**Table 1. Patient, Disease, and Transplantation Characteristics According to Stem Cell Source and Number of Mismatched Loci**

| | Bone Marrow Transplant | | | |
|---|---|---|---|---|
| | Class II One Locus Mismatch | Class I One Locus Mismatch | Two Loci Mismatch | Cord Blood Transplantation |
| | N (%) | N (%) | N (%) | N (%) |
| Number of transplantations | 248 | 424 | 356 | 351 |
| Patient age at transplantation | | | | |
| Median (range) | 36 (16-60) | 34 (16-67) | 34 (16-59) | 37 (16-58) |
| Patient sex | | | | |
| Male | 151 (61) | 241 (57) | 210 (59) | 162 (46) |
| Female | 97 (39) | 183 (43) | 146 (41) | 189 (54) |
| Sex matching | | | | |
| Matched | 145 (58) | 268 (63) | 217 (61) | 170 (48) |
| Male to female | 52 (21) | 82 (19) | 73 (21) | 97 (28) |
| Female to male | 50 (20) | 71 (17) | 64 (18) | 84 (24) |
| Unknown | 1 (<1) | 3 (1) | 2 (1) | 0 (0) |
| Diagnosis | | | | |
| AML | 135 (54) | 204 (48) | 172 (48) | 193 (55) |
| ALL | 78 (31) | 149 (35) | 135 (38) | 113 (32) |
| MDS | 35 (14) | 71 (17) | 49 (14) | 45 (13) |
| Disease status | | | | |
| Standard | 124 (50) | 214 (50) | 168 (47) | 147 (42) |
| Advanced | 114 (46) | 195 (46) | 169 (47) | 174 (50) |
| Unknown | 10 (4) | 15 (4) | 19 (5) | 30 (9) |
| ABO matching | | | | |
| Matched | 119 (48) | 184 (43) | 153 (43) | 114 (32) |
| Minor mismatch | 53 (21) | 108 (25) | 85 (24) | 99 (28) |
| Major mismatch | 67 (27) | 116 (27) | 97 (27) | 73 (21) |
| Bidirectional | 8 (3) | 12 (3) | 14 (4) | 64 (18) |
| Unknown | 1 (<1) | 4 (1) | 7 (2) | 1 (<1) |
| HLA-mismatched number and direction | | | | |
| Matched | | | | 20 (6) |
| One locus mismatched | | | | 87 (25) |
| HVG direction | 16 (6) | 38 (9) | | 8 (9) |
| GVH direction | 17 (7) | 30 (7) | | 8 (9) |
| Both directions | 215 (87) | 356 (84) | | 71 (82) |
| Two loci mismatched | | | | 244 (70) |
| Two HVG direction | | | 4 (1) | 2 (1) |
| One HVG direction and one GVH direction | | | 6 (2) | 4 (2) |
| Two GVH direction | | | 4 (1) | 3 (1) |
| One both directions and one HVG direction | | | 42 (12) | 40 (16) |
| One both directions and one GVH direction | | | 29 (8) | 28 (11) |
| Two both directions | | | 271 (76) | 167 (68) |
| No. of nucleated cells infused ($\times 10^7$/kg) | | | | |
| Median | 25.0 | 24.5 | 23 | 2.46 |
| Range | 2.40-59.8 | 2.10-97.5 | 1.5-66.0 | 1.41-6.01 |
| Preparative regimen | | | | |
| CY + TBI | 94 (38) | 168 (40) | 151 (42) | 109 (31) |
| CY + CA + TBI | 46 (19) | 78 (18) | 74 (21) | 124 (35) |
| CY + BU + TBI | 20 (8) | 39 (9) | 27 (8) | 15 (4) |
| Other TBI regimen | 45 (18) | 70 (17) | 61 (17) | 80 (23) |
| BU + CY | 34 (14) | 54 (13) | 30 (8) | 21 (6) |
| Other non-TBI regimen | 9 (4) | 15 (4) | 13 (4) | 2 (1) |
| GVHD prophylaxisis | | | | |
| Cycrosporine A + sMTX | 87 (35) | 221 (52) | 150 (42) | 213 (61) |
| Cyclosporine A ± other | 1 (<1) | 5 (1) | 5 (1) | 24 (7) |
| Tacrolimus + sMTX | 152 (61) | 191 (45) | 193 (54) | 76 (22) |
| Tacrolimus ± other | 8 (3) | 5 (1) | 6 (2) | 35 (10) |
| Others | 0 (0) | 2 (<1) | 2 (<1) | 3 (1) |

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; BU, oral busulfan; CA, citarabine; CY, cyclophosphamide; GVH, graft-versus-host; HVG, host-versus-graft; MDS, myelodysplastic syndomes; sMTX, short-term methotrexate.

## Outcome

### *OS and RFS*

OS and RFS for CB recipients were similar when compared with that of single-HLA-DRB1-mismatched BM recipients (relative risk [RR] = 0.85, 95% confidence interval [CI], 0.68-1.06; $P$ = .149 for OS and RR = 0.97, 95% CI, 0.92-1.35; $P$ = .747) (Table 2).

The adjusted probabilities of survival at 3 years posttransplantation of CB recipients (47%) were not different from those of single HLA-DRB1 mismatched BM recipients (41%; $P$ = .19) or single HLA class I-mismatched BM recipients (47%; $P$ = .96), but superior to those of 6 of 8 BM recipients (38%; $P$ = .014) (Figure 1A). Figure 1B shows adjusted RFS curves (42% for CB recipients, 36% for single HLA-DRB1-mismatched BM, 44% for single HLA class I-mismatched BM, and 36% for 6 of 8 BM recipients, at 3 years posttransplant) ($P$ values of comparison between CB and single HLA-DRB1-mismatched BM, CB, and single HLA

**Table 2. Multivariate Analyses of Overall Survival, Relapse-Free Survival, Relapse, and Transplant-Related Mortality**

| | Degree of HLA Mismatch | N | Overall Survival | | | Relapse-Free Survival | | | Relapse | | | Transplant-Related Mortality | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | RR | (95% CI) | P value | RR | (95% CI) | P value | RR | (95% CI) | P value | RR | (95% CI) | P value |
| Bone marrow transplant | Single DRB1 (7/8) | 248 | 1.00 | | | 1.00 | | | 1.00 | | | 1.00 | | |
| | Single A or B (7/8) | 137 | 0.84 | (0.64-1.11) | .216 | 0.82 | (0.63-1.08) | .158 | 0.65 | (0.41-1.01) | .056 | 1.07 | (0.77-1.49) | .698 |
| | Single C (7/8) | 287 | 0.89 | (0.72-1.12) | .324 | 0.86 | (0.69-1.07) | .170 | 0.60 | (0.41-0.87) | .007 | 1.13 | (0.86-1.48) | .391 |
| | C + DRB1 (6/8) | 144 | 0.97 | (0.74-1.27) | .831 | 0.95 | (0.73-1.24) | .726 | 0.76 | (0.49-1.17) | .208 | 1.10 | (0.78-1.55) | .600 |
| | A/B + C (6/8) | 122 | 1.22 | (0.94-1.59) | .143 | 1.15 | (0.88-1.49) | .300 | 0.70 | (0.44-1.10) | .12 | 1.42 | (1.03-1.96) | .032 |
| | Other two loci (6/8) | 90 | 1.25 | (0.92-1.68) | .146 | 1.13 | (0.84-1.53) | .409 | 0.60 | (0.35-1.02) | .061 | 1.48 | (1.03-2.13) | .035 |
| Cord blood transplant | | 351 | 0.85 | (0.68-1.06) | .149 | 0.97 | (0.92-1.35) | .747 | 1.28 | (0.93-1.76) | .125 | 0.68 | (0.50-0.92) | .011 |

RR indicates relative risk; CI, confidence interval.
Adjusted by patient age at transplantation >40 versus ≤40, patient sex, donor-patient sex mismatch versus matched, ABO major mismatch versus others, advanced versus standard disease status at transplantation, cyclophosphamide and total-body irradiation or busulfan and cyclophosphamide for conditioning versus other conditioning regimen, and cyclosporine-based versus tacrolimus-based prophylaxis against graft-versus-host disease.

class I-mismatched BM, and CB and 6 of 8 BM recipients were 0.80, 0.12, and 0.43, respectively).

### Relapse and TRM

There was no significant increase of relapse rates among CB recipients when compared with DRB1 single-mismatched BM recipients (RR = 1.28, 95% CI, 0.93-1.76; $P = .125$). The risk of TRM was lower in CB recipients compared with that of single HLA-DRB1-mismatched BM recipients (RR = 0.68, 95% CI, 0.50-0.92; $P = .011$) (Table 2). The risk of TRM was also lower in CB recipients when compared with 6 of 8 BM recipients (RR = 0.52, 95% CI, 0.39-0.68; $P < .001$).

### Hematologic recovery

Neutrophil and platelet recovery was inferior in CB recipients, as shown in Table 3 (RR = 0.50, 95% CI, 0.42-0.60; $P < .001$ for neutrophil recovery, RR = 0.52, 95% CI, 0.42-0.63; $P < .001$ for platelet recovery).

### Acute GVHD and chronic GVHD

The risk of grade 2 to 4 or severe (grades 3-4) aGVHD was lower in CB recipients than that of single HLA-DRB1-mismatched BM recipients (RR = 0.55, 95% CI, 0.42-0.72; $P < .001$ for grade 2 to 4 aGVHD and RR = 0.43, 95% CI, 0.27-0.58; $P < .001$ for severe aGVHD) (Table 4). Unadjusted cumulative incidence of severe aGVHD was 9% for CB, 19% for single HLA-DRB1-mismatched BM, 18% for single HLA

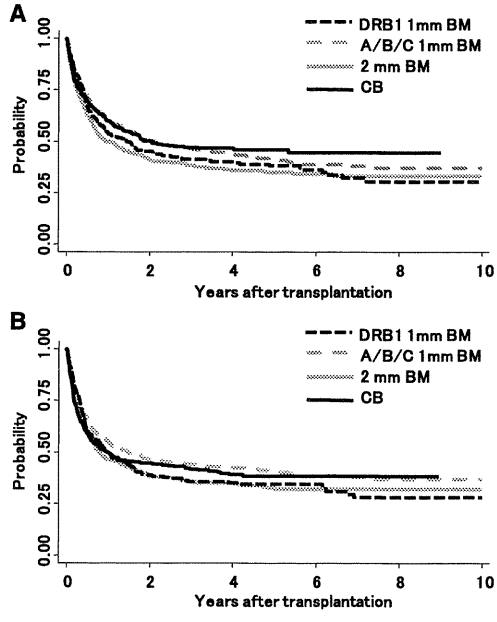


**Figure 1.** Adjusted probabilities of OS (A) and RFS (B). The adjusted 3-year probabilities of OS for unrelated cord blood recipients, single-HLA-DRB1-mismatched unrelated bone marrow (UBM) recipients, single-HLA-class-I-mismatched UBM, and 6 of 8 UBM recipients were 47%, 41%, 47%, and 38%, respectively (A). The adjusted 3-year probabilities of RFS were 42%, 36%, 44%, and 36%, respectively (B).

**Table 3. Multivariate Analyses of Neutrophil and Platelet Recovery**

| | | | Neutrophil Recovery | | | Platelet Recovery | | |
|---|---|---|---|---|---|---|---|---|
| | Degree of HLA Mismatch | N | RR | (95% CI) | *P* value | RR | (95% CI) | *P* value |
| Bone marrow transplantation | Single DRB1 (7/8) | 248 | 1.00 | | | 1.00 | | |
| | Single A or B (7/8) | 137 | 1.31 | (1.04-1.65) | .021 | 1.31 | (1.01-1.70) | .039 |
| | Single C (7/8) | 287 | 1.19 | (0.98-1.43) | .069 | 0.98 | (0.79-1.21) | .840 |
| | C + DRB1 (6/8) | 144 | 0.96 | (0.77-1.20) | .735 | 0.79 | (0.62-1.02) | .065 |
| | A/B + C (6/8) | 122 | 1.14 | (0.89-1.45) | .307 | 0.84 | (0.63-1.13) | .255 |
| | Other two loci (6/8) | 90 | 0.89 | (0.68-1.14) | .346 | 0.80 | (0.58-1.10) | .174 |
| Cord blood transplantation | | 351 | 0.50 | (0.42-0.60) | <.001 | 0.52 | (0.42-0.63) | <.001 |

RR indicates relative risk; CI, confidence interval.

Adjusted by patient age at transplantation >40 versus <40, patient sex, donor-patient sex mismatch versus matched, ABO major mismatch versus others, advanced versus standard disease status at transplant, cyclophosphamide, and total-body irradiation or busulfan and cyclophosphamide for conditioning versus other conditioning regimen, and cyclosporine-based versus tacrolimus-based prophylaxis against graft-versus-host disease.

class I-mismatched BM, and 22% for 6 of 8 BM at 100 days posttransplantation ($P < .001$ between CB and single HLA-DRB1-mismatched BM) (Figure 2A).

Among recipients who survived at least 100 days posttransplantaton, the risk of developing cGVHD and extensive-type cGVHD was not significantly increased in all HLA disparity groups of CB recipients when compared with that of HLA-DRB1-allele/antigen-mismatched BM recipients (RR = 1.36, 95% CI, 0.99-1.88; $P = .057$ for cGVHD, and RR = 0.86, 95% CI, 0.55-1.34; $P = .500$ for extensive-type cGVHD). The unadjusted cumulative incidence of extensive-type cGVHD was 17% for CB recipients, 20% for single HLA-DRB1-mismatched BM, 25% for single HLA class I-mismatched BM, and 30% for 6 of 8 BM recipients at year posttransplantation ($P = .34$ between CB and single HLA-DRB1-mismatched BM) (Figure 2B).

## DISCUSSION

Our main objective was to compare OS after transplantation of UCBT and single-HLA-mismatched UBMT and to provide useful data for selection of an appropriate donor and graft source in second stem cell source/donor selection for adults with hematologic malignancy. To the best of our knowledge, this is the first study to involve mismatched allele/antigen-specific analyses including CB for the process of donor selection. Our results suggest that 0 to 2 HLA-mismatched UCB is a reasonable second alternative of choice for adult patients with leukemia, with similar survival to that of single DRB1-mismatched or other 7 of 8 UBM recipients, the current first choice for second alternative donor/stem cells.

Neutrophil and platelet recovery was slower in CB recipients than BM recipients, consistent with the results of previous reports [7-10,12]. This is the major limitation of the use of UCB, and several strategies have been studied to reduce the neutropenic period, such as screening for patients' pretransplantation anti-HLA antibodies and their specificity, transplantation of 2 UCB units if a single UCB unit with an adequate cell dose is not available, or direct infusion of UCB into bone marrow [22-26].

Despite higher HLA disparity at the antigen level (69% 2 antigen mismatch, 25% antigen mismatch, and 6% matched), UCB recipients showed lower incidence of severe aGVHD than single DRB1-mismatched UBM recipients, consistent with other reports that compared UCB with single-mismatched UBM (7 of 8) [8,11,12]. In our study, tacrolimus and short-term methotrexate were used preferentially in BM recipients, whereas cyclosporine A was used in 68% of CB recipients. Prior studies have shown reduced severe aGVHD with tacrolimus, and this difference may have underscored the improved aGVHD control of UCB over mismatched BM in unadjusted analyses [27,28]. It is likely that decreased risk of grade 2 to 4 aGVHD in UCB recipients contributed to decreased risk of TRM among UCB recipients.

Increasing the number of HLA mismatches from 7 of 8 to 6 of 8 was associated with an approximately 10% reduction in survival in UBM recipients, which was quite similar to the results from the National Marrow Donor Program [3]. Because we eliminated data from the first 3 pioneering years of unrelated BMT, most of the bone marrow recipients and donors were allele-typed for at least HLA-A, -B, and -DRB1 before transplantation. Survival outcomes of single class I mismatch were not significantly different from those of single class II mismatch in the current analyses. We believe that allele typing of HLA-A, -B, and -DRB1 before transplantation led to better selection of the donor compared with that in the first several years of UBMT. This study includes a large number of fully typed BM and CB recipients, but there are limitations. The choice of stem cell source is influenced by many unmeasured factors that can affect outcome. It is also influenced by the availability of acceptable HLA disparity for unrelated donors and mainly cell dose for cord blood units. Although we have adjusted for known risk factors and disparities between groups, we cannot rule out the influence of potential selection bias, which can only be excluded in a randomized controlled trial. Transplantation years

**Table 4. Multivariate Analyses of Acute (Grades 2 to 4 and Grades 3 to 4), Chronic, and Extensive-Type Chronic Graft-versus-Host Disease**

| | Degree of HLA Mismatch | N | Grade 2-4 acute GVHD | | | Grade 3-4 acute GVHD | | | Chronic GVHD | | | Extensive cGVHD | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | RR | (95% CI) | *P*-value | RR | (95% CI) | *P*-value | N | RR | (95% CI) | RR | (95% CI) | *P* value |
| Bone marrow transplantation | Single DRB1 (7/8) | 248 | 1.00 | | | 1.00 | | | 199 | 1.00 | | 1.00 | | |
| | Single A or B (7/8) | 137 | 0.76 | (0.55-1.06) | .103 | 0.91 | (0.56-1.47) | .698 | 111 | 0.91 | (0.61-1.36) | 0.89 | (0.52-1.50) | .651 |
| | Single C (7/8) | 287 | 0.93 | (0.72-1.20) | .584 | 0.91 | (0.61-1.35) | .635 | 227 | 1.56 | (1.15-2.10) | 1.79 | (1.22-2.63) | .003 |
| | C + DRB1 (6/8) | 144 | 0.85 | (0.60-1.18) | .320 | 0.88 | (0.54-1.44) | .610 | 109 | 1.44 | (1.01-2.05) | 1.47 | (0.93-2.32) | .097 |
| | A/B + C (6/8) | 122 | 1.40 | (1.04-1.90) | .028 | 1.90 | (1.25-2.87) | .003 | 87 | 1.64 | (1.14-2.34) | 2.26 | (1.46-3.50) | <.001 |
| | Other two loci (6/8) | 90 | 0.88 | (0.60-1.28) | .501 | 0.65 | (0.34-1.22) | .183 | 60 | 1.35 | (0.86-2.12) | 1.15 | (0.62-2.13) | .652 |
| Cord blood transplantation | | 351 | 0.55 | (0.42-0.72) | <.001 | 0.43 | (0.27-0.58) | <.001 | 252 | 1.36 | (0.99-1.88) | 0.86 | (0.55-1.34) | .500 |

GVHD indicates graft-versus-host disease; cGVHD, chronic graft-versus-host disease.
Adjusted by patient age at transplantation >40 versus <40, patient sex, donor-patient sex mismatch versus matched, ABO major mismatch versus others, advanced versus standard disease status at transplantation, cyclophosphamide, and total-body irradiation or busulfan and cyclophosphamide for conditioning versus other conditioning regimen, and cyclosporine-based versus tacrolimus-based prophylaxis against graft-versus-host disease.




**Figure 2.** Cumulative incidence of grade 3 to 4 aGVHD (A) and extensive-type cGVHD (B). The cumulative incidences of grade 3 to 4 aGVHD at 100 days posttransplantation for unrelated cord blood recipients, single HLA-DRB1-mismatched unrelated bone marrow (UBM) recipients, and single HLA class I-mismatched UBM were 9%, 19%, 18%, and 22% (A). The cumulative incidences of extensive-type cGVHD at 1-year posttransplantation were 17%, 20%, 25%, and 30% (B).

of UBM recipients included from 1996 and 1999, for which there were no significant outcome differences between UBMT performed in 1996 to 1999 and after 2000. In these periods, there were advances including in supportive care and nutritional management, introduction of new antifungal agents, and more frequent use of tacrolimus, which may have affected transplantation outcomes [27-32].

In conclusion, we suggest that 0 or 2 HLA-mismatched UCB is a comparable second alternative for adult patients with leukemia in the absence of the first alternative, an 8 of 8 UBM donor, with survival similar to that of single DRB1-mismatched or other 7 of 8 UBM recipients. UCB may be preferred over single mismatched UBM when a transplantation is needed urgently, considering the short time needed for UCBT.

## ACKNOWLEDGMENTS

The authors are grateful for the assistance and cooperation of all the staff members of the collaborating institutes of the Japan Cord Blood Bank Network and Japan Marrow Donor Program. This work was supported by a Research Grant for Tissue Engineering (H17-014), a Research Grant for Allergic Disease and Immunology (H20-015), a Research Grant for Cancer (H19-1), and a Research Grant for Allergic Disease and Immunology (H23-010) from the Japanese Ministry of Health, Labor, and Welfare.

*Financial disclosure:* The authors have nothing to disclose.

## AUTHORSHIP STATEMENT

Contributions: Y.A., Y.M., R.S., and S. Kato designed the study, and wrote the article; Y.A. analyzed results and created the figures; T.N.I., H.A, and M. Takanashi reviewed and cleaned the Japan Cord Blood Bank Network data, and reviewed the results; S. Taniguchi, S. Takahashi, S. Kai., H.S., Y. Kouzai., N.K., T.M., T.F., and Y. Kodera submitted and cleaned the data; M. Tsuchida, K.K., T.K., and Y.M. reviewed and cleaned the Japan Marrow Donor Program data, and reviewed the results.

## SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2011.10.008.

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# Related transplantation with HLA-1 Ag mismatch in the GVH direction and HLA-8/8 allele-matched unrelated transplantation: a nationwide retrospective study

Junya Kanda,[1,2] Hiroh Saji,[3] Takahiro Fukuda,[4] Takeshi Kobayashi,[5] Koichi Miyamura,[6] Tetsuya Eto,[7] Mineo Kurokawa,[8] Heiwa Kanamori,[9] Takehiko Mori,[10] Michihiro Hidaka,[11] Koji Iwato,[12] Takashi Yoshida,[13] Hisashi Sakamaki,[5] Junji Tanaka,[14] Keisei Kawa,[15] Yasuo Morishima,[16] Ritsuro Suzuki,[17] Yoshiko Atsuta,[17] and Yoshinobu Kanda[1]

[1]Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama, Japan; [2]Adult Stem Cell Transplant Program, Division of Cellular Therapy, Department of Medicine, Duke University Medical Center, Durham, NC; [3]HLA Laboratory, Kyoto, Japan; [4]Stem Cell Transplantation Division, National Cancer Center Hospital, Tokyo, Japan; [5]Hematology Division, Tokyo Metropolitan Cancer and Infectious Diseases Center, Komagome Hospital, Tokyo, Japan; [6]Department of Hematology, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; [7]Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan; [8]Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [9]Department of Hematology, Kanagawa Cancer Center, Yokohama, Japan; [10]Department of Medicine, Division of Hematology, Keio University School of Medicine, Tokyo, Japan; [11]Department of Internal Medicine, National Hospital Organization, Kumamoto Medical Center, Kumamoto, Japan; [12]Department of Blood Transfusion, Hiroshima Red Cross and Atomic Bomb Survivors Hospital, Hiroshima, Japan; [13]Department of Internal Medicine, Toyama Prefectural Central Hospital, Toyama, Japan; [14]Stem Cell Transplantation Center, Hokkaido University Hospital, Sapporo, Japan; [15]Department of Haematology/Oncology, Osaka Medical Centre and Research Institute for Maternal and Child Health, Osaka, Japan; [16]Department of Hematology and Cell Therapy, Aichi Cancer Center Hospital, Nagoya, Japan; and [17]Department of Hematopoietic Stem Cell Transplantation Data Management/Biostatistics, Nagoya University School of Medicine, Nagoya, Japan

**To clarify which is preferable, a related donor with an HLA-1 Ag mismatch at the HLA-A, HLA-B, or HLA-DR loci in the graft-versus-host (GVH) direction (RD/1AG-MM-GVH) or an HLA 8/8-allele (HLA-A, HLA-B, HLA-C, and HLA-DRB1)–matched unrelated donor (8/8-MUD), we evaluated 779 patients with acute leukemia, chronic myelogenous leukemia, or myelodysplastic syndrome who received a T cell–replete graft from an RD/1AG-MM-GVH or 8/8-MUD. The use of an RD/1AG-MM-GVH donor was significantly associated with a higher overall mortality rate than the use of an 8/8-MUD in a multivariate analysis (hazard ratio, 1.49; $P < .001$), and this impact was statistically significant only in patients with standard-risk diseases ($P = .001$). Among patients with standard-risk diseases who received transplantation from an RD/1AG-MM-GVH donor, the presence of an HLA-B Ag mismatch was significantly associated with a lower overall survival rate than an HLA-DR Ag mismatch because of an increased risk of treatment-related mortality. The HLA-C Ag mismatch or multiple allelic mismatches were frequently observed in the HLA-B Ag-mismatched group, and were possibly associated with the poor outcome. In conclusion, an 8/8-MUD should be prioritized over an RD/1AG-MM-GVH donor during donor selection. In particular, an HLA-B Ag mismatch in the GVH direction has an adverse effect on overall survival and treatment-related mortality in patients with standard-risk diseases. (*Blood.* 2012;119(10):2409-2416)**

## Introduction

An HLA-matched unrelated donor (MUD) is considered to be an alternative donor in hematopoietic stem cell transplantation (SCT) for patients who lack an HLA-identical sibling. However, it is difficult to find an MUD for patients with rare HLA haplotypes. SCT from a related donor with 1 Ag mismatch at HLA-A, HLA-B, or HLA-DR loci in the graft-versus-host (GVH) direction results in a higher but acceptable incidence of acute GVHD and outcomes comparable to that of SCT from a matched related donor (MRD) in patients with high-risk diseases because it reduces the risk of relapse via a graft-versus-leukemia (GVL) effect.[1-3] In previous studies, HLA mismatches in the host-versus-graft (HVG) direction were associated with higher graft failure and lower overall survival (OS).[1,2,4] However, strategies to reduce the risk of graft failure might have been improved by the use of conditioning regimens that strongly suppress recipient immune system.[5] Therefore, in current clinical practice in Japan, SCT from a related donor with 1 Ag mismatch in the GVH direction and accepting multiple Ag mismatches in the HVG direction without specific stem cell manipulation is being performed,[1,2] although such an approach has not yet been evaluated in a large cohort.

Our previous study showed that SCT from an HLA-1 Ag-mismatched donor in the GVH or HVG direction is comparable to that from an HLA-A, HLA-B, or HLA-DR Ag-MUD.[1] However, this study is relatively old (1991-2000) and may not reflect current practice. Furthermore, the analysis was mainly performed based on serological matching, because information on HLA allele matching in unrelated transplantation was insufficient at that time. The importance of allele matching at the HLA-A, HLA-B, and HLA-DRB1 loci in unrelated donor transplantation has been established previously.[6-8] In addition, the importance of allele matching at the HLA-C locus has been highlighted in several recent studies of unrelated transplantation, although HLA-C matching is, in general,

Submitted August 8, 2011; accepted October 20, 2011. Prepublished online as *Blood* First Edition paper, October 31, 2011; DOI 10.1182/blood-2011-08-372573.

There is an Inside *Blood* commentary on this article in this issue.

The online version of this article contains a data supplement.

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still not considered in related transplantation.[9-12] Therefore, we conducted a nationwide retrospective study to compare the clinical outcomes of transplantation from a related donor with an HLA-1 Ag mismatch at the HLA-A, HLA-B, or HLA-DR loci in the GVH direction (RD/1AG-MM-GVH) with an HLA 8/8-allele (HLA-A, HLA-B, HLA-C, and HLA-DRB1)–MUD (8/8-MUD).

## Methods

### Data collection

Data for patients 16-70 years of age with acute myelogenous leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), or chronic myelogenous leukemia (CML) who received a first allogeneic transplantation from a related donor or HLA-6/6–Ag-MUD between January 1, 2001 and December 31, 2008 were obtained from the Transplant Registry Unified Management Program,[13] which includes data from the Japan Society for Hematopoietic Cell Transplantation and the Japan Marrow Donor Program. Our analysis included 344 patients who received a graft from an RD/1AG-MM-GVH donor and 453 patients who received a graft from an 8/8-MUD. The following patients were excluded: 11 patients who lacked data on survival status, survival date, sex of recipient and donor, stem cell source, GVHD prophylaxis, or performance status; 2 patients who received both BM and peripheral blood in related transplantation; and 5 patients who received stem cells manipulated by ex vivo T-cell depletion or CD34 selection. Finally, 327 patients who received a graft from an RD/1AG-MM-GVH donor and 452 patients who received a graft from an 8/8-MUD fulfilled the criteria. The data on 2318 patients who received transplantation from an MRD were also collected on the basis of similar inclusion and exclusion criteria to compare the OS rate. The study was approved by the data management committees of Transplant Registry Unified Management Program and by the institutional review board of Saitama Medical Center (Jichi Medical University, Saitama, Japan), where this study was organized.

### Histocompatibility

Histocompatibility data for serological and genomic typing for the HLA-A, HLA-B, HLA-C, and HLA-DR loci were obtained from reports obtained from the institution at which the transplantation was performed. To reflect current practice in Japan, HLA matching in RD/1AG-MM-GVH donors was assessed by serological data for HLA-A, HLA-B, and HLA-DR loci, whereas that in 8/8-MUD was assessed by genomic data for HLA-A, HLA-B, HLA-C, and HLA-DR loci. When the recipient's Ags or alleles were not shared by the donor, this was considered an HLA mismatch in the GVH direction; when the donor's Ags or alleles were not shared by the recipient, this was considered a mismatch in the HVG direction. SCT from a related donor with 1 Ag mismatch in the GVH direction has been performed by accepting multiple Ag mismatches in the HVG direction,[1,2] and therefore was included in this study.

### End points and statistical analyses

The primary end point of the study was to compare OS rates between the RD/1AG-MM-GVH and 8/8-MUD groups. For exploratory purposes, OS, treatment-related mortality (TRM), relapse, acute and chronic GVHD, and cumulative incidences of neutrophil engraftment were analyzed in a subset of cohorts. The physicians who performed transplantation at each center diagnosed and graded acute and chronic GVHD according to standard criteria.[14,15] The incidence of chronic GVHD was evaluated in patients who survived for at least 100 days. Neutrophil recovery was considered to have occurred when the absolute neutrophil count exceeded $0.5 \times 10^9$/L for 3 consecutive days after transplantation.

Descriptive statistics were used to summarize variables related to patient characteristics. Comparisons between groups were performed with the $\chi^2$ statistic or extended Fisher exact test as appropriate for categorical variables and the Mann-Whitney $U$ test or the Kruskal-Wallis test as appropriate for continuous variables. The probability of OS was estimated according to the Kaplan-Meier method, and the groups were compared with the log-rank test. The probabilities of TRM, relapse, acute and chronic GVHD, and neutrophil engraftment were estimated on the basis of cumulative incidence curves to accommodate the following competing events[16]: death for relapse, relapse for TRM, death without GVHD for acute and chronic GVHD, and death without engraftment for neutrophil engraftment; the groups were compared with a Gray test.[17] Cox proportional-hazards regression was used to evaluate variables that may affect OS, whereas the Fine and Gray proportional-hazard model was used to evaluate variables that may affect TRM, relapse, acute and chronic GVHD, and neutrophil engraftment.[18] For patients for whom conditioning intensity (myeloablative or reduced-intensity) was not reported, we reclassified the conditioning regimen as either myeloablative or reduced-intensity according to the National Marrow Donor Program/Center for International Blood and Marrow Transplant Research operational definitions.[19] To be consistent with our previous study, acute leukemia in the first or second remission, CML in the first or second chronic phase, and MDS without leukemic transformation were defined as standard-risk diseases, and others were defined as high-risk diseases.[1] The following variables were considered: the recipient's age group ($\leq 50$ years or $> 50$ years at transplantation), recipient's sex, presence of female (donor) to male (recipient) sex mismatch, performance status (0-1 or 2-4), disease (AML, ALL, CML, or MDS), disease status before transplantation (standard- or high-risk), type of conditioning regimen (myeloablative or reduced-intensity), type of GVHD prophylaxis (cyclosporine-based, tacrolimus-based, or other), use of antithymocyte globulin or alemtuzumab, and the time from diagnosis to transplantation ($< 6$ months or $\geq 6$ months). In addition, a variable of graft source (BM or peripheral blood) was also considered in the analysis specific to related donors. Factors with $P < .10$ in the univariate analysis were used in the first multivariate model without donor type and deleted in a stepwise manner from the model by backward selection. We added donor type to the final model. All tests were 2-sided, and $P < .05$ was considered to indicate statistical significance. All statistical analyses were performed with STATA Version 11 software (StataCorp) and R Version 2.12.0 software (The R Foundation for Statistical Computing).

## Results

### Patient characteristics

Compared with recipients of an 8/8-MUD, recipients of an RD/1AG-MM-GVH were more likely to be younger, to be male receiving a transplantation from a female donor, to have a shorter duration from diagnosis to transplantation, to have a high-risk disease, to receive cyclosporine for GVHD prophylaxis, to receive antithymocyte globulin or alemtuzumab, and to have a longer follow-up period (Table 1). Approximately half of the recipients in the RD/1AG-MM-GVH group received peripheral blood stem cells, whereas during this period in Japan, the source of transplantation from an MUD was restricted to BM. In the RD/1AG-MM-GVH group, the number of Ag mismatches in the HVG direction was 0 in 11%, 1 in 67%, 2 in 20%, and 3 in 2%. HLA-A, HLA-B, and HLA-DRB1 allelic information in both recipients and donors was available in 148 of 327 transplantations from an RD/1AG-MM-GVH donor and information on HLA-C Ag mismatch in either the GVH or HVG direction was available in 123 of 327.

### OS

The 2-year OS rates in the 8/8-MUD and RD/1AG-MM-GVH groups were 0.59 (95% confidence interval [CI], 0.53-0.64) and 0.44 (95% CI, 0.38-0.49), respectively (log-rank test; $P < .001$; Figure 1A). Multivariate analysis revealed that, compared with the use of an 8/8-MUD, the use of an RD/1AG-MM-GVH was a significant adverse factor for OS (hazard ratio [HR], 1.49; 95% CI,

**Table 1. Patient characteristics**

| Variable | RD/1AG-MM-GVH (n = 327) | 8/8 MUD (n = 452) | P |
|---|---|---|---|
| Median age at transplantation, y (range) | 45 (16-69) | 48 (16-68) | .043 |
| **Recipient sex, n (%)** | | | |
| Male | 184 (56%) | 267 (59%) | .434 |
| Female | 143 (44%) | 185 (41%) | |
| **Sex combination of donors and recipients, n (%)** | | | |
| Female to male | 91 (28%) | 73 (16%) | < .001 |
| Other combinations | 236 (72%) | 379 (84%) | |
| **Performance status, n (%)** | | | |
| 0/1 | 298 (91%) | 415 (92%) | .736 |
| 2/3/4 | 29 (9%) | 37 (8%) | |
| **Disease, n (%)** | | | |
| AML | 167 (51%) | 249 (55%) | .512 |
| ALL | 90 (28%) | 107 (24%) | |
| CML | 19 (6%) | 21 (5%) | |
| MDS | 51 (16%) | 75 (17%) | |
| **Duration from diagnosis to transplantation, n (%)** | | | |
| < 6 mo | 124 (38%) | 102 (23%) | < .001 |
| ≥ 6 mo | 191 (58%) | 350 (77%) | |
| Unknown | 12 (4%) | 0 (0%) | |
| **Disease risk, n (%)** | | | |
| Standard | 175 (54%) | 317 (70%) | < .001 |
| High | 133 (41%) | 129 (29%) | |
| Unknown | 19 (6%) | 6 (1%) | |
| **Source of stem cells, n (%)** | | | |
| BM | 142 (43%) | 452 (100%) | < .001 |
| Peripheral blood | 185 (57%) | | |
| **HLA compatibility in the HVG direction, n (%)*** | | | |
| Matched | 36 (11%) | 452 (100%) | < .001 |
| 1-antigen mismatch | 218 (67%) | | |
| 2-antigen mismatch | 65 (20%) | | |
| 3-antigen mismatch | 8 (2%) | | |
| **HLA compatibility in the GVH direction, n (%)*** | | | |
| Matched | 0 (0%) | 452 (100%) | < .001 |
| 1-allele mismatch | 111 (34%) | | |
| 2-allele mismatch | 36 (11%) | | |
| 3-allele mismatch | 1 (0%) | | |
| Uncertain/missing | 179 (55%) | | |
| **Conditioning regimen, n (%)** | | | |
| Myeloablative | 243 (74%) | 338 (75%) | .883 |
| Reduced-intensity | 84 (26%) | 114 (25%) | |
| **GVHD prophylaxis, n (%)** | | | |
| Cyclosporine-based | 113 (35%) | 108 (24%) | 0.004 |
| Tacrolimus-based | 209 (64%) | 338 (75%) | |
| Others | 5 (2%) | 6 (1%) | |
| **Use of ATG/alemtuzumab, n (%)** | | | |
| Yes | 33 (10%) | 13 (3%) | < .001 |
| No | 294 (90%) | 439 (97%) | |
| Median follow-up of survivors, mo (range) | 36.2 (3.0-95.7) | 13.5 (1.7-62.8) | < .001 |

*HLA compatibility was defined according to the HLA-A, HLA-B, and HLA-DR loci.

1.19-1.86; $P < .001$; Table 2). Age > 50 years, performance status ≥ 2, and high-risk disease were also found to be significant adverse factors, whereas other variables, such as the time from diagnosis to transplantation, were not.

Because our previous study showed that the impact of an HLA-1 Ag mismatch in a related transplantation on OS differed according to whether patients had standard-risk or high-risk diseases,[1] the survival rates were compared separately in each disease-risk group. The OS rates of patients with standard-risk diseases in the 8/8-MUD group were significantly higher than those in the RD/1AG-MM-GVH group ($P = .003$), whereas there was no significant difference in high-risk patients ($P = .090$; Figure 1B-C). Although the interaction between the donor type and disease risk did not reach statistical significance ($P = .140$), multivariate analyses in each disease-risk group showed that the adverse impact of the use of an RD/1AG-MM-GVH donor was significant in standard-risk patients (HR, 1.72; 95% CI, 1.24-2.39; $P = .001$), but not in high-risk patients (Table 2).

To visually compare MRDs and other stem-cell sources, the OS rate for MRDs was layered on those for MUDs and RD/1AG-MM-GVHs (Figure 1). The OS curve of transplantation from an MRD

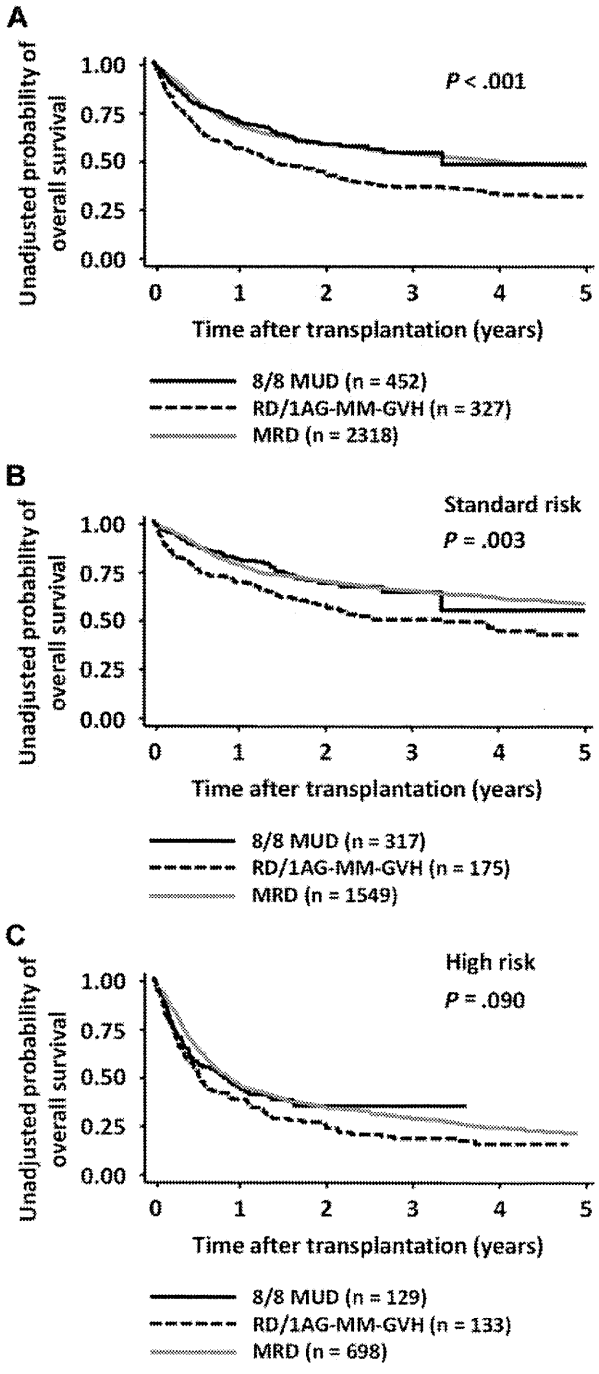


**Figure 1. OS according to donor type and risk of disease.** OS after transplantation from an RD/1AG-MM-GVH donor, an 8/8-MUD, and HLA-MRD in patients with both-risk (A), standard-risk (B), or high-risk diseases (C). Survival rates in the 8/8-MUD and RD/1AG-MM-GVH groups were compared with the log-rank test.

**Table 2. Multivariate analysis of OS**

| | Total (n = 779) | | Standard-risk (n = 492) | | High-risk (n = 262) | |
|---|---|---|---|---|---|---|
| **Variable** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** |
| **Donor type** | | | | | | |
| 8/8 MUD | 1.00 | | 1.00 | | 1.00 | |
| RD/1AG-MM-GVH | 1.49 (1.19-1.86) | < .001 | 1.72 (1.24-2.39) | .001 | 1.30 (0.96-1.76) | .095 |
| **Age, y** | | | | | | |
| ≤ 50 | 1.00 | | 1.00 | | | |
| > 50 | 1.44 (1.16-1.79) | .001 | 1.55 (1.13-2.15) | .007 | | |
| **Performance status** | | | | | | |
| 0/1 | 1.00 | | | | 1.00 | |
| 2/3/4 | 1.79 (1.30-2.48) | < .001 | | | 1.76 (1.24-2.52) | .002 |
| **Disease risk** | | | | | | |
| Standard | 1.00 | | | | | |
| High | 2.41 (1.92-3.03) | < .001 | | | | |
| Unknown | 1.38 (0.82-2.33) | .227 | | | | |

Only variables that remained after backward selection in the multivariate analysis are shown.

was superimposed on that from an MUD in both standard- and high-risk patients (MRD vs MUD: standard-risk group, $P = .895$, and high-risk group, $P = .581$). Multivariate analysis confirmed that OS in the MRD group was comparable to the MUD group (MRD vs MUD: standard-risk group, HR, 1.02; 95% CI, 0.79-1.32; $P = .878$; high-risk group, HR, 0.98; 95% CI, 0.76-1.26; $P = .865$).

### Effect of HLA mismatches on OS

To identify factors that may contribute to the inferior OS in standard-risk patients in the RD/1AG-MM-GVH group compared with those in the 8/8-MUD group, we evaluated the impact of each HLA-A, HLA-B, or HLA-DR Ag mismatch in the GVH direction and the number of Ag mismatches in the HVG direction on OS rates in the RD/1AG-MM-GVH group.

In the RD/1AG-MM-GVH group, the OS rate for patients who received a transplantation from a related donor with an HLA-B Ag mismatch in the GVH direction and that from a donor with 2 or 3 Ag mismatches in the HVG direction were significantly lower than those in other groups (log-rank test for HLA-A Ag mismatch vs HLA-B Ag mismatch vs HLA-DR Ag mismatch in the GVH direction, $P < .001$, and 0-1 mismatches vs 2-3 mismatches in the HVG direction, $P = .003$; Figure 2). However, multivariate analysis revealed that only the presence of an HLA-B Ag mismatch in the GVH direction (HR, 1.57; 95% CI, 1.13-2.18; $P = .007$) was significantly associated with a lower OS (Table 3).

The OS rates were also compared separately in the standard-risk and high-risk disease groups (Figure 2). Although the interaction between the presence of an HLA-B Ag mismatch and disease risk did not reach statistical difference ($P = .232$), the adverse impact of an HLA-B Ag mismatch in the GVH direction was observed in the standard-risk group (HR, 1.86 95% CI, 1.14-3.01; $P = .012$), but not in the high-risk group (Table 3). Conversely, the survival curve for the HLA-A Ag or HLA-DR Ag-mismatched group was almost superimposed on that for 8/8-MUDs (Figure 2; standard-risk group: for the HLA-A Ag-mismatched group vs the 8/8-MUD group, HR, 1.26; 95% CI, 0.73-2.19; $P = .411$; for the HLA-DR Ag-mismatched group vs the 8/8-MUD group, HR, 1.37; 95% CI, 0.89-2.11; $P = .154$; high-risk group: for the HLA-A Ag-mismatched group vs the 8/8-MUD group, HR, 1.26; 95% CI, 0.80-2.00; $P = .320$; and for the HLA-DR Ag-mismatched group vs the 8/8-MUD group, HR, 1.03; 95% CI, 0.67-1.59]; $P = .880$). The impact of 2 or 3 Ag mismatches in the HVG direction was not significant in either the standard-risk or high-risk group (Table 3).

### Effect of an HLA-B mismatch on TRM, relapse, GVHD, and neutrophil engraftment in patients with standard-risk diseases

Our findings showed that an HLA-B Ag mismatch in the GVH direction strongly contributed to the low survival rate in standard-risk patients, which can explain the inferior survival rates in the RD/1AG-MM-GVH group compared with the 8/8-MUD group. Therefore, we evaluated the impact of an HLA-B Ag mismatch in the GVH direction on other outcomes in patients with standard-risk diseases in the RD/1AG-MM-GVH group.

First, we compared the characteristics of patients with standard-risk diseases who received transplantation from a related donor with an HLA-A, HLA-B, and HLA-DR Ag mismatch in the GVH direction (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Two or 3 Ag mismatches in the HVG direction were observed more frequently in the HLA-B Ag-mismatched group (28%) than in the HLA-A Ag-mismatched group (2%) or the HLA-DR Ag-mismatched group (17%). Although there was no information available on allelic mismatch or HLA-C Ag mismatch in more than half of the patients, an HLA-C Ag mismatch in either the GVH or HVG direction was observed more frequently in the HLA-B Ag-mismatched group (61% among the available data) than in the HLA-A Ag-mismatched group (25%) or the HLA-DR Ag-mismatched group (17%).

The incidence of TRM was higher in the HLA-B Ag-mismatched group (3-year mortality rate: HR, 0.47; 95% CI, 0.32-0.60) than in the HLA-A Ag-mismatched group (HR, 0.28; 95% CI, 0.14-0.44) or the HLA-DR Ag-mismatched group (HR, 0.27; 95% CI, 0.17-0.38; Figure 3A; log-rank test, $P = .030$). The presence of an HLA-B Ag mismatch in the GVH direction was an independent significant adverse factor that affected TRM in the RD/1AG-MM-GVH group (Table 4). Conversely, the incidence of relapse did not significantly differ among the 3 groups (Figure 3B and Table 4).

The incidence of grade 2-4 acute GVHD in the HLA-B Ag-mismatched group was higher than that in the HLA-A Ag-mismatched group, but comparable to that in the HLA-DR Ag-mismatched group (supplemental Figure 1 and supplemental Table 2). There was no significant difference in the incidence of grade 3-4 acute GVHD among the 3 groups. Regarding neutrophil engraftment, multivariate analysis showed that an HLA-B Ag mismatch was significantly associated with inferior neutrophil engraftment and 2 or 3 Ag mismatches in the HVG direction were

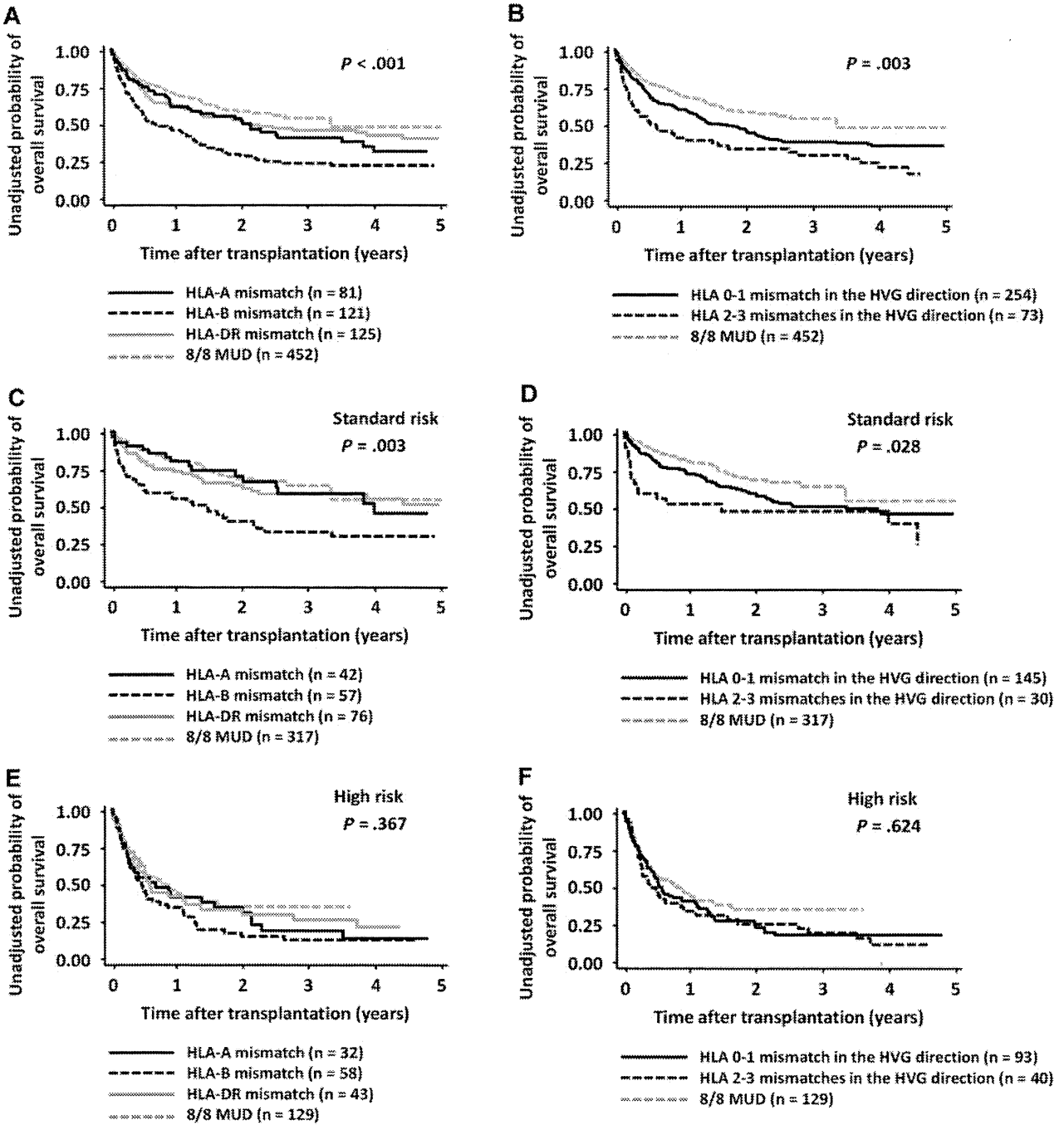


**Figure 2. OS in patients with both-risk, standard-risk, or high-risk diseases according to the locus of HLA mismatch in the GVH direction and the number of mismatches in the HVG direction.** Survival rates in patients with HLA-A, HLA-B, and HLA-DR Ag mismatches in the GVH direction were compared with the log-rank test (A,C,E). Survival rates in patients with 0-1 and 2-3 mismatches in the HVG direction were compared with the log-rank test (B,D,F). Survival rates of the 8/8-MUD group are shown for visual comparison.

associated with inferior neutrophil engraftment, with marginal significance (supplemental Table 2).

## Discussion

In this nationwide retrospective study, we found that the survival rate of the RD/1AG-MM-GVH group was significantly inferior to that of the 8/8-MUD group, and this significant difference was observed only in patients with standard-risk diseases, although the interaction between donor type and disease risk did not reach statistical significance. We reported previously that transplantation from a related donor with 1 Ag mismatch in the GVH or HVG direction gave a clinical outcome comparable to that of transplantation from a 6/6-Ag-MUD in patients with either standard-risk or high-risk diseases.[1] However, because HLA matching at the allelic level in unrelated transplantation significantly reduces the risk of GVHD, in the present study, the survival curve of transplantation from an 8/8-MUD was substantially improved, and could be superimposed on a curve corresponding to that from an MRD.

**Table 3. Multivariate analysis of OS in patients receiving transplantation from a related donor with a 1-antigen mismatch in the GVH direction**

| | Total (n = 327) | | Standard-risk (n = 175) | | High-risk (n = 133) | |
|---|---|---|---|---|---|---|
| **Variable** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** |
| **HLA mismatch in the GVH direction** | | | | | | |
| HLA-DR mismatch | 1.00 | | 1.00 | | 1.00 | |
| HLA-A mismatch | 1.07 (0.73-1.56) | .737 | 0.98 (0.54-1.81) | .966 | 1.11 (0.65-1.89) | .701 |
| HLA-B mismatch | 1.57 (1.13-2.18) | .007 | 1.86 (1.14-3.01) | .012 | 1.36 (0.86-2.17) | .193 |
| **HLA mismatch in the HVG direction** | | | | | | |
| 0-1 mismatches | 1.00 | | 1.00 | | 1.00 | |
| 2-3 mismatches | 1.27 (0.91-1.76) | .154 | 1.67 (0.98-2.85) | .061 | 1.06 (0.69-1.61) | .799 |
| **Age, y** | | | | | | |
| ≤ 50 | 1.00 | | 1.00 | | | |
| > 50 | 1.52 (1.14-2.03) | .004 | 1.87 (1.21-2.91) | .005 | | |
| **Disease risk** | | | | | | |
| Standard | 1.00 | | | | | |
| High | 2.06 (1.53-2.78) | < .001 | | | | |
| Unknown | 1.00 (0.53-1.89) | .989 | | | | |

Only variables that remained after backward selection in the multivariate analysis are shown.

Consistent with our findings, several studies have shown that the clinical outcomes of transplantation from an 8/8-10/10 MUD are comparable to those from an MRD.[20,21] The significant difference in survival rates between transplantation from an RD/1AG-MM-GVH donor and an 8/8-MUD disappeared in patients with high-risk diseases, probably because the adverse impact of acute GVHD on survival might be offset by the potential GVL effect in transplantation from an RD/1AG-MM-GVH donor.[1,2,22]

We evaluated factors that may contribute to the inferior OS in patients with standard-risk diseases in the RD/1AG-MM-GVH group and found that, compared with the presence of an HLA-DR Ag mismatch, the presence of an HLA-B Ag mismatch in the GVH direction was significantly associated with lower OS and higher TRM. Conversely, the rates of OS and TRM in the HLA-A Ag- or HLA-DR Ag-mismatched group were superimposed on those in the MUD group. However, HLA-A, HLA-B, and HLA-DR Ag mismatches had similar effects on the incidence of severe acute GVHD; consequently, the causal relationship between an HLA-B Ag mismatch in the GVH direction and higher TRM remains unknown. In contrast to our findings, Valcarcel et al reported that there was no significant difference in OS between the use of 1-Ag–mismatched related donors (n = 89) and 8/8-MUDs (n = 700) in transplantation for AML and ALL during the first or second complete remission.[23] This difference from our results can be partly

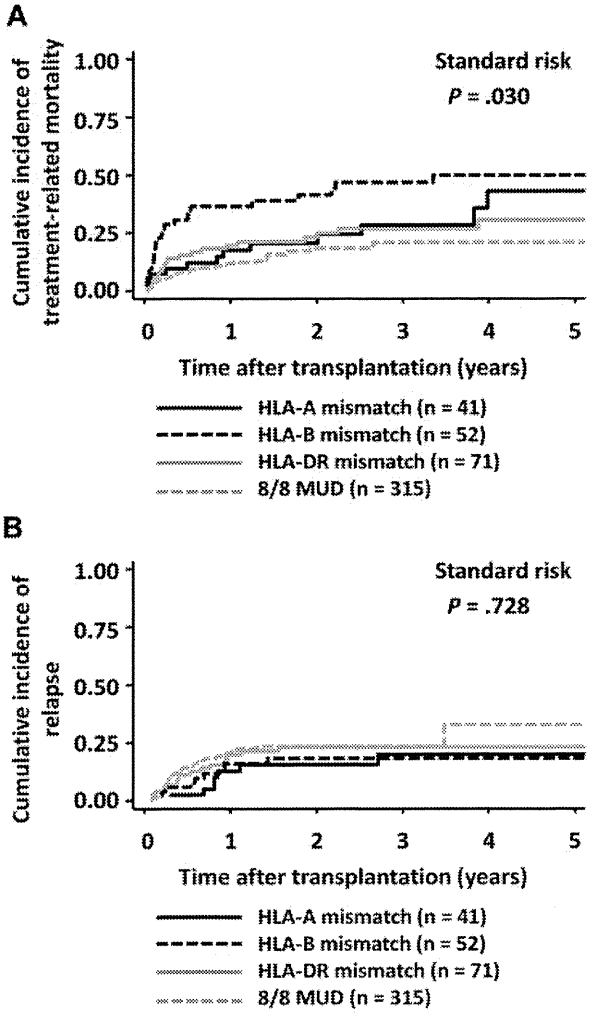


**Figure 3. Cumulative incidence according to the locus of HLA mismatch in the GVH direction in patients with standard-risk diseases.** Cumulative incidences in the related transplantation groups were compared with the Gray test. (A) TRM. (B) Relapse.

**Table 4. Multivariate analysis of TRM and relapse in patients with standard-risk diseases receiving transplantations from a related donor with a 1-antigen mismatch in the GVH direction**

| | TRM (n = 164) | | Relapse (n = 164) | |
|---|---|---|---|---|
| **Variable** | **HR (95% CI)** | ***P*** | **HR (95% CI)** | ***P*** |
| **HLA mismatch in the GVH direction** | | | | |
| HLA-DR mismatch | 1.00 | | 1.00 | |
| HLA-A mismatch | 1.22 (0.59-2.52) | .587 | 0.70 (0.29-1.67) | .418 |
| HLA-B mismatch | 2.00 (1.09-3.65) | .025 | 0.80 (0.34-1.87) | .605 |
| **HLA mismatch in the HVG direction** | | | | |
| 0-1 mismatches | 1.00 | | 1.00 | |
| 2-3 mismatches | 2.21 (1.14-4.28) | .019 | 0.67 (0.23-1.98) | .467 |
| **Age, y** | | | | |
| ≤ 50 | 1.00 | | | |
| > 50 | 2.08 (1.18-3.65) | .011 | | |
| **Duration from diagnosis to transplantation** | | | | |
| < 6 mo | 1.00 | | | |
| ≥ 6 mo | 2.40 (1.19-4.82) | .014 | | |
| Unknown | 2.23 (0.77-6.48) | .140 | | |

Only variables that remained after backward selection in the multivariate analysis are shown.

explained by the fact that the MUD group in their study included a significantly smaller number of ALL patients with low-risk cytogenetics. In addition, in our study, genetic homogeneity in the Japanese population might affect the lower incidence of severe acute GVHD in MUD transplantation because of the less frequent mismatches in minor histocompatibility Ags.[24,25]

The frequency of an HLA-C Ag mismatch was substantially higher in the HLA-B Ag-mismatched group than in the HLA-A or HLA-DR Ag-mismatched groups. This finding may represent linkage disequilibrium between the HLA-B and HLA-C genes, which are located at a very close physical proximity within the major histocompatibility complex.[26,27] Therefore, the impact of HLA-B-Ag might be affected by the co-presence of HLA-C Ag mismatch. We could not evaluate the impact of HLA-C Ag mismatch on OS rates because of the limited information on HLA-C Ag mismatch; therefore, an analysis with larger cohorts with complete HLA-C Ag information will be needed to evaluate the impact of HLA-C and/or HLA-B mismatch in transplantation from an RD/1AG-MM-GVH donor. Accordingly, we could not evaluate the impact of the KIR ligand mismatch. Although the impact of KIR ligand mismatch is still controversial, several studies analyzing T cell–replete transplantation showed that KIR ligand mismatch is associated with lower OS.[12,28,29] The analysis of KIR matching would be helpful in elucidating the mechanism underlying the adverse effect of HLA-B mismatch in T cell–replete transplantation from an RD/1AG-MM-GVH donor.

Whether the presence of allelic mismatches in addition to the 1-Ag mismatch (2 or more allelic mismatches in total) affects transplantation outcome is also an important clinical question in transplantation from an RD/1AG-MM-GVH donor. A high frequency of 2-allele mismatches in the GVH direction was seen in the HLA-B Ag-mismatched group, suggesting a possible association between 2-allele mismatches and low OS. However, we did not observe a significant effect of the number of allelic mismatches on OS after transplantation from an RD/1AG-MM-GVH donor, possibly because of the small sample size.

Our study has several limitations. First, because several months are required to arrange unrelated transplantations, patients at low risk for relapse may more often be selected for these procedures. To minimize this bias, we included the duration from diagnosis to transplantation in the multivariate analysis; however, this variable did not have a significant effect in the multivariate analysis. Second, heterogeneous backgrounds may have resulted in a bias. In particular, the stem-cell source in unrelated transplantation was exclusively BM. However, the analysis of OS in the subgroup of patients who received a BM graft from an RD/1AG-MM-GVH donor or an 8/8-MUD showed similar results. Third, because we have incomplete Ag and allele information on the HLA-C and -DQB1 loci, we may have underestimated the degree of mismatching in transplantation from an RD/1AG-MM-GVH donor. Fourth, the difference in the impact of donor type between standard- and high-risk diseases should be cautiously interpreted, because the interaction between the donor type and disease risk did not reach statistical significance. This may be partly because of the lower statistical power to detect the interaction than the main effect.

In conclusion, our findings suggest that an 8/8-MUD, if available, should be prioritized over an RD/1AG-MM-GVH donor for patients without an MRD if an immediate transplantation is not necessary. In particular, the presence of an HLA-B Ag mismatch in the GVH direction has an adverse effect on OS because of treatment-related complications. This may be because of the high frequencies of additional mismatches of HLA-C Ag or allele in the HLA-B Ag-mismatched group. To elucidate the mechanism of the adverse outcomes in RD/1AG-MM-GVH donors with an HLA-B Ag mismatch, HLA Ag/allele matching including HLA-C should be performed in transplantations from an RD/1AG-MM-GVH donor.

## Acknowledgments

The authors are indebted to all of the physicians and data managers at the centers who contributed valuable data on transplantation to the Japan Society for Hematopoietic Cell Transplantation and the Japan Marrow Donor Program. They also thank all of the members of the data management committees of the Japan Society for Hematopoietic Cell Transplantation and the Japan Marrow Donor Program for their management of data.

J.K. is a Research Fellow of the Japan Society for the Promotion of Science. This work was supported in part by a grant-in-aid for Japan Society for the Promotion of Science (to J.K.).

## Authorship

Contribution: Y.K. designed the research and organized the project; J.K., H. Saji, and Y.K. reviewed and analyzed the data and wrote the manuscript; J.K. and Y.K. performed the statistical analysis; H. Sakamaki, J.T., R.S., and Y.A. collected data from Japan Society for Hematopoietic Cell Transplantation; K.K. and Y.M. collected data from Japan Marrow Donor Program; and all authors interpreted the data and reviewed and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Yoshinobu Kanda, MD, Division of Hematology, Saitama Medical Center, Jichi Medical University, 1-847 Amanuma Town, Omiya Ward, Saitama City, Saitama, Japan 330-8503; e-mail: ycanda-tky@umin.ac.jp.

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Leukemia Research 35 (2011) 1205–1211

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

# Predictability of the response to tyrosine kinase inhibitors via *in vitro* analysis of Bcr-Abl phosphorylation

Masaru Shibata[a], Sachiko Ezoe[a,*], Kenji Oritani[a], Keiko Matsui[a], Masahiro Tokunaga[a], Natsuko Fujita[a], Yuri Saito[a], Takayuki Takahashi[b], Masayuki Hino[c], Itaru Matsumura[d], Yuzuru Kanakura[a]

[a] *Hematology and Oncology, Osaka University, Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[b] *Kobe City Medical Center General Hospital, Kobe, Japan*
[c] *Department of Hematology, Graduate School of Medicine, Osaka City University, Osaka, Japan*
[d] *Department of Internal Medicine, Kinki University School of Medicine, Sayama, Japan*

ARTICLE INFO

*Article history:*
Received 16 December 2010
Received in revised form 12 January 2011
Accepted 14 January 2011
Available online 26 March 2011

*Keywords:*
CML
Tyrosine kinase inhibitor
Bcr-Abl
Crkl
Phosphorylation
Immunoblot

ABSTRACT

It would be of great value to predict the efficacy of tyrosine kinase inhibitors (TKIs) in the treatment of individual CML patients. We propose an immunoblot system for detecting the phosphorylation of Crkl, a major target of Bcr-Abl, in blood samples after *in vitro* incubation with TKIs. When the remaining phosphorylated Crkl after treatment with imatinib was evaluated as the "residual index (RI)", high values were found in accordance with imatinib resistance. Moreover, RI reflected the outcome of imatinib- as well as second generation TKIs with a high sensitivity and specificity. Therefore, this system should be useful in the selection of TKIs.



## 1. Introduction

The introduction of tyrosine kinase inhibitors (TKIs) targeting Bcr-Abl have dramatically improved the treatment of CML. Imatinib mesylate (Gleevec; Novartis Pharmaceuticals, East Hanover, NJ) was shown to induce high rates of cytogenetic and molecular responses, resulting in greatly prolonged survival in CML patients [1,2]. However, despite the remarkable improvement in survival and responsiveness with imatinib-treatment, a considerable proportion of the patients treated with imatinib have been reported to exhibit either primary or secondary resistance or intolerance [3–5]. Clinical resistance to imatinib can result from mutations in the Abl kinase domain at residues that directly contact imatinib or that influence imatinib binding [6]. As resistance can also arise in the absence of Bcr-Abl mutations, other mechanisms of resistance and disease progression may exist, including Bcr-Abl-independent signaling in CML cells [7]. To overcome the resistance and intolerance to imatinib, efforts have been made to develop second- and third-generation TKIs. Examples of such inhibitors include nilotinib (Tasigna, Novartis) [8], dasatinib (Sprycel, Bristol-Myers Squibb) [9] and other TKIs under clinical investigation such as bosutinib [10] and INNO-406 [11]. These TKIs are significantly more potent than imatinib and have exhibited efficacy against many types of imatinib-resistant Bcr-Abl mutants. Furthermore, they are also candidates for first-line therapy, as there is a need to improve the results achieved with imatinib [12–14]. In parallel with the entrance of new therapeutic compounds, an important question is which TKI is the most appropriate to each CML patient.

To establish a system with which we can predict the response of each patient to TKIs, we investigated in this study the phosphorylation of Crkl, a major target of Bcr-Abl, after *in vitro* incubation with or without TKIs in peripheral blood (PB) samples from patients either newly diagnosed or resistant to imatinib. It is demonstrated that this *in vitro* analysis system is highly reflective of the clinical response to TKIs of CML patients, and these data should prove useful in selecting TKIs in individual cases.

## 2. Patients, materials and methods

### 2.1. Patient blood samples

Thirty-one patients with CML in the chronic phase (CP) were included in this study (Table 1). The optimal response, response and resistance were defined in accordance with the European Leukemia Net (ELN) recommendations [15,16]. Briefly, an "optimal response" to imatinib means achieving a complete hematological response (CHR) at 3 months or complete cytogenetic response (CCyR) at

* Corresponding author. Tel.: +81 6 6879 3871; fax: +81 6 6879 3879.
*E-mail address:* sezoe@bldon.med.osaka-u.ac.jp (S. Ezoe).

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doi:10.1016/j.leukres.2011.01.012

6 months after the induction of imatinib, and resistance means failure to achieve such a response. On the other hand, in nilotinib- or dasatinib-treated patients, a "response" means a minor cytogenetic response (mCyR) at 3 months or partial cytogenetic response (PCyR) at 6 months after the induction of the second generation TKI, and resistance means failure to achieve this response.

Ten microliters of the PB samples were obtained from patients with informed consent at the beginning or before the initiation of imatinib, nilotinib or dasatinib. Half of each sample was used for examination of the Bcr-Abl sequence, which was performed by the SRL Co. (Tokyo, Japan), and the other half was used for immunoblot analysis.

Approvals for the study were obtained from the institutional review boards of all the participating facilities.

### 2.2. Reagents

Imatinib, methanesulfonate salt was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland), and nilotinib and dasatinib were purchased from LC laboratories (Boston, MA). The antibodies used in this study were as follows: anti-Lyn, anti-phospho-Crkl, anti-phospho-c-Abl from Cell Signaling Technology (Beverly, MA), anti-phospho-Lyn(Y396) from Epitomics (Burlingame, CA), anti-Crkl, anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA), and the secondary antibodies, anti-Rabbit IgG HRP and anti-Goat IgG HRP were from Promega (Madison, WI). Pervanadate was purchased from Sigma–Aldrich (St. Louis, MO).

### 2.3. Cell line

A Bcr-Abl positive human cell line, K562, was used in the preliminary experiments in this study. K562 cells were maintained in RPMI1640 (nacalai tesque, Kyoto, Japan) supplemented with 10% fetus bovine serum (FBS) (EQUITECH-BIO, Kerrville, TX).

### 2.4. Immunoblot assays of patients' samples

Whole blood cell samples from patients were used within 3 h after blood had been drawn. Red cells were lysed with Whole Blood Lysing Reagents (Beckman Coulter, Brea, CA), and white blood cells were cultured with or without imatinib, nilotinib or dasatinib. After 5-h incubation, the cell lysates were collected and subjected to immunoblot assays. Gel electrophoresis and immunoblot assays were performed according to methods described previously [17,18]. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (PerkinElmer Life Sciences, Boston, MA).

### 2.5. Evaluation of phosphorylation intensity and determination of the "residual index (RI)"

The intensity of each blot of immunoreactive protein was quantified using ChemiDoc XRS+ with Image Lab Software (Bio Rad, Tokyo Japan). The RI values of each patient to TKIs were determined in accordance with the numerical expression, as indicated in Fig. 2A.

### 2.6. Statistical analysis

Analysis of variance was used to assess data reproducibility. The Mann–Whitney rank sum was used to define differences between groups.

## 3. Results

### 3.1. Immunoblot analysis of phosphorylated Crkl in CML patients

To assess the drug response of the CML patients, we performed immunoblot assays detecting phosphorylated Crkl, a direct target of Bcr-Abl kinase. To establish the experimental procedures, preliminary experiments were performed with K562, a CML blast crisis cell line, or blood sample from a newly diagnosed CML patient (Patient A), 98% of whose PB cells were Bcr-Abl-positive on fluorescence *in situ* hybridization (FISH). First, to determine the optimum incubation period for the TKIs, PB cells were incubated with or without TKIs for varying time periods. A two-hour incubation was not sufficient because imatinib did not completely suppress the phosphorylation of Crkl, while 24-h incubation was too long because the PB neutrophils appeared to die (Fig. 1A, left panel). A five-hour incubation completely eliminated the phosphorylation of Crkl without cell death. On the other hand, simultaneous treatment with a phosphatase inhibitor sustained the phosphorylation of Crkl even after treatment for 24 h (Fig. 1A, right panel). Thus, we decided to incubate cells for 5 h without phosphatase inhibitors. Next, to build an *in vitro* simulation model for the estimation of the activities of TKIs in the body, we fixed the concentrations of TKIs at the peak value of plasma concentrations in patients (Cmax) after administration of the recommended dose of TKIs. The Cmax of imatinib in CML patients after taking orally 400 mg of the drug is 3.0–4.8 μM, and that of nilotinib after taking 400 mg is 2.9–4.0 μM. In the case of

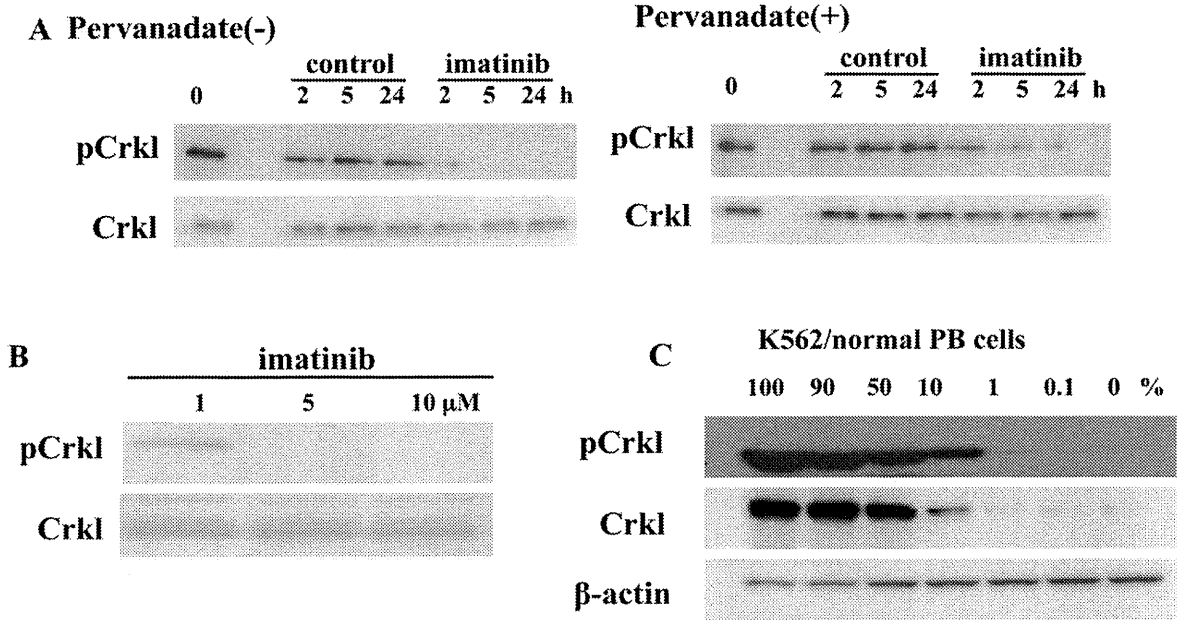


**Fig. 1.** Optimization of western blot after TKI-incubation. (A and B) Blood sample from Patient A was incubated with or without 5 μM imatinib supplemented with (right panel) or without (left panel) 10 μM of pervanadate for the indicated periods (A) or incubated with imatinib at the indicated concentrations for 5 h (B). The treated cells were lysed and subjective to immunoblot analysis using the indicated antibodies. (C) K562 cells were mixed into normal human PB cells at the indicated ratios. Then the samples were subjective to immunoblot analysis.

dasatinib, the Cmax after the ingestion of 100 mg dasatinib was 100nM [19–21]. In terms of pharmacokinetics, we fixed the concentrations of these TKIs (imatinib, nilotinib and dasatinib) at 5 μM, 5 μM, and 0.1 μM, respectively. As shown in Fig. 1B, 1 μM of imatinib did not eliminate the phosphorylation of Crkl in the examined sample of patient A who are newly diagnosed and well responded to imatinib, but 5 μM and 10 μM of imatinib did, indicating that 1 μM is too low concentration for estimation of clinical outcome. Finally, to estimate the sensitivity of this system, K562 cells were mixed with normal PB cells at variable ratios, as indicated. Fig. 1C shows that the phosphorylated Crkl at the lowest 1% was detectable in K562 cells. Thus, we analyzed patients having more than 10% Bcr-Abl-positive cells in PB by FISH.

### 3.2. Iimmunoblot analysis

To quantify the *in vitro* responsiveness to TKIs, we measured the density of each blot using a densitometric method. We then defined "residual index (RI)" for each TKI by the numerical expression as shown in Fig. 2A. Triplicate measurements were performed on 3 individual patients (Patient B, C and D). There were no significant variations among the RIs in each patient. Standard error for each sample set was less than 5% (4.6%, 1.2% and 3.4%, respectively) (Fig. 2B).

### 3.3. Responses to the TKIs in patients with various stages of CML

Fig. 3A represents typical results of the immunoblot analyses in 2 patients with newly diagnosed CML (Patient 1 and 2), and 2 patients who were receiving imatinib but were displaying resistance (Patient 16 and 17). Although all of these samples exhibited apparent phosphorylation of Crkl without TKIs, the phosphorylated Crkl disappeared from the samples of Patients 1 and 2 when incubated with imatinib, nilotinib or dasatinib. In the case of Patients 16 and 17, on the other hand, weak bands remained in the imatinib and/or nilotinib-incubated samples, but disappeared in the dasatinib-treated ones. Thus, this immunoblot analysis appeared to be useful in evaluating Crkl phosphorylation after *in vitro* TKI-incubation. All patients were divided into two groups: one being newly diagnosed and another receiving imatinib-therapy but showing resistance. The imatinib-RIs of the samples from the imatinib-resistant group (median RI: 34.2%) were much higher than those of the samples from newly diagnosed patients (median RI: 4.2%) (Fig. 3B).

### 3.4. Sequential examinations using the residual index

RI values were analyzed sequentially in the course of the different TKI-treatments in 2 imatinib-resistant patients (Patient 23 and 27).

Patient 23 (Fig. 4A): after six months of treatment with imatinib, the drug was changed to dasatinib because of a failure to achieve an optimal response (72% $Ph1^+$ in FISH). Six months after the start of dasatinib, $Ph1^+$ cells were disappeared. The samples were obtained twice: prior to the treatment with imatinib, and at the time of change to dasatinib. Immunoblot analysis showed that neither imatinib nor nilotinib eliminated the phosphorylation of Crkl at the initiation of treatment, but dasatinib did. Furthermore the RI values were under 10% only in the sample incubated with dasatinib.

Patient 27 (Fig. 4B): when the first sample was obtained, the percentage of $Ph1^+$ cells was 93% after 7-year treatment with imatinib.

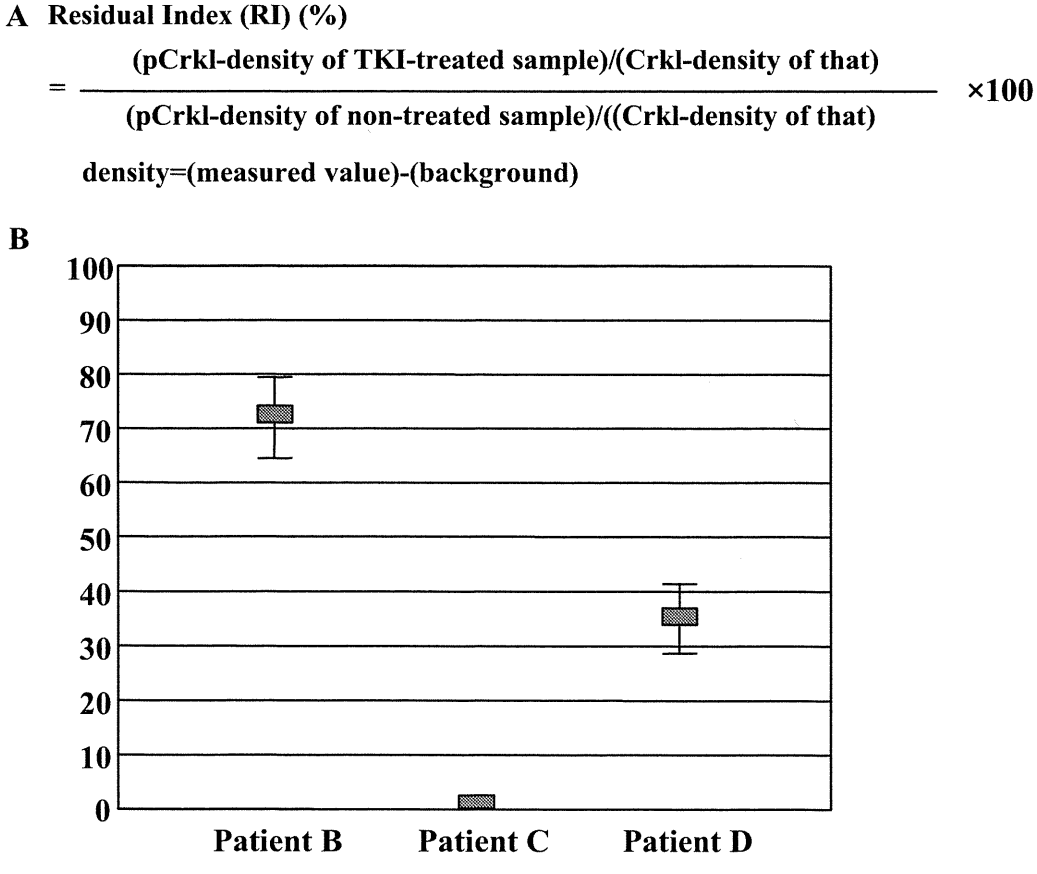


**Fig. 2.** "Residual index (RI)". (A) The numerical expression of RI. "Measured value" means the density of each blot measured by densitometric method. (B) The reproducibility of RIs for imatinib treatment. Means and standard errors, representing triplicate assays in 3 patients, are shown.

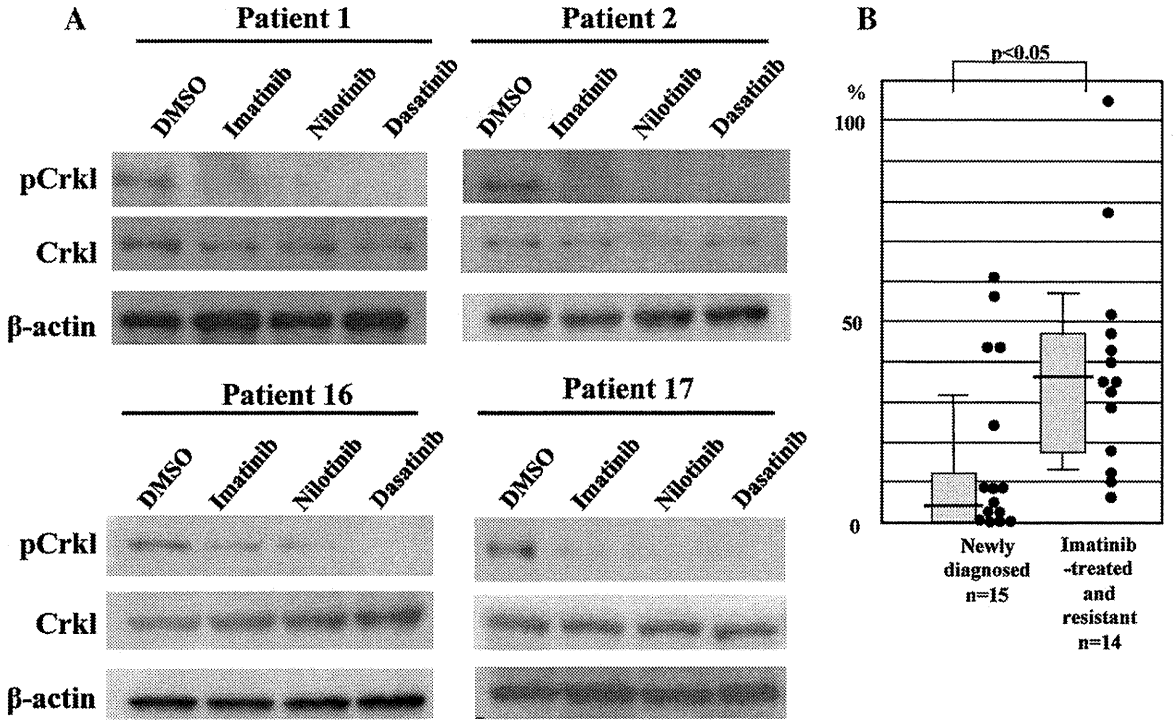


**Fig. 3.** Different RI values against imatinib between patients at diagnosis and patients showing imatinib-resistance. (A) Four typical data of immunoblots were represented. PB cells from newly diagnosed patients (Patient 1 and 2) or patients (Patient 16 and 17) who had been receiving imatinib-therapy but showed its resistance were incubated for 5 h *in vitro* with or without indicated TKIs. The concentration of imatinib, nilotinib, and dasatinib are 5 μM, 5 μM, and 0.1 μM, respectively. The incubated cells were lysed and subjected to immunoblot analysis using the indicated antibodies. (B) RIs against imatinib were calculated in 15 patients at diagnosis and 14 patients who had been receiving imatinib-therapy and showed its resistance. The distribution of RIs in each group was plotted. Representative box plots show values within the 25th to 75th percentile. Medians are indicated in crossbar. Fifth and 95th percentiles are shown by error bars. The statistical difference was $p < 0.05$.

Then the treatment was changed to dasatinib, which was stopped because of a strong pancytopenia. The patient was then treated with nilotinib, but the percentage of $Ph1^+$ cells again increased. The second sample was obtained at the time of the change from dasatinib to nilotinib. In both samples, the incubation with the three TKIs did not eliminate the phosphorylation of Crkl. Although the second sample exhibited a strong sensitivity only to dasatinib (RI = 4.1%), the remaining CML cells additionally displayed continuous Lyn-phosphorylation (Fig. 4B).

### 3.5. RIs in patients with Bcr-Abl point mutations

The most important issue in TKIs resistance is the acquisition of point mutations in Bcr-Abl. Bcr-Abl mutations were detected in 4 samples (Table 2). The RI values of Patient 28, with a threonine-to-isoleucine mutation at codon 315 (T315I), were higher than 10% in all the TKI-treated samples. In accordance with the *in vitro* results, the disease was refractory to both imatinib and dasatinib. A phenylalanine-to-leucine mutation at codon 317 (F317L) and a methionine-to-threonine at codon 351 (M351T) were detected in Patient 27. F317L is reported to confer high responsiveness to nilotinib, while M351T does the same to dasatinib. The RI values of this patient were over 10% in all of the samples treated with TKIs, which conformed the outcome of failing to achieve CHR after nilotinib or dasatinib treatment. Next, the RI value in the sample with the phenylalanine-to-valine mutation at codon 359 (F359V) (Patient 23) was less than 10% only in the dasatinib-treated sample, which does not conflict with the reported IC50 data. Finally, although the F317L mutation is reported to be highly sensitive to nilotinib, the RI value for nilotinib in Patient 19, who later proved to be resistant to nilotinib but responded to dasatinib, was higher than 10%, and lower than 10% for dasatinib. Therefore, RIs are likely to be highly correled with the favorability of Bcr-Abl mutations to TKIs, and in some cases, to predict the responsiveness with higher sensitivity than mutations.

### 3.6. Correlation of RI with patient outcome

To analyze whether the RIs correlate with the clinical response to TKIs, newly diagnosed patients ($n = 15$) were separated into two groups in accordance with the most recent outcome, imatinib-sensitive ($n = 13$), who achieved an optimal response after the sample collection, and imatinib-resistant ($n = 2$), who did not. The median RI of the patients in the sensitive group was 4.2% and that in the resistant group was 43.2% ($p < 0.05$) (Fig. 5, left panel). We also assessed the predictability of the response to nilotinib. Eight patients imatinib resistant had undergone nilotinib-therapy. Among them, 4 achieved optimal responses and the others failed. The median RI in the nilotinib-sensitive group was 3.5% in contrast to 31.2% in the resistant group (Fig. 5, middle panel). Although the sample size was too small to conduct statistical analysis, the RIs were clearly separated between dasatinib-sensitive and -resistant groups (Fig. 5, right panel).

When the cut-off value of RI was set at 10%, the specificities, sensitivities and predicted values were all 100% in terms of nilotinib and dasatinib responsiveness (Table 3). Also, in the evaluation of imatinib-treatment, the specificity and sensitiveness were more than 77%. Therefore, it is suggested that the RIs (cut-off value: 10%) are useful as a novel predictor for clinical utility of TKIs, especially in imatinib-resistant cases.

## 4. Discussion

Imatinib, the first approved TKI for CML, frequently induces durable cytogenetic remission and thus occupies an important position as the current standard of care. Now, second-generation

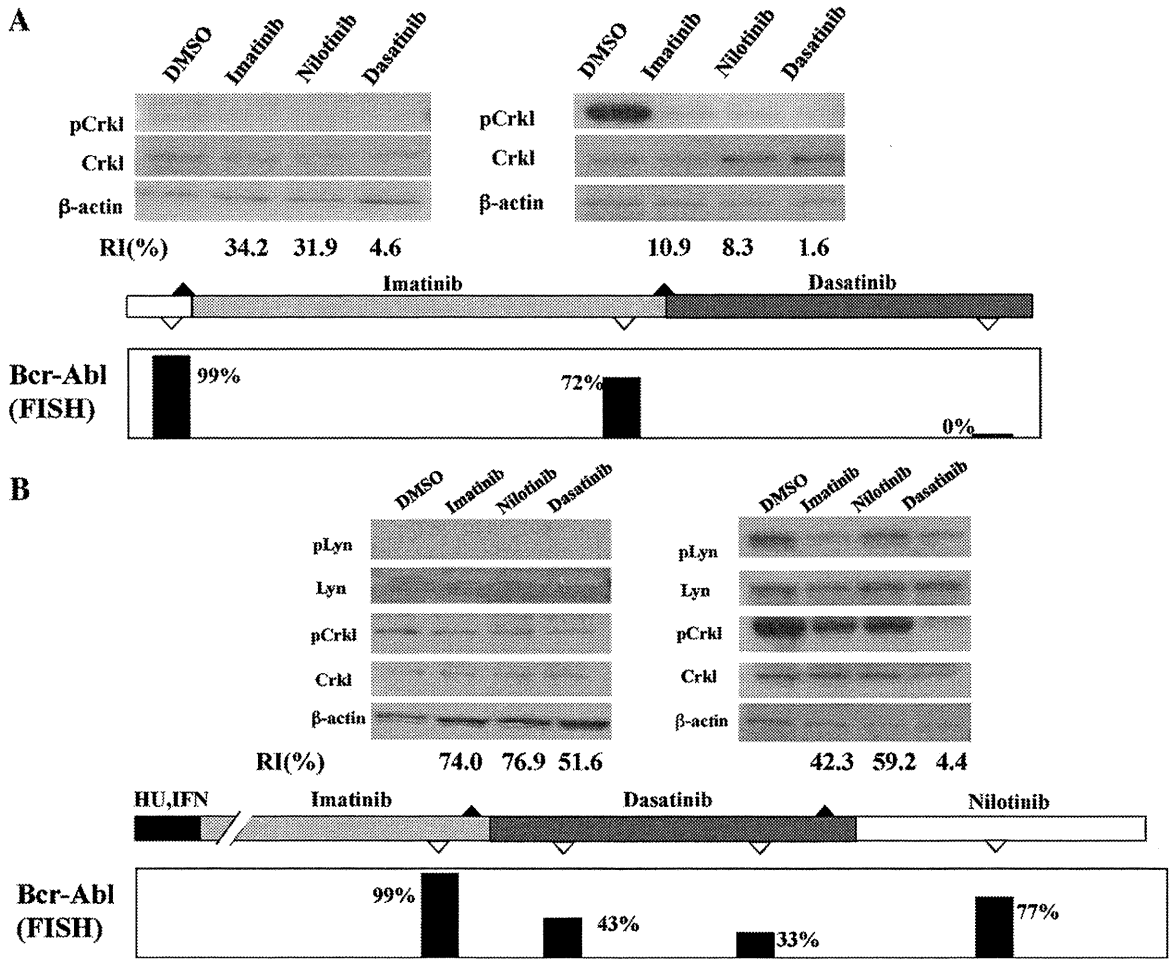


**Fig. 4.** Sequential examinations of RI values during clinical treatments in two patients. Immunoblots were sequentially analyzed during CML-treatment in two patients who showed resistance to TKIs. Data of immunoblots using the indicated antibodies are shown with their clinical course. FISH analyses are indicated by open triangles, and immunoblot analysis by closed triangles.

**Table 1**
Patient characteristics.

| Characteristic | |
|---|---|
| No. of patients | 31 |
| Median age, y (range) | 55 (20–89) |
| Sex (male/female) | 14/17 |
| Treatment before sample collection | |
| No | 13 |
| IFN | 3 |
| TKI | 18 |
| Bcr-Abl mutation | 4 |
| Median follow-up, months (range) | 6 (3–14) |

TKIs, such as nilotinib and dasatinib, have now been made available [12,13]. Although these TKIs are significantly more potent and show higher sensitivity against some imatinib-resistant mutations, there are no useful guidelines for the proper choice of second-generation TKIs in imatinib-resistant patients. Furthermore, second-generation TKIs have recently been recommended as first-line therapies based on the evidence that an earlier achievement of remission may provide a better clinical outcome or less disease progression. There is still a need for indicators pointing to the proper drug choice for individual patients. The *in vitro* responsiveness to TKIs in terms of cell proliferation has been demonstrated to be a predictor of clinical response. The IC50, a cell based screen for resistance determining the drug concentration that can induce 50% of growth suppression, is a potent predictor of the responsiveness to drugs. In patients with *de novo* CML, the $IC50^{imatinib}$ was reported to possess a high predictive value [22]. However, determination of the IC50 for each TKI requires so much effort and time that an application suitable for all patients may be quite a distant prospect. Furthermore, as the optimal concentration varies for each TKI, comparing the efficacy between different TKIs is difficult. Although the cellular IC50 s for the effect of TKIs on Bcr-Abl point mutations have been reported [23–26], this information

**Table 2**
Patients with BCR-ABL mutations, and their RI values.

| Patient | Mutation | RIs | | | Clinical outcome |
|---|---|---|---|---|---|
| | | Imatinib | Nilotinib | Dasatinib | |
| Patient 19 | F317L | 40.0 | 30.8 | 3.9 | Imatinib and nilotinib resistant, and dasatinib respond |
| Patient 23 | F359V | 15.8 | 11.9 | 1.4 | Imatinib resistant, and nilotinib and dasatinib intolerant |
| Patient 27 | M351T/F317L | 74.0 | 76.9 | 51.6 | imatinib resistant, and nilotinib and dasatinib intolerant |
| Patient 28 | T315I | 104.2 | 88.0 | 93.0 | Imatinib and dasatinib resistant |

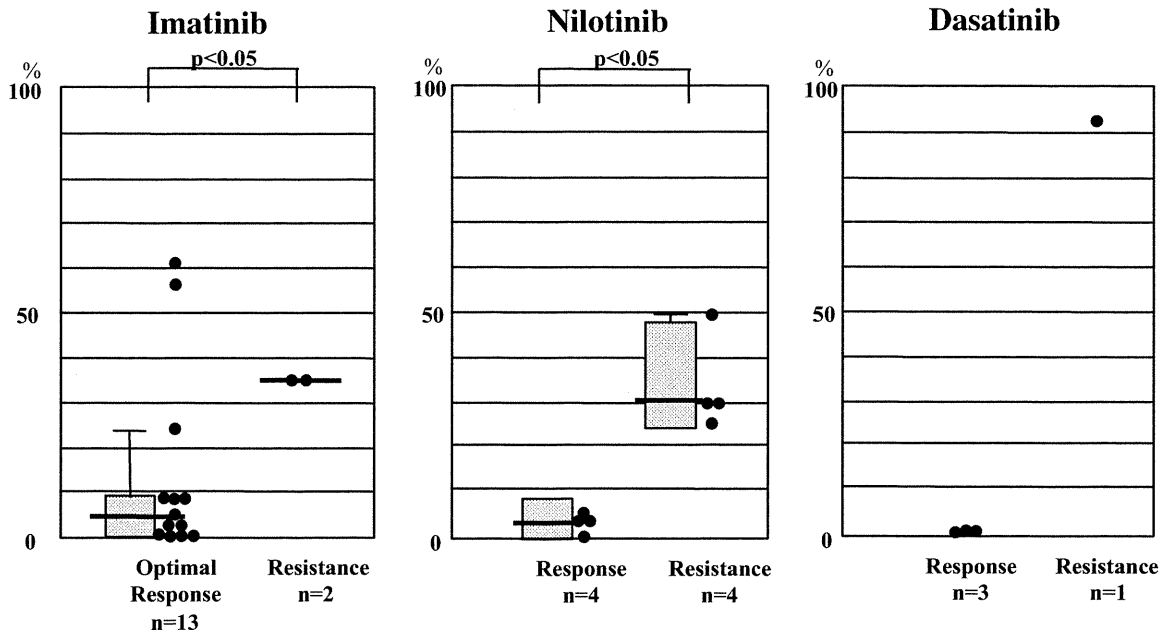


**Fig. 5.** RI values in patients grouped by clinical response to each TKI-therapy. Fifteen patients were newly diagnosed as CML, and their PB cells were obtained just before the beginning of imatinib-therapy. The patients were divided into two groups: "optimal response" in imatinib-treated patients means *de novo* CML patients who later proved to achieve optimal response, and "Resistance" means patients who later failed to achieve optimal response. Among 12 patients who had showed imatinib-resistance, 8 patients received nilotinib-therapy and 4 patients received dasatinib-therapy at a stretch of imatinib-therapy. Their PB cells were obtained just before the change of therapy. The patients were divided into two groups: that of responsive patients and of resistant patients to each TKI. Dot plots demonstrate the RI values of patients to each TKI. Representative box plots show values within the 25th to 75th percentile. Medians are indicated in crossbar. Fifth and 95th percentiles are shown by error bars.

**Table 3**
Sensitivity and specificity.

| | Optimal response | Resistance | Predicted value |
|---|---|---|---|
| Newly diagnosed and Imatinib-treated patients ($n=15$) | | | |
| RI < 10 | 10 | 0 | 100% |
| RI ≥ 10 | 3 | 2 | 40% |
| Specificity/sensitivity | 77% | 100% | |
| Imatinib-resistant and Nilotinib-treated patient ($n=8$) | | | |
| RI<10 | 4 | 0 | 100% |
| RI ≥ 10 | 0 | 4 | 100% |
| Specificity/sensitivity | 100% | 100% | |
| Imatinib-resistant and Dasatinib-treated patients ($n=4$) | | | |
| RI < 10 | 3 | 0 | 100% |
| RI ≥ 10 | 0 | 1 | 100% |
| Specificity/sensitivity | 100% | 100% | |
| | Newly diagnosed and later achieved optimal response | Imatinib-treated and showed resistance to Imatinib | Predicted value |
| All included and evaluable patients ($n=27$) | | | |
| RI < 10 | 10 | 1 | 91% |
| RI ≥ 10 | 3 | 13 | 81% |
| Specificity/sensitivity | 77% | 93% | |

is only useful when the mutated subclone is the predominant cell population.

In this study, we evaluated the effect of TKIs on Crkl phosphorylation as a "residual index". It is noteworthy that the samples from patients who had shown resistance to imatinib had much higher RIs than the samples from newly diagnosed patients. In the case of newly diagnosed patients, most samples responsive to imatinib *in vitro*, but two patients whose samples displayed markedly high RIs *in vitro* proved not to achieve an optimal response to the drug. Although substantial accordance was later detected in the immunoblot data between the responsiveness and resistance to imatinib, a few samples had markedly high RIs in patients who later achieved optimal responses to imatinib. These exceptional cases will have to be followed for a longer period. The data showed 100% of sensitivity and 77% of specificity when the RIs were separated at 10%. On the other hand, in imatinib-resistant patients, the results of the tests did reflect the patient outcome. Although the sample size was small, the immunoblot analysis was able to predict the clinical responsiveness to nilotinib or dasatinib treatment with 100% sensitivity and specificity. Thus, this system can be a useful tool for selecting TKIs, especially in imatinib-resistant patients. It may be inferred that the lower confidence in

the case of the untreated patients might due to a multiplicity of CML subclones.

CML patients develop imatinib resistance through either Bcr-Abl dependent or independent mechanisms. The most characterized and frequent mechanism is the acquisition of point mutations within the kinase domain of the Bcr-Abl gene, and some of the mutations such as T315I are potent predictors for outcome. However, even in those patients who have some mutations other than a few restricted mutations such as T315I and F317L, we cannot accurately predict the efficacy of TKIs. Furthermore, nearly half of the patients resistant to imatinib have no mutations in Bcr-Abl, which indicates that other mechanisms are also important for the acquisition of drug-resistance. Thus, we need other information for selecting TKIs. In this study, 4 patients carried point mutations in this region. Samples from 3 of them had RI values compatible with the predictive outcomes from the mutations. Notably, the RI values of the other sample contradicted the response of the mutation, but accorded with the actual response of the patient. From these points of view, the system described here can be utilized as another powerful predictor than IC50s for Bcr-Abl mutations.

The immunoblot system described here has the capacity to detect TKI-resistant subclones, including CML cells with Bcr-Abl mutations. In addition, our strategy seems to evaluate Bcr-Abl activity more directly than the cellular IC50 and require smaller population of TKI-resistant subclones than Bcr-Abl sequence analysis. Thus, when used together with the cellular IC50 values and Bcr-Abl sequence, this immunoblot system should help improve the treatment of patients with CML.

## Conflict of interest

The authors state that they have no conflict of interest.

## Acknowledgements

We would like to thank Shibano M. (Sakai Munincipal Hospital, Sakai, Japan), Sugahara H. (Sumitomo Hospital, Osaka, Japan), Moriyama Y. (Ikeda City Hospital, Ikeda, Japan), Azenishi Y. (Minoh City Hospital, Minoh, Japan), Ishida N. (Itami City Hospital, Itami, Japan), and Yamada M. (Suita Munincipal Hospital, Suita, Japan), who kindly provided blood samples.

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