 (0.906–1.031)	0.295	0.978 (0.916–1.045)	0.517
KIR2DL1 positive/*HLA-C2* positive	10 (13.2)	2.521 (1.209–5.258)	0.014	1.915 (0.890–4.122)	0.097
KIR2DL2 positive/*HLA-C1* positive	12 (15.8)	1.243 (0.607–2.546)	0.553	1.115 (0.534–2.330)	0.772
KIR3DL1 positive/*HLA-Bw* positive	64 (84.2)	0.539 (0.276–1.055)	0.071	0.725 (0.368–1.430)	0.353
*KIR2DL4*005/011* or **008*	40 (52.6)	1.942 (1.160–3.250)	0.012	1.797 (1.052–3.067)	0.032
*KIR2DS4*003* or **007/010*	20 (26.3)	3.878 (2.125–7.075)	<0.001	3.348 (1.773–6.323)	<0.001
*KIR3DL1*005*	14 (18.4)	3.634 (1.884–7.013)	<0.001	2.746 (1.403–5.376)	0.003
*KIR3DL2*009 or *010*	22 (28.9)	1.864 (1.067–3.258)	0.029	1.980 (1.109–3.524)	0.021
Activating KIR positive	35 (46.1)	1.383 (0.830–2.304)	0.214	1.094 (0.636–1.881)	0.745
KIR haplotype A/A	41 (53.9)	0.723 (0.434–1.205)	0.214	0.914 (0.532–1.572)	0.745
Female sex	29 (37.8)	1.709 (1.028–2.842)	0.039	N/A	
Age 65 years and over	22 (28.9)	1.262 (0.718–2.219)	0.419	1.180 (0.651–2.138)	0.586
Sokal score low vs. high	Low: 39 (51.3%)	0.635 (0.306–1.316)	0.222	0.462 (0.218–0.982)	0.045
Sokal score intermediate vs. high	Int: 24 (31.6%)	0.594 (0.271–1.302)	0.193	0.384 (0.168–0.881)	0.024
Sokal score low vs. intermediate	High: 10 (13.2%)	1.001 (0.566–1.770)	0.997	1.033 (0.582–1.833)	0.913
First-line dasatinib or nilotinib	46 (60.5)	7.305 (3.377–15.803)	<0.001	N/A	

KIR haplotypes, activating KIRs, their matching ligands and $MR^{4.0}$

KIR haplotype A consists of several inhibitory KIRs and one activating KIR (*KIR2DS4*), and 41 patients (53.9%) were homozygous for this haplotype. All but one patient carried the *KIR2DS4* gene, and 35 patients (46.1%) had activating KIRs other than *KIR2DS4* (Table 3). Homozygosity for KIR haplotype A and the presence of activating *KIR* genes other than *KIR2DS4* were not associated with achievement of $MR^{4.0}$ (Fig. 2A and Table 3; haplotype A: HR = 0.723, 95% CI = 0.434−1.205, $P = 0.214$; presence of activating *KIR* genes other than KIR2DS4: HR = 1.383, 95% CI = 0.830–2.304, $P = 0.214$).

We also investigated the impacts of functional activating *KIR* and *HLA* genotype combinations. We focused on the pairs of *KIR2DS1/S3* and *HLA-C2*, and *KIR2DS2* and *HLA-C1*, which have been well-investigated activating *KIR/HLA* pairs. No association between the presence of these combinations with achievement of $MR^{4.0}$ was found (present: $n = 24$; absent: $n = 52$; HR = 0.918, 95% CI = 0.566–1.67, $P = 0.958$; Fig. 2B).

Inhibitory *KIR* genes and their matching ligands are not associated with $MR^{4.0}$

Combinations of inhibitory KIRs and their cognate ligands are important in modulation of NK cell cytotoxicity during their development (42). Therefore, we investigated whether inhibitory KIRs and their matching ligands were associated with achievement of $MR^{4.0}$. Because all the patients were positive for *HLA-C1* and its matched ligand gene *KIR2DL3*, this pair was excluded from further analyses. No association between the presence of a single functional combination of an inhibitory *KIR–HLA* genotype was found (*KIR2DL1*/*HLA-C2*, *KIR2DL2*/*HLA-C1*, or *KIR3DL1*/*HLA-Bw4*) with achievement of $MR^{4.0}$ (Table 3). We then analyzed whether carrying multiple functional *KIR–HLA* genotype combinations was associated with achievement of $MR^{4.0}$ (43). Patients carried between zero and three functional pairs of inhibitory KIRs and HLAs (zero: $n = 7$; one: $n = 23$; two: $n = 43$; three: $n = 3$; Fig. 2C and D); none of which was associated with clinical outcome.

Selected *KIR* alleles are positively associated with $MR^{4.0}$ achievement

We next investigated whether allelic polymorphisms of *KIR* genes were associated with achievement of $MR^{4.0}$. Because four *KIR* genes *KIR2DL4*, *KIR2DS4*, *KIR3DL1*, and *KIR3DL2* have abundant allelic polymorphisms (Table 2), the relationships of these alleles with disease outcome were investigated. The presence of several *KIR* alleles was positively correlated with the achievement of $MR^{4.0}$ in univariate analysis (Table 3 and Fig. 3), which included *KIR2DL4*005/011*, *KIR2DL4*008*, *KIR2DS4*003*, *KIR2DS4*007/010*, *KIR3DL1*005*, *KIR3DL2*009*, and *KIR3DL2*010* (*KIR2DL4*005/011* or **008*: HR = 1.942, 95% CI = 1.160–3.250, $P = 0.012$; *KIR2DS4*003* or **007/010*: HR = 3.878, 95% CI = 2.125–7.075, $P < 0.001$; *KIR3DL1*005:* HR = 3.634, 95% CI = 1.884–7.013, $P < 0.001$; *KIR3DL2*010* or **009*: HR = 1.864, 95% CI = 1.067–3.258, $P = 0.029$; Table 3). Even after adjustment for the two prognostic factors, second-generation TKIs as first-line therapy and patient sex (female), these alleles remained significantly correlated with achievement of $MR^{4.0}$ (*KIR2DL4*011/005* or **008*: HR = 1.797, 95% CI = 1.052–3.067, $P = 0.032$; *KIR2DS4*003* or **007/010*: HR = 3.348, 95% CI = 1.773–6.323, $P < 0.001$; *KIR3DL1*005:* HR = 2.746, 95% CI = 1.403–5.376, $P = 0.003$; *KIR3DL2*009* or **010*: HR = 1.980, 95% CI = 1.109–3.524, $P = 0.021$; Table 3). Estimation of *KIR2DL4–3DL1/3DS1–2DS4–3DL2* haplotypes at the allele level suggested that *KIR3DL1*005* and several other favorable alleles were linked (Supplementary Table S1). As shown in haplotypes #8, #9, #14, and #15 (Supplementary Table S1), alleles *KIR2DL4*005/011*, *KIR2DS4*003*, *KIR2DS4*007/010*, and *KIR3DL2*009/010* were present in several *KIR* haplotypes that had *KIR3DL1*005* in common, which were associated with improved therapeutic effects of TKIs (Table 3 and Fig. 3). These results

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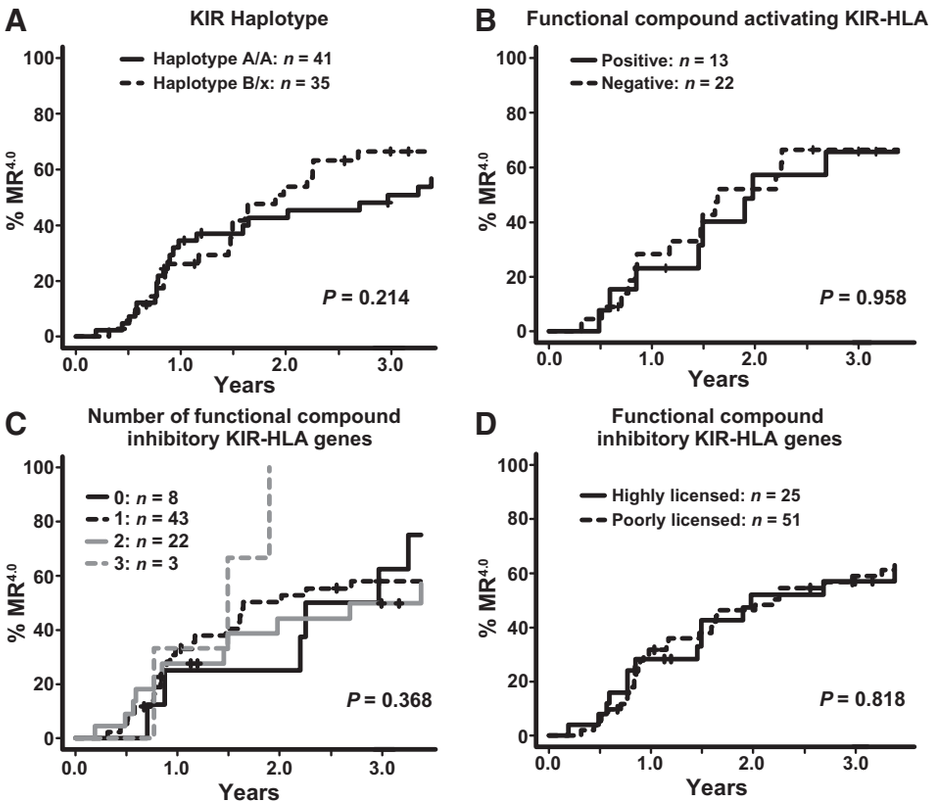

Figure 2.
Cumulative incidences of $MR^{4.0}$ according to *KIR* haplotypes and activating and inhibitory KIRs. **A,** Cumulative incidences of $MR^{4.0}$ ($\%MR^{4.0}$) over time are presented according to different *KIR* haplotypes. Solid line, patients homozygous for KIR haplotype A (A/A); dashed line, B/x haplotypes (A/B and B/B; $P = 0.214$). **B,** $\%MR^{4.0}$ are presented according to the presence or absence of functional combinations of activating *KIR* and *HLA* genes. Solid line, patients with activating KIRs and their ligands; dashed line, patients without those combinations ($P = 0.918$). **C** and **D,** $\%MR^{4.0}$ are presented according to the number of combinations of inhibitory KIRs and their ligands. **C,** Black solid line, patients without any combinations; black dashed line, one combination; gray solid line, two combinations; gray dashed line, three combinations ($P = 0.368$). **D,** Patients were divided into highly licensed (number of combinations of inhibitory KIRs and their ligands: two or three) and poorly licensed (zero or one). Solid line, highly licensed patients; dashed line, poorly licensed patients ($P = 0.818$). All curve comparisons were completed using log-rank tests for time to achievement of $MR^{4.0}$, and $P < 0.05$ were considered statistically significant. Number of patient indicted in **A–D**.

provide evidence that the *KIR3DL1*005* allele, in particular, may be associated with superior prognosis, and, therefore, we next focused on the impact of allelic polymorphisms of *KIR3DL1* and its ligand *HLA-B*.

Interaction avidity between *KIR3DL1* and HLA-B contributes to TKI responses

KIRs encoded by *KIR3DL1* alleles were classified into high (KIR3DL1-h), low (KIR3DL1-l), and null (KIR3DL1-n) subtypes based on protein expression levels and sequence homology (refs. 17, 29, 30; Supplementary Table S2). *KIR3DL1*015* and **001* belonged to the KIR3DL1-h isoform, and *KIR3DL1*005* and **007* to the KIR3DL1-l isoform. Although *HLA-B* genotypes were segregated into Bw4 and Bw6 subtypes, only the former can act as a ligand of KIR3DL1. HLA-Bw4 is dimorphic at position 80 (isoleucine (80I) or threonine (80T); ref. 36; Supplementary Table S2). *KIR3DL1* and *Bw4/6* polymorphisms defined *KIR3DL1-H/L/N* subtypes and *Bw4/6* subtypes, respectively (Supplementary Table S3), and their combination defined three subgroups based on the biological avidity of the interaction between their respective proteins (refs. 31, 33, 36; Supplementary Table S4). KIR/HLA combinations with high avidity were recognized as strongly interacting KIR/HLA combinations, which included KIR3DL1-H/Bw4-80I and KIR3DL1-L/Bw4-80T. KIR/HLA combinations with low avidity were recognized as weakly interacting combinations, which included KIR3DL1-H/Bw4-80T and KIR3DL1-L/Bw4-80I. KIR/HLA combinations without avidity were recognized as noninteracting combinations, which included KIR3DL1-N/HLA-B and KIR3DL1/Bw6.

We analyzed associations of the KIR/HLA combinations with clinical outcome. The analyses distributed 35 (46.1%) patients to strong interactors, 29 (38.2%) patients to weak interactors, and 12 (15.8%) patients to noninteractors. Noninteractors were more likely to achieve $MR^{4.0}$ than strong interactors (HR = 2.208, 95% CI = 1.034–4.713, $P = 0.041$; Fig. 3E). Among weak interactors, carriers of *KIR3DL1*005* (11 of 29 patients) had better outcomes than those without this allele (HR = 14.22, 95% CI = 3.648–55.44, $P < 0.001$; Fig. 3F).

NK cell degranulation is associated with $MR^{4.0}$ and patient sex

K562 is a human erythromyeloblastoid leukemia cell line that lacks HLA class I expression. These cells activate NK cells, resulting in IFNγ secretion and degranulation. We investigated whether the magnitude of NK cell activation *in vitro* was associated with achievement of $MR^{4.0}$ by analyzing patient PBMC samples. Patients who achieved $MR^{4.0}$ exhibited higher CD107a, a marker for degranulation (Fig. 4A and B), and their levels of IFNγ release also tended to be higher (Fig. 4A and C). Higher CD107a and IFNγ release was also observed in female patients compared with male patients (Fig. 4D and E). These results suggest that achievement of deep molecular response in CML patients depended on NK cell activation.

Discussion

Our findings demonstrated that first-line treatment with second-generation TKIs and patient sex, specifically female, were associated with the achievement of $MR^{4.0}$, consistent with

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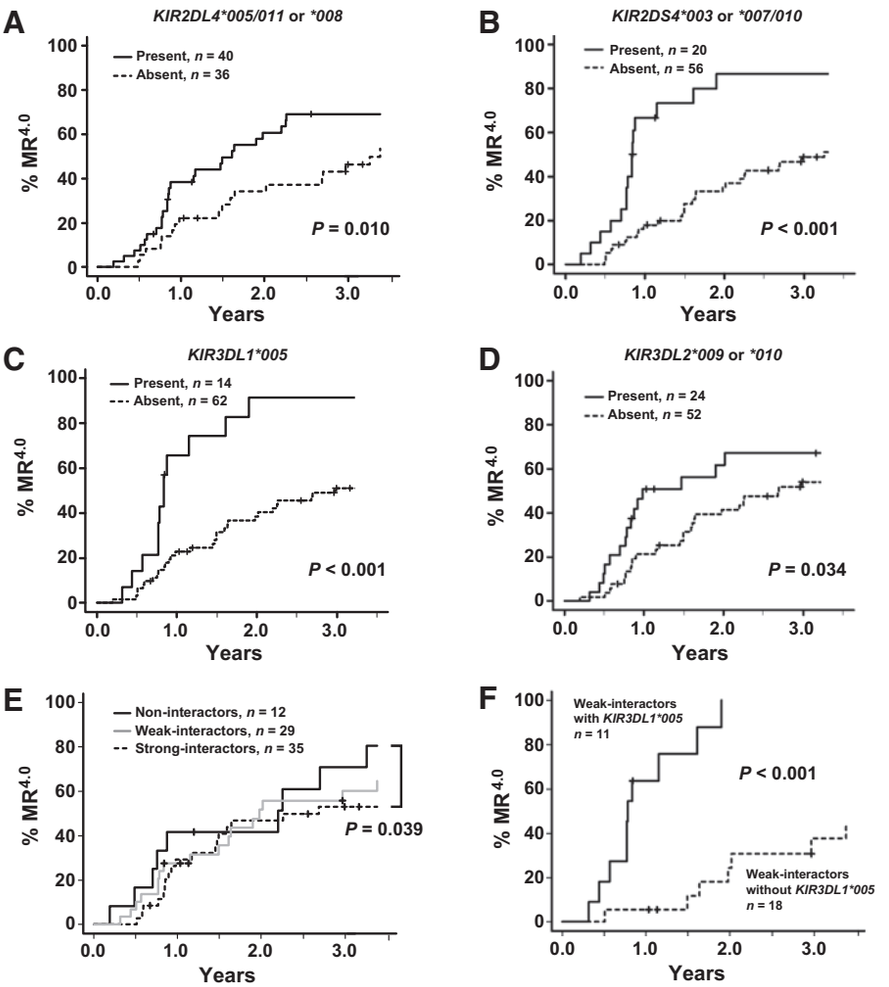

Figure 3.

Cumulative incidences of $MR^{4.0}$ achievement according to selected *KIR* alleles and combinations of *KIR3DL1* and HLA-B alleles. Cumulative incidences of $MR^{4.0}$ are shown segregated according to the presence or absence of (**A**) *KIR2DL4*005/011* or **008;* (**B**) *KIR2DS4*003 or 007/010* (**C**) *KIR3DL1*005;* and (**D**) *KIR3DL2*009* or **010*. **E** and **F**, To investigate associations with the avidity of interactions between *KIR3DL1* and HLA-B and patient outcome, patients were classified into three subgroups based on *KIR3DL1* alleles and *HLA-B* subtypes: strong interactors ($n = 35$, black dashed line), weak interactors ($n = 29$, gray solid line), and noninteractors ($n = 12$, black solid line). **E,** Cumulative incidences of $MR^{4.0}$ are shown for the three subgroups. Noninteractors exhibited higher levels of $MR^{4.0}$ achievement compared with strong interactors ($P = 0.039$). **F,** Cumulative incidences of $MR^{4.0}$ are shown for weak interactors, further segregated depending on the presence of *KIR3DL1*005* using log-rank test, and $P < 0.05$ were considered statistically significant; $P < 0.001$. Number of patient indicted in **A–F**.

previous reports (44, 45). Second-generation TKIs inhibit BCR–ABL and induce apoptosis of CML cells over 100 times more potently than imatinib (46), resulting in early and deep molecular remission in patients with CML. Of our patients, 82.5% achieved $MR^{3.0}$ and 48% achieved $MR^{4.0}$, consistent with previous reports that dasatinib achieved $MR^{3.0}$ in 85% and $MR^{4.0}$ in 42% of Japanese CML patients after 2 years (47). Female CML patients are reported to achieve early and deep molecular responses (45), and to sustain TFR, also consistent with our results. Together, these findings indicate that our patient group was representative and supportive of previously generated data.

Although early molecular response (*BCR-ABL* $\leq$ 10% at 3 months) is now recognized as an important favorable prognostic factor in TKI-treated CML, Sokal scores do not always predict outcome, especially because of the advent of TKIs (48). Our analysis indicated that patients with high Sokal scores achieve $MR^{4.0}$ more frequently than those with intermediate or low scores. However, because only ten patients were assigned high Sokal scores, we cannot conclude that high Sokal score is inversely correlated with outcome, and we do not think that our results are sufficient to contradict the previous consensus. Further investigation is needed to elucidate the association between Sokal score and treatment response in the second-generation TKI era.

Higher CD107a degranulation of NK cells was observed in female patients and those who achieved $MR^{4.0}$, implying that the magnitude of NK cell activation may contribute to the efficacy of TKIs. We found that several alleles, *KIR2DL4*, *KIR3DL1*, and *KIR2DS4*, were associated with achievement of $MR^{4.0}$, and all three of these *KIR*s contributed to haplotype A. Specifically, we identified favorable subpopulations carrying specific *KIR* alleles among CML patients with KIR haplotype A. Previous reports indicate that homozygosity for KIR haplotype A correlates with superior molecular response (27). Our analysis did not directly support these observations, which may be due to the different *KIR* allele frequencies among Caucasian and Japanese populations (33). Compared with Caucasians, the frequency of functional *KIR2DL4* and *KIR2DS4* alleles in the Japanese population is much higher (33), and therefore, in Japanese patients, KIR haplotype A may be more suppressive, resulting in less NK cell cytotoxicity and antitumor immunity than in Caucasians.

*KIR3DL1*005* was strongly associated with specific alleles of three other KIR genes: *KIR2DL4*, *KIR2DS4*, and *KIR3DL2*. We identified several known KIR allelic haplotypes, such as *KIR2DL4*005/011-KIR2DS4*007/010-KIR3DL2*010*, *KIR2DL4*005/011-KIR2DS4*007/010-KIR3DL2*002*, and *KIR2DL4*005/011-KIR2DS4*007/010-KIR3DL2*009*, representing recognized haplotypes that have

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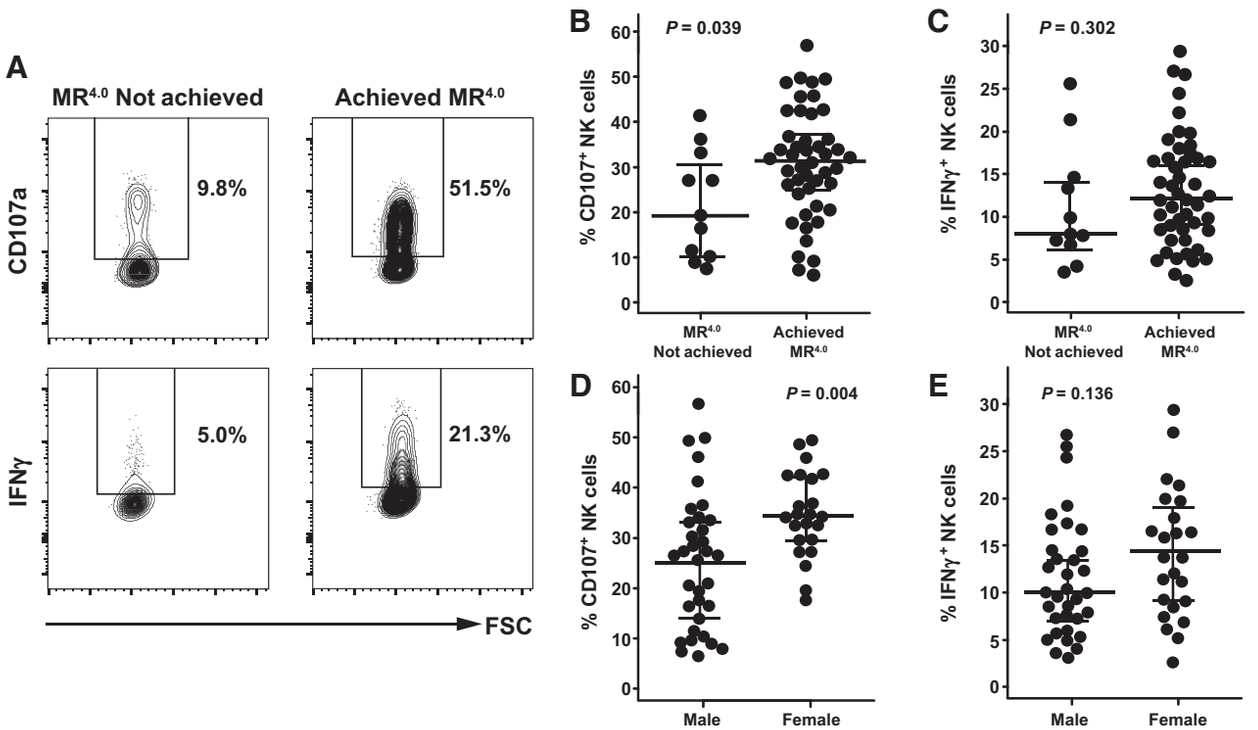

Figure 4.

Activation status of NK cells is associated with therapeutic effects of TKIs. PBMCs from CML patients were cocultured with HLA class I–deficient K562 cells for 6 hours, and CD107a degranulation and intracellular IFNγ release from NK cells were analyzed by flow cytometry (E:T ratio 10:1). Representative plots from (**A**) patients who did and did not achieve $MR^{4.0}$ are shown and (**B** and **C**) aggregated results are also plotted. Achieved $MR^{4.0}$: $n = 46$; did not achieve $MR^{4.0}$: $n = 11$). **D** and **E**, Data from patients segregated into male ($n = 34$) and female ($n = 23$) are also presented. Statistical comparisons were made using the Mann–Whitney test, and $P < 0.05$ were considered statistically significant.

the *KIR3DL1*005* allele in common (33). These haplotypes could affect therapeutic responses to TKIs by generating increased NK cell activity, which suggests the particular significance of the *KIR3DL1*005* allele. Of these KIRs, polymorphism of *KIR3DL1* has been most investigated, demonstrating differential expression and functions for each allele at this locus (29, 31, 33, 49). Some reports have indicated clinical impacts of allelic polymorphisms in *KIR3DL1* in HIV infection (36), acute myelogenous leukemia relapse after hematopoietic stem cell transplantation (38, 50), and the therapeutic effects of anti-GD2 against neuroblastoma (37). Given that *KIR3DL1*005* is associated with low expression levels and a low suppressive capacity of NK cells (33, 36), this allele may be associated with more potent antitumor NK cell immunity against CML.

Both *KIR3DL1* and *HLA-B* exhibit allelic polymorphisms. *KIR3DL1* alleles are divided into three subtypes (*KIR3DL1*-H/L/N), as are those of *HLA-B* (Bw4-80T, Bw4-80I, and Bw6; refs. 29, 31). It is assumed that combinations of *KIR3DL1* and *HLA-B* alleles define the differential avidity of interactions between them, which may determine the potency of NK cell immunity (21, 51). Weakly interactive combinations of *KIR3DL1* and *HLA-B* hardly suppress NK cell cytotoxicity, resulting in higher NK cell activation and superior therapeutic effects of anti-GD2 antibody (37). In our study, noninteracting combinations and weakly interacting combinations involving *KIR3DL1*005* were associated with improved achievement of $MR^{4.0}$. These results are in accordance with previous reports relating to anti–GD2-treated neuroblastoma and acute myelogenous leukemia after allo-HSCT (37, 38). However, this report indicated a clinical impact of *KIR3DL1*/*HLA-B* interactions in TKI-treated CML. Allelic polymorphisms of *KIR2DL4*/*2DS4*/*3DL2* also exhibited statistically significant associations. We cannot comment regarding their biological impact on NK cell immunity or CML at present, as it is unclear whether these polymorphisms are associated with immunological function. However, these results warrant further investigation.

Our results from NGS of *KIR2DL4*, *KIR3DL1*, *KIR2DS4*, and *KIR3DL2* demonstrated that specific *KIR* alleles are associated with superior therapeutic effects of TKIs in CML. *KIR3DL1*005* exhibited strong linkage with *KIR2DL4*011/005* and *2DS4*007*, indicating that these alleles compose favorable KIR haplotypes. These results suggest that allelic genotyping of KIRs may enable prediction of treatment responses in TKI-treated CML patients and allow identification of patients likely to exhibit unfavorable responses who may require salvage therapy. Because $MR^{4.0}$ is essential to achieve TFR, allelic genotyping of *KIR*s may enable successful withdrawal of TKIs, although TFR was not evaluated in this study. Such an approach would contribute to reducing the cost of safe treatment for CML. These data reveal new aspects of CML through analysis of NK cell antitumor immunity.

Disclosure of Potential Conflicts of Interest

S. Kimura reports receiving commercial research grants and speakers bureau honoraria from Bristol-Myers Squibb, Novartis Pharmaceuticals, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: H. Saji, A. Kawaguchi, S. Kimura
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Ureshino, T. Shindo, Y. Kusunoki, H. Tanaka, H. Saji
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Ureshino, T. Shindo, H. Kojima, Y. Kusunoki, Y. Miyazaki, H. Tanaka, H. Saji, A. Kawaguchi
Writing, review, and/or revision of the manuscript: H. Ureshino, T. Shindo, H. Tanaka, H. Saji, A. Kawaguchi, S. Kimura
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Tanaka, H. Saji, S. Kimura
Study supervision: H. Tanaka, H. Saji

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