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Pretreatment evaluation of fluorescence resonance energy transfer-based drug sensitivity test for patients with chronic myelogenous leukemia treated with dasatinib

Takeshi Kondo[1] | Mari Fujioka[2] | Masumi Tsuda[3] | Kazunori Murai[4] | Kohei Yamaguchi[5] | Takuto Miyagishima[6] | Motohiro Shindo[7] | Takahiro Nagashima[8] | Kentaro Wakasa[9] | Nozomu Fujimoto[10] | Satoshi Yamamoto[11] | Masakatsu Yonezumi[12] | Souichi Saito[13] | Shinji Sato[14] | Kazuei Ogawa[15] | Takaaki Chou[16] | Reiko Watanabe[17] | Yuichi Kato[18] | Shuichiro Takahashi[19] | Yoshiaki Okano[20] | Joji Yamamoto[21] | Masatsugu Ohta[22] | Hiroaki Iijima[23] | Koji Oba[24] | Satoshi Kishino[25] | Junichi Sakamoto[26] | Yoji Ishida[4] | Yusuke Ohba[2] | Takanori Teshima[1] | on behalf of the Inter-Michinoku Dasatinib Study Group (IMIDAS)

[1]Department of Hematology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

[2]Department of Cell Physiology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

[3]Department of Cancer Pathology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

[4]Division of Hematology and Oncology, Department of Internal Medicine, Iwate Medical University School of Medicine, Morioka, Japan

[5]Division of Hematology, Aomori Prefectural Central Hospital, Aomori, Japan

[6]Department of Internal Medicine, Japan Labour Health and Welfare Organization, Kushiro Rosai Hospital, Kushiro, Japan

[7]Division of Gastroenterology and Hematology/Oncology, Asahikawa Medical University, Asahikawa, Japan

[8]Department of Internal Medicine/General Medicine, Kitami Red Cross Hospital, Kitami, Japan

[9]Division of Hematology, Hokkaido P.W.F.A.C. Obihiro-Kosei General Hospital, Obihiro, Japan

[10]Division of Hematology, Kaisei Hospital, Sapporo, Japan

[11]Department of Hematology, Sapporo City General Hospital, Sapporo, Japan

[12]Department of Hematology, Hokkaido Cancer Center, Sapporo, Japan

[13]Department of Internal Medicine, Nihonkai General Hospital, Sakata, Japan

[14]Department of Hematology, Okitama Public General Hospital, Okitama, Japan

[15]Department of Cardiology and Hematology, Fukushima Medical University, Fukushima, Japan

[16]Department of Internal Medicine, Niigata Cancer Center Hospital, Niigata, Japan

[17]Department of Hematology, Saitama Medical Center, Saitama Medical University, Kawagoe, Japan

[18]Department of Hematology, Faculty of Medicine, Yamagata University, Yamagata, Japan

[19]Department of Hematology, Yamagata Prefectural Central Hospital, Yamagata, Japan

[20]Department of Hematology, Iwate Prefectural Miyako Hospital, Miyako, Japan

[21]Department of Hematology, Sendai City Hospital, Sendai, Japan

[22]Department of Hematology, Aizu Medical Center, Fukushima Medical University, Aizuwakamatsu, Japan

[23]Hokkaido University Hospital Clinical Research and Medical Innovation Center, Sapporo, Japan

[24]Department of Biostatistics, School of Public Health, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

[25]Department of Medication Use Analysis and Clinical Research, Meiji Pharmaceutical University, Kiyose, Japan

[26]Tokai Central Hospital, Kakamigahara, Japan

Correspondence
Takeshi Kondo, Department of Hematology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan.
Email: t-kondoh@med.hokudai.ac.jp

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Tyrosine kinase inhibitors (TKI) are used for primary therapy in patients with newly diagnosed CML. However, a reliable method for optimal selection of a TKI from the viewpoint of drug sensitivity of CML cells has not been established. We have developed a FRET-based drug sensitivity test in which a CrkL-derived fluorescent biosensor efficiently quantifies the kinase activity of BCR-ABL of living cells and sensitively evaluates the inhibitory activity of a TKI against BCR-ABL. Here, we validated the utility of the FRET-based drug sensitivity test carried out at diagnosis for predicting the molecular efficacy. Sixty-two patients with newly diagnosed chronic phase CML were enrolled in this study and treated with dasatinib. Bone marrow cells at diagnosis were subjected to FRET analysis. The ΔFRET value was calculated by subtraction of FRET efficiency in the presence of dasatinib from that in the absence of dasatinib. Treatment response was evaluated every 3 months by the *BCR-ABL1* International Scale. Based on the ΔFRET value and molecular response, a threshold of the ΔFRET value in the top 10% of FRET efficiency was set to 0.31. Patients with ΔFRET value ≥0.31 had significantly superior molecular responses (MMR at 6 and 9 months and both MR4 and MR4.5 at 6, 9, and 12 months) compared with the responses in patients with ΔFRET value <0.31. These results suggest that the FRET-based drug sensitivity test at diagnosis can predict early and deep molecular responses. This study is registered with UMIN Clinical Trials Registry (UMIN000006358).

KEYWORDS
BCR-ABL, chronic myeloid leukemia, drug sensitivity test, fluorescence resonance energy transfer, tyrosine kinase inhibitor

1 | INTRODUCTION

Chronic myeloid leukemia (CML) is one of the most well-established types of leukemia in terms of not only the molecular mechanism of the disease but also the development of molecularly targeted therapy. Generation of constitutively active tyrosine kinase BCR-ABL by reciprocal translocation between chromosome 9 and chromosome 22 plays a pathogenic role in the disease.[1] After the introduction of imatinib, a first-generation tyrosine kinase inhibitor (TKI), the prognosis of patients with chronic phase CML (CML-CP) was dramatically improved.[2,3] However, despite the efficacy of imatinib for treatment of CML-CP, many patients could not continue treatment with imatinib because of intolerance or resistance.[4] To overcome these clinical problems, second-generation TKI including dasatinib and nilotinib have been approved and have been shown to be highly effective not only for imatinib-resistant or imatinib-intolerant patients but also for newly diagnosed patients.[5–11] Therefore, 3 TKI, imatinib, dasatinib and nilotinib, are widely used for treatment of patients with newly diagnosed CML-CP. Moreover, bosutinib and ponatinib are approved for second-line or later treatment for patients who are intolerant or resistant to prior treatment.[12–14] These TKI show a therapeutic effect by inhibiting BCR-ABL kinase activity, although they inhibit not only BCR-ABL kinase activity but also the activities of other off-target kinases. The off-target effect may be associated with potential adverse events such as cardiovascular, metabolic and pulmonary toxicities, and the spectrum of adverse events varies among these TKI.[15] In the current situation, the choice of a TKI for first-line treatment is generally based on the patient's comorbidities and disease status.[16] The Sokal or Hasford risk score is generally used to estimate disease status, and a high risk is associated with a low rate of cytogenetic and molecular remission and with a high rate of disease progression. Second-generation TKI (ie, dasatinib and nilotinib) are favored for patients with a high risk as these TKI induce more rapid and deeper responses and thus minimize the risk of disease progression compared with imatinib.[10,11,16] Although the Sokal and Hasford risk scores are widely used, they do not provide information indicating which drug might be most effective. In addition, it is not clear whether these scoring systems can predict the outcomes of patients treated with a second-generation TKI.[17] Therefore, a new method for risk stratification of patients with newly diagnosed CML that is more sensitive than the conventional risk scores and is applicable for second-generation TKI should be developed.

CrkL is a major substrate phosphorylated by BCR-ABL, and the level of phospho-CrkL, as analyzed by western blotting or flow

cytometry, has thus been used as a biomarker of BCR-ABL activity and drug responses.[18–20] We have developed a FRET-based drug sensitivity test in which Pickles, a CrkL-derived fluorescent biosensor, efficiently quantifies the kinase activity of BCR-ABL of living cells and sensitively evaluates the inhibitory activity of a TKI against BCR-ABL. In this method, the sensitivity for detection of BCR-ABL activity in the CML-derived cell line K562 by the FRET biosensor is much higher than that by western blotting or flow cytometry, which detects phosphorylated CrkL: the FRET biosensor could detect a significant effect of imatinib at a concentration ≥0.1 μmol/L, whereas western blotting and flow cytometry required at least 1 and 0.5 μmol/L imatinib, respectively, to detect a significant decrease in the phosphorylation status of endogenous CrkL.[21] In addition, FRET-based analysis enables visualization of BCR-ABL activity in individual cells and discrimination of cells with high BCR-ABL activity from cells with low BCR-ABL activity.[21] Thus, the FRET-based drug sensitivity test carried out at diagnosis might be able to predict the clinical response to a TKI in patients with CML. The aim of the present study was to validate the utility of the FRET-based drug sensitivity test carried out at diagnosis for predicting the molecular efficacy of dasatinib.

2 | MATERIALS AND METHODS

2.1 | Patient population and treatment

The clinical study was approved by the institutional review boards of Hokkaido University Hospital and each participating hospital, and written informed consent was obtained from all patients engaged in this study. This study is registered in the University Medical Information Network (UMIN000006358). Criteria for inclusion of patients were: (i) diagnosed as having CML-CP; (ii) age 15 years or older; (iii) Eastern Cooperative Oncology Group performance status (ECOG PS) of 0-2; (iv) no severe dysfunctions in primary organs; and (v) no previous treatment for CML except for treatment with hydroxyurea. The definition of CML-CP was described previously.[22] Sixty-two patients with newly diagnosed CML-CP were enrolled into this study. After diagnosis of CML-CP, the patients were treated with 100 mg dasatinib once daily. Study treatment was continued unless protocol-defined disease progression or unacceptable toxicity was observed. Treatment interruption and dose reduction were permitted for managing adverse events. Dose intensity was calculated as follows: actual total dose of dasatinib intake divided by scheduled total dose of dasatinib during treatment.

2.2 | Molecular analysis of *BCR-ABL1* transcripts

Quantification of the *BCR-ABL1* transcript by real-time quantitative polymerase chain reaction analysis was carried out to assess the molecular response. Patient peripheral blood samples were obtained before and at 3, 6, 9, and 12 months after starting dasatinib treatment. The *BCR-ABL1* International Scale (*BCR-ABL1* IS) in peripheral blood was measured by a central laboratory center (BML, Tokyo, Japan) with the conversion factor 0.87 as previously described.[23] For validation of *BCR-ABL1* IS, *ABL1* was used as a reference gene. Molecular responses were defined as major molecular response (MMR; *BCR-ABL1* IS of 0.1% or less), molecular response 4 (MR4; *BCR-ABL1* IS of 0.01% or less), and molecular response 4.5 (MR4.5; *BCR-ABL1* IS of 0.0032% or less). When *BCR-ABL1* was undetectable, total gene number of *ABL1* was used to determine molecular response. Missing data were dealt with as an unachieved molecular response.

2.3 | Fluorescence resonance energy transfer-based drug sensitivity test

The FRET-based drug sensitivity test was carried out as described previously.[21] Bone marrow samples, which were primarily taken for diagnosis of CML, were subjected to analysis, as our previous study suggested that cells with high FRET efficiency are more abundant in bone marrow than in peripheral blood.[21] Briefly, fresh bone marrow samples were collected prior to starting dasatinib treatments, and mononuclear cells were isolated using Lymphoprep (Nycomed) transfected with an expression vector for the CrkL-modified biosensor Pickles by nucleofection (program number T-020 and Solution V; Amaxa Biosystems), and maintained in RPMI1640 supplemented with 10% FBS. After 24 hours of transfection, cells expressing Pickles were cultured in phenol red-free RPMI1640 (Invitrogen, Carlsbad, CA, USA) buffered with 15 mmol/L HEPES (pH 7.4; to avoid CO_2 control) and treated with 0.1 μmol/L dasatinib or not treated. Simultaneously, cells expressing Pickles were treated with 4 μmol/L nilotinib. Cell images were acquired as previously described.[21] Following background subtraction, FRET/enhanced cyan fluorescent protein (ECFP) ratio images were created using MetaMorph software (Molecular Devices, San Jose, CA, USA), and the images were used to illustrate FRET efficiency. In the dot plots, the absolute values for emission ratio (FRET/ECFP) were calculated and plotted, 1 dot representing the FRET efficiency of a single cell.

2.4 | Optimal threshold for FRET analysis and statistical analysis

To evaluate the sensitivity of CML cells to dasatinib, FRET efficiency without dasatinib treatment was subtracted from FRET efficiency with dasatinib treatment and designated as ΔFRET. Mean value of the top 10% FRET efficiency in analyzed cells was used to calculate ΔFRET, and ΔFRET in the top 10% FRET efficiency ($\Delta FRET^{top10\%}$) was used to evaluate drug sensitivity. One-sided unpaired *t* test and logistic regression analysis were carried out to determine whether ΔFRET is associated with achievement of MMR, MR4 and MR4.5. Receiver operating characteristic (ROC) curves were generated on the basis of $\Delta FRET^{top10\%}$ value and molecular responses. Optimal threshold of $\Delta FRET^{top10\%}$ to predict molecular response was calculated using the Youden index. Based on the optimal threshold of $\Delta FRET^{top10\%}$, we classified patients into 2 groups, a high $\Delta FRET^{top10\%}$ group and a low $\Delta FRET^{top10\%}$ group. Achievement of molecular responses in these groups was examined by the 1-sided Fisher's exact test. Multivariate

logistic regression analysis was carried out to evaluate clinical factors that may affect the efficacy of dasatinib in terms of molecular response. Analysis for achievement of molecular response was based on the modified intention-to-treat method. Calculation of halving time with dasatinib treatment was carried out as previously described,[24,25] and the relevance of halving time to the $\Delta FRET^{top10\%}$ value and pharmacokinetic parameters of dasatinib are described in Doc S1 in Supplementary Information. Collinearity of the $\Delta FRET^{top10\%}$ value between dasatinib and nilotinib was evaluated by Pearson's correlation coefficient, and a regression line was determined by a simple linear regression analysis. All the statistical tests were conducted under the significance levels of .05 (2-sided) and .025 (1-sided).

3 | RESULTS

3.1 | Patients' characteristics and molecular responses

Sixty-two patients were subjected to FRET analysis. Table 1 shows the patients' characteristics. Forty-three patients were male and 19 patients were female, and the median age of patients was 63 years. According to the Sokal risk score, 32 patients (51.6%) were at low risk, 21 patients (33.9%) were at intermediate risk, 5 patients (8.1%) were at high risk, and the risk for 4 patients (6.4%) was undetermined. Treatment was interrupted or dasatinib dose was reduced in 44 patients for managing adverse events. Median dose intensity of dasatinib in all of the patients was 88.3% (range, 38.9%-100%) during treatment. Molecular response was calculated by modified intent-to-treat analysis. MMR rates were 33.9% by 3 months, 71.0% by 6 months, 79.0% by 9 months, and 83.9% by 12 months. MR4 rates were 4.8% by 3 months, 32.3% by 6 months, 50.0% by 9 months, and 53.2% by 12 months, and MR4.5 rates were 1.6% by 3 months, 22.6% by 6 months, 38.7% by 9 months, and 46.8% by 12 months. Treatment was discontinued as a result of hematological toxicities in 3 patients, non-hematological toxicities in 3 patients, disease progression in 1 patient, and gastric cancer in 1 patient.

3.2 | Fluorescence resonance energy transfer analysis and calculation of the optimal threshold to determine drug sensitivity

Fluorescence resonance energy transfer efficiency was variable in cells without dasatinib treatment, and only a small fraction of the cells showed high FRET efficiency (Figure 1A,B, left panels). This meant that we needed to determine the population of analyzed cells that should be used for analysis. Initially, we classified the cells into 10% fractions in the order of descending FRET efficiency and calculated mean values of FRET efficiency and ΔFRET. The value of ΔFRET in cells with the top 10% FRET efficiency was higher than the values in other fractions of cells and thus most efficiently reflected the effect of dasatinib (Figure 1C). Therefore, we used the top 10% FRET efficiency to calculate the ΔFRET value for further analysis. The value was designated as $\Delta FRET^{top10\%}$.

TABLE 1 Characteristics of patients and clinical responses to dasatinib

	Total
Patient number	62
Gender (Male/Female)	43/19
Age at diagnosis, median y.o. (range)	63 (33-80)
Sokal Score, n (%)	
Low	32 (51.6)
Intermediate	21 (33.9)
High	5 (8.1)
Unknown	4 (6.4)
ECOG PS, n (%)	
0	59 (95.2)
1	2 (3.2)
2	1 (1.6)
BCR-ABL IS prior to treatment, median (%)	48.7
(range)	(7.8-221.0)
Median intensity of dasatinib (%)	88.3
(range)	38.9-100
Discontinuation, n (%)	
3 mo	0 (0.0)
6 mo	2 (3.2)
9 mo	7 (11.3)
12 mo	8 (12.9)
Cumulative MMR achievement, n (%)	
3 mo	21 (33.9)
6 mo	44 (71.0)
9 mo	49 (79.0)
12 mo	52 (83.9)
Cumulative MR4 achievement, n (%)	
3 mo	3 (4.8)
6 mo	20 (32.3)
9 mo	31 (50.0)
12 mo	33 (53.2)
Cumulative MR4.5 achievement, n (%)	
3 mo	1 (1.6)
6 mo	14 (22.6)
9 mo	24 (38.7)
12 mo	29 (46.8)

ECOG PS, Eastern Cooperative Oncology Group performance status; IS, International Scale; MMR, major molecular response; MR4, molecular response 4; MR4.5, molecular response 4.5.

Next, we investigated whether $\Delta FRET^{top10\%}$ is associated with molecular response. We compared $\Delta FRET^{top10\%}$ values in patients who achieved MMR, MR4, and MR4.5 with those in patients who did not achieve MMR, MR4, and MR4.5. $\Delta FRET^{top10\%}$ values in patients who achieved MR4 by 6 months or MR4.5 by 12 months were significantly higher than those in patients who did not achieve those molecular responses (Figure 2; Table S1). These results suggested that FRET

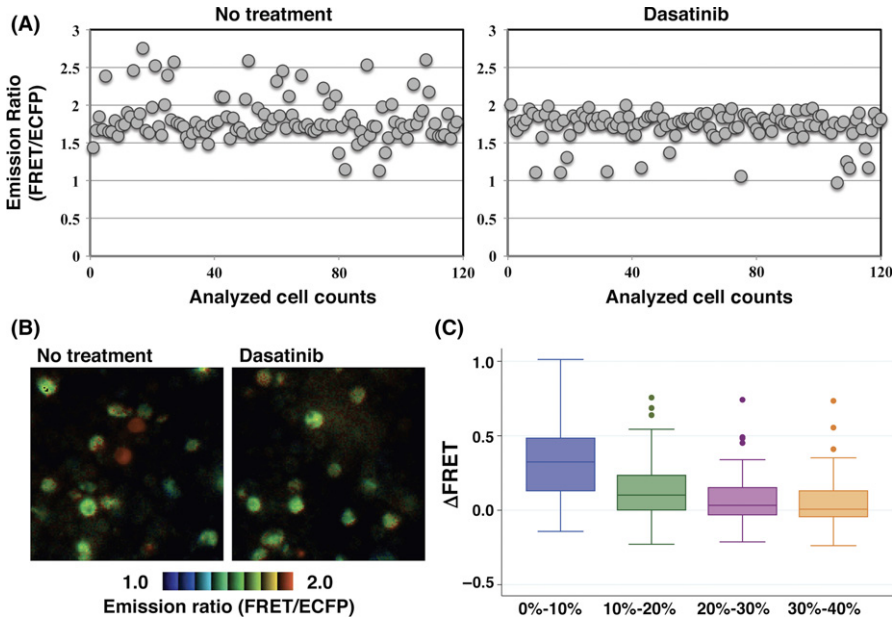

FIGURE 1 Observation of FRET efficiency in individual CML cells that is suppressed by treatment with dasatinib. A, Analysis of FRET efficiency in a representative case. CML cells were transfected with an expression vector for the FRET biosensor Pickles. At 24 hours after transfection, the cells were incubated in the presence or absence of 0.1 μmol/L dasatinib and then subjected to microscopic analysis. Each dot shows FRET efficiency of individual cells. The ordinate represents emission ratio (FRET/enhanced cyan fluorescent protein [ECFP]) efficiency and the abscissa indicates the order of the cells analyzed. B, Fluorescence images of a representative case are presented. A limited fraction of cells in the absence of dasatinib showed high FRET efficiency. C, ΔFRET values in every 10% fraction of cells in the order of descending FRET efficiency are plotted in box and whisker plots

analysis can be used to identify CML-CP patients treated with dasatinib who will rapidly achieve deep molecular responses in the clinical course. In addition, logistic regression analysis suggested that $\Delta FRET^{top10\%}$ value was significantly associated with achievement of MR4 by 6 months and achievement of MR4.5 by 12 months (Table S2). Using ROC curve analysis and the Youden index, the optimal thresholds of $\Delta FRET^{top10\%}$ value for achieving molecular response were 0.32 for MR4 by 6 months and 0.31 for MR4.5 by 12 months (Figure S1). Therefore, we provisionally selected the optimal ΔFRET threshold of 0.31 for further analysis.

3.3 | Molecular responses stratified by FRET analysis

According to the threshold of ΔFRET value of 0.31, patients in the present study were classified into a high $\Delta FRET^{top10\%}$ group ($\Delta FRET^{top10\%} \geq 0.31$, n = 32) and a low $\Delta FRET^{top10\%}$ group ($\Delta FRET^{top10\%} < 0.31$, n = 30). In the high $\Delta FRET^{top10\%}$ group, MMR rates were 40.6% by 3 months, 87.5% by 6 months, 90.6% by 9 months, and 93.8% by 12 months, MR4 rates were 3.1% by 3 months, 50.0% by 6 months, 68.8% by 9 months, and 68.8% by 12 months, and MR4.5 rates were 0.0% by 3 months, 34.4% by 6 months, 56.3% by 9 months, and 65.6% by 12 months. In the low $\Delta FRET^{top10\%}$ group, MMR rates were 26.7% by 3 months, 53.3% by 6 months, 66.7% by 9 months, and 73.3% by 12 months, MR4 rates were 6.7% by 3 months, 13.3% by 6 months, 30.0% by 9 months, and 36.7% by 12 months, and MR4.5 rates were 3.3% by 3 months, 10.0% by 6 months, 20.0% by 9 months, and 26.7% by 12 months. As a result, MMR rates by 6 months, 9 months, or MR4 rates and MR4.5 rates by 6 months, 9 months, and 12 months in the high $\Delta FRET^{top10\%}$ group were significantly higher than those in the low $\Delta FRET^{top10\%}$ group (Figure 3). These results suggested that the FRET-based drug sensitivity test can predict the molecular responses of patients with CML-CP prior to treatment with dasatinib.

3.4 | Clinical factors at diagnosis and treatment responses

We carried out multivariate analysis for clinical factors at diagnosis that may be associated with clinical outcomes. In addition to $\Delta FRET^{top10\%}$ value, we incorporated patient's age, gender, performance status, Sokal score and *BCR-ABL1* IS at diagnosis into analysis. As a result, $\Delta FRET^{top10\%}$ value remained as the only significant factor among the factors analyzed that was associated with achievement of MR4 by 6 months and MR4.5 by 9 and 12 months (Table 2). No significant correlation was found with Sokal score. Therefore, $\Delta FRET^{top10\%}$ value seemed to be the most reliable factor among the analyzed factors for predicting an early and deep molecular response in patients with CML-CP prior to treatment with dasatinib.

3.5 | Further stratification by combination of $\Delta FRET^{top10\%}$ value and halving time

Although our results suggest a clinical utility of the $\Delta FRET^{top10\%}$ value of dasatinib for predicting molecular responses, several patients having a high $\Delta FRET^{top10\%}$ value failed to achieve MMR,

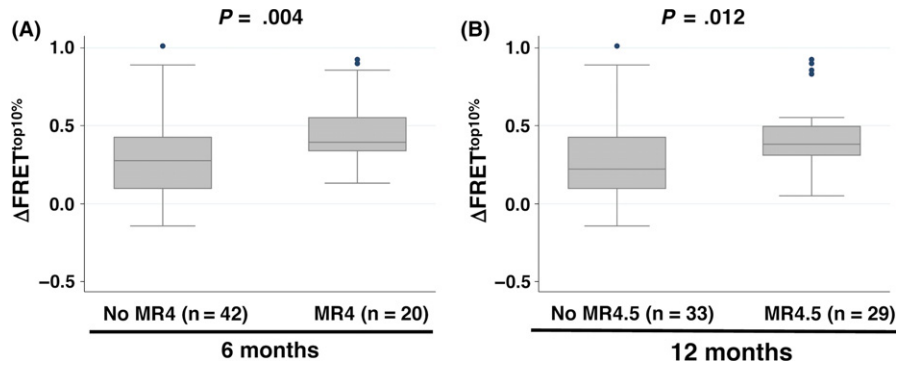

FIGURE 2 $\Delta FRET^{top10\%}$ values in patients with molecular response 4 (MR4) and molecular response 4.5 (MR4.5) and patients without MR4 and MR4.5. $\Delta FRET^{top10\%}$ values in patients who achieved MR4 by 6 months (A) and MR4.5 by 12 months (B) were significantly higher than those in patients who failed to achieve those responses. $\Delta FRET^{top10\%}$ values were plotted in box and whisker plots and statistically examined by the 1-sided unpaired *t* test

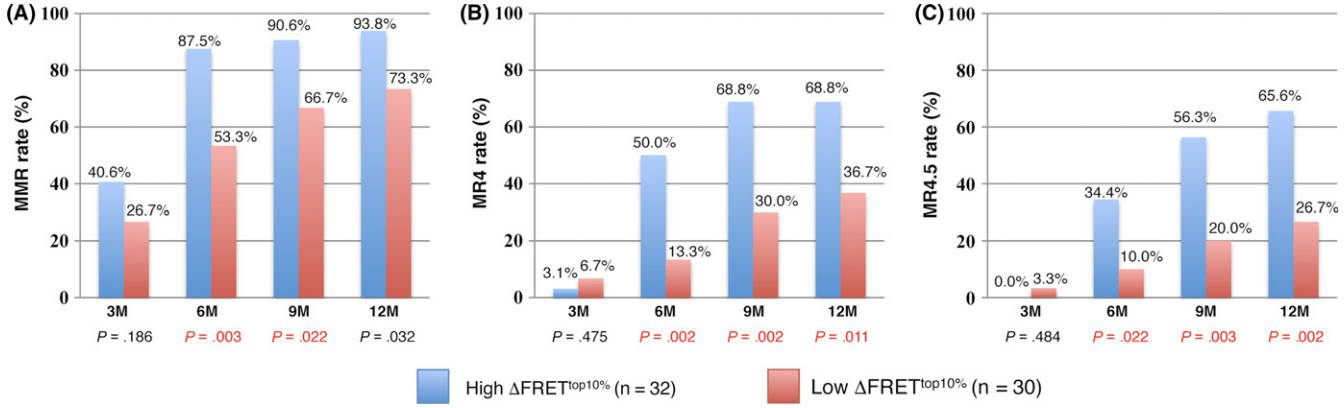

FIGURE 3 Cumulative major molecular response (MMR), molecular response 4 (MR4), and molecular response 4.5 (MR4.5) rates are stratified by the $\Delta FRET^{top10\%}$ threshold of 0.31. A, Cumulative MMR rate, B, MR4 rate, and C, MR4.5 rate were significantly different between patients with a high $\Delta FRET^{top10\%}$ value and those with a low $\Delta FRET^{top10\%}$ value. Differences of molecular responses were statistically examined by 1-sided Fisher's exact test

MR4, or MR4.5 by 12 months. After starting treatment with the TKI, patients with CML-CP were stratified at 3 months by achievement of 10% of *BCR-ABL1* IS. The rate of *BCR-ABL1* IS decline, so-called halving time, has been shown to have a significant predictive value for MMR and MR4 at 12 months.[24,25] In the 62 patients in this study, 59 patients achieved 10% of *BCR-ABL1* IS at 3 months, 2 patients failed to achieve 10% of *BCR-ABL1* IS at 3 months, and 1 patient had missing data. Based on the data of *BCR-ABL1* IS before treatment and at 3 months, the optimal halving time threshold for MMR at 12 months was calculated to be 14.76 days (Doc S1; Figure S2). Patients with a short halving time (≤14.76 days) had significantly higher MMR, MR4 and MR4.5 rates than did patients with a longer halving time (>14.76 days) (Figure S3). In addition, there was no significant association between $\Delta FRET^{top10\%}$ value and halving time (Doc S1). We carried out multivariate analysis for achievement of MMR, MR4 and MR4.5 in which halving time was incorporated into the analysis. As dose modification was carried out for 44 patients, we also incorporated dose intensity into the analysis. Although halving time was the strongest factor among the factors analyzed and was associated with achievement of MMR and MR4 after 6 months and MR4.5 after 9 months, $\Delta FRET^{top10\%}$ value remained as a significant factor for achievement of MR4 by 6 months and MR4.5 by 12 months (Table S3). These results suggest that the combination of $\Delta FRET^{top10\%}$ value and halving time can further stratify patients. Therefore, we divided patients into 4 groups: high $\Delta FRET^{top10\%}$ (≥0.31)/short halving time (≤14.76 days) group (n = 25); high $\Delta FRET^{top10\%}$ (≥0.31)/long halving time (>14.76 days) group (n = 6); low $\Delta FRET^{top10\%}$ (<0.31)/short halving time (≤14.76 days) group (n = 16); and low $\Delta FRET^{top10\%}$ (<0.31)/long halving time (>14.76 days) group (n = 14). MMR rates at 12 months in patients with high $\Delta FRET^{top10\%}$/short halving time and patients with low $\Delta FRET^{top10\%}$/short halving time were 100%, and they were significantly higher than MMR rate in patients with low $\Delta FRET^{top10\%}$/long halving time (42.9%). As expected, the rate of MR4.5 in patients with high $\Delta FRET^{top10\%}$/short halving time was significantly higher than those in other groups, including patients with low $\Delta FRET^{top10\%}$/short halving time (Figure 4).

TABLE 2 Multivariate analysis of pretreatment factors affecting MMR, MR4, and MR4.5

		6 months			9 months			12 months		
		Odds Ratio	95% CI	*P*-value	Odds Ratio	95% CI	*P*-value	Odds Ratio	95% CI	*P*-value
MMR achievement	$\Delta FRET^{top10\%}$	8.061	0.721-90.091	.090	3.496	0.289-42.227	.325	3.124	0.206-47.315	.411
	Age	1.039	0.978-1.103	.217	1.03	0.965-1.099	.37	1.021	0.949-1.099	.571
	Male patient	0.341	0.0683-1.702	.190	0.641	0.128-3.208	.588	1.032	0.190-5.623	.971
	ECOG PS	0.374	0.039-3.593	.394	0.457	0.049-4.236	.491	0.491	0.051-4.715	.538
	Int. & high Sokal Score	0.668	0.050-9.006	.761	0.581	0.042-7.981	.684	0.504	0.033-7.614	.621
	BCR-ABL1 IS	1.000	0.985-1.015	.959	0.998	0.983-1.014	.800	0.995	0.979-1.012	.576
MR4 achievement	$\Delta FRET^{top10\%}$	25.360	1.437-447.517	.027	8.547	0.860-84.983	.067	6.574	0.703-61.446	.099
	Age	1.025	0.962-1.092	.445	1.002	0.950-1.058	.931	1.013	0.960-1.069	.625
	Male patient	1.003	0.227-4.427	.997	1.241	0.340-4.529	.744	1.423	0.394-5.138	.590
	ECOG PS	1.105	0.122-9.966	.929	1.175	0.147-9.406	.879	1.105	0.139-8.804	.925
	Int. & high Sokal Score	9.304	0.659-131.345	.099	4.086	0.328-50.835	.274	3.281	0.264-40.786	.356
	BCR-ABL1 IS	1.009	0.994-1.024	.237	0.999	0.986-1.012	.875	0.999	0.987-1.012	.924
MR4.5 achievement	$\Delta FRET^{top10\%}$	4.721	0.324-68.756	.256	17.323	1.139-263.461	.040	26.503	1.896-370.563	.015
	Age	1.035	0.963-1.112	.350	1.039	0.978-1.104	.217	1.028	0.971-1.089	.345
	Male patient	0.449	0.106-1.903	.277	2.263	0.497-10.308	.291	2.651	0.613-11.458	.192
	ECOG PS	0.925	0.133-6.447	.937	1.205	0.144-10.073	.863	1.066	0.128-8.879	.953
	Int. & high Sokal Score	3.263	0.375-28.412	.284	7.657	0.509-115.107	.141	5.65	0.371-86.017	.213
	BCR-ABL1 IS	1.003	0.989-1.018	.657	1.005	0.991-1.018	.499	0.999	0.986-1.012	.849

ΔFRET, FRET efficiency without dasatinib treatment was subtracted from FRET efficiency with dasatinib treatment.
ECOG PS, Eastern Cooperative Oncology Group performance status; FRET, fluorescence resonance energy transfer; IS, International Scale; MMR, major molecular response; MR4, molecular response 4; MR4.5, molecular response 4.5.

3.6 | ΔFluorescence resonance energy transfer analysis value of nilotinib in the analyzed patients

We also compared the $\Delta FRET^{top10\%}$ value of dasatinib with that of nilotinib using the same bone marrow samples. It was thought that this comparison would provide some information about the relationships of ΔFRET with dasatinib and nilotinib, although patients were not treated with nilotinib. An overall comparison of $\Delta FRET^{top10\%}$ values showed that $\Delta FRET^{top10\%}$ of dasatinib was highly associated with that of nilotinib based on simple linear regression analysis ($P < .0001$). This result implies that $\Delta FRET^{top10\%}$ of dasatinib is almost equal to $\Delta FRET^{top10\%}$ of nilotinib in most patients. Interestingly, some samples strayed off greatly from the expected values (Figure 5).

4 | DISCUSSION

In the present study, we examined the feasibility of applying the FRET-based drug sensitivity test to predict the efficacy of dasatinib for treatment of patients with CML. FRET efficiency in bone marrow mononuclear cells isolated from patients with CML was quite variable. These observations are consistent with the results of a previous study showing that the expression levels of *BCR-ABL1* transcripts varied among CML patients.[26] Our previous study also indicated that only a limited number of cells showed high CrkL phosphorylation along with high BCR-ABL expression, despite the fact that most of the cells analyzed were BCR-ABL-positive.[21] Therefore, initially we tried to determine the cells that should be assigned to analysis. As a result, we focused on the top 10% FRET efficiency and calculated the $\Delta FRET^{top10\%}$ value, which could include high FRET efficiency cells and presumably reflect drug sensitivity. Although cells with high FRET efficiency should be further characterized, one candidate might be immature cells including CML stem cells, which were reported to express high levels of functional BCR-ABL.[27]

Based on the relations of $\Delta FRET^{top10\%}$ with MR4 rate by 6 months and MR4.5 rate by 12 months, we provisionally calculated 0.31 as an optimal threshold value of $\Delta FRET^{top10\%}$. This threshold value efficiently stratified patients by molecular responses after 6 months. Further study is needed to establish a more definitive threshold, as this study is based on a limited number of patients.

Recently, it was reported that leukemic stem cell quantification at diagnosis of CML is a strong predictive marker for molecular responses by imatinib, dasatinib and nilotinib.[28,29] In those studies, leukemic stem cell burden was correlated with other biological

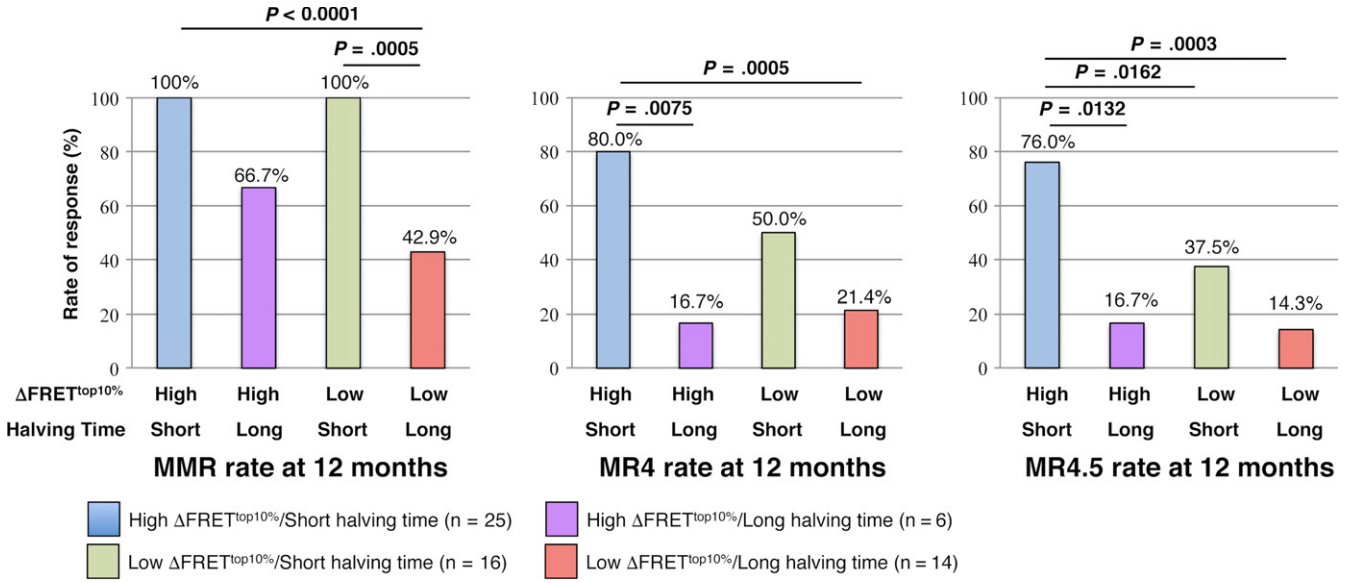

FIGURE 4 Combination of $\Delta FRET^{top10\%}$ value with halving time identifies the most dasatinib-sensitive patients. Patients were divided into 4 groups according to $\Delta FRET^{top10\%}$ value and halving time. Achievement of molecular responses in these groups was examined by 1-sided Fisher's exact test. Although major molecular response (MMR) rates were the same in patients with high $\Delta FRET^{top10\%}$ value/short halving time and patients with low $\Delta FRET^{top10\%}$ value/short halving time (left panel), MR4 rate and MR4.5 rate by 12 months in patients with high $\Delta FRET^{top10\%}$ value/short halving time were higher than those in other groups (middle and right panels, respectively). Post hoc analyses compared response rates by the 1-sided Fisher's exact test; therefore, *P*-values are descriptive and unadjusted for multiple comparisons

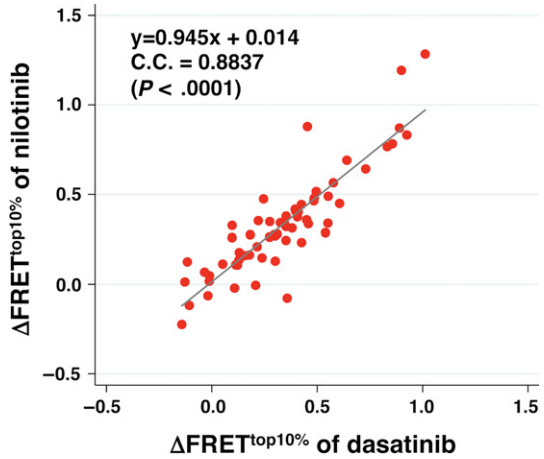

FIGURE 5 Collinearity of $\Delta FRET^{top10\%}$ value between dasatinib and nilotinib. Relationship between the $\Delta FRET^{top10\%}$ value of dasatinib and the $\Delta FRET^{top10\%}$ value of nilotinib was examined by a simple linear regression test. The $\Delta FRET^{top10\%}$ value of dasatinib was strongly correlated with the $\Delta FRET^{top10\%}$ value of nilotinib (correlation coefficient: 0.8837, $P < .0001$)

factors such as white blood cell count, blast percentage and spleen size. Moreover, patients with a low leukemic stem cell burden at diagnosis showed less hematological toxicity by the TKI and achieved higher rates of cytogenetic and molecular responses than did patients with a high leukemic stem cell burden. In those studies, rates of early molecular response of *BCR-ABL1* IS ≤10% at 3 months and *BCR-ABL1* IS ≤1% at 6 months were significantly higher in patients with a low leukemic stem cell burden than in those with a high leukemic stem cell burden. In contrast, the $\Delta FRET^{top10\%}$ value was a predictive factor for achievement of early and deep molecular responses (ie, MMR, MR4, and MR4.5 rates after 6 months). In our analysis, Sokal score was not associated with the achievement of MMR, MR4, or MR4.5. Although the population of patients with Sokal high risk was quite limited in our analysis, this was consistent with a recent report from Japan.[25]

Although $\Delta FRET^{top10\%}$ values could be a predictive biomarker for molecular response, some patients with high $\Delta FRET^{top10\%}$ values failed to achieve MMR, MR4, or MR4.5 by 12 months. One possible explanation is that the molecular response by treatment with dasatinib is greatly affected by pharmacokinetic and pharmacodynamic parameters of dasatinib, which are highly variable in patients.[30,31] Therefore, we assumed that the halving time would refine stratification of patients evaluated by the $\Delta FRET^{top10\%}$ value, because the halving time may reflect not only the drug sensitivity of CML cells but also pharmacokinetic parameters (Doc S1; Figure S4). As expected, rates of MR4 and MR4.5 by 12 months in patients with high $\Delta FRET^{top10\%}$/long halving time were significantly lower than those in patients with high $\Delta FRET^{top10\%}$/short halving time. Moreover, patients with high $\Delta FRET^{top10\%}$/short halving time showed a higher rate of MR4.5 by 12 months than did patients with low $\Delta FRET^{top10\%}$/short halving time, although patients with a short halving time achieved an MMR rate of 100% by 12 months regardless of the $\Delta FRET^{top10\%}$ value. Thus, the $\Delta FRET^{top10\%}$ value combined with halving time effectively stratified patients from the viewpoint of achievement of MR4 and MR4.5 by 12 months. Our results suggest some implications. The FRET-based drug sensitivity test could be a reliable prognostic marker at diagnosis. This prognostic marker would

be corrected by halving time at 3 months after treatment. Moreover, patients who have a short halving time could be further stratified by the results of the FRET-based drug sensitivity test. Currently, stopping treatment with a TKI in patients with a sustained deep molecular response (ie, MR4, MR4.5 or deeper) has been attempted, and a substantial number of patients could achieve treatment-free remission.[32] The combination of the FRET-based drug sensitivity test and halving time may provide information about the probability of patients achieving a deep molecular response, which is a prerequisite for treatment-free remission.

One may imagine that patients who are estimated to be dasatinib-sensitive by FRET analysis would also be sensitive to nilotinib. As shown in Figure 5, $\Delta FRET^{top10\%}$ values of nilotinib were similar to those of dasatinib, suggesting that both dasatinib and nilotinib are equally effective for most patients with CML. Interestingly, some samples strayed off greatly from the expected values. The underlying mechanism causing such differences should be further clarified. This result may imply that drug sensitivity of nilotinib is different from that of dasatinib in such patients. Although validation of FRET analysis is still required for TKI other than dasatinib, the FRET-based drug sensitivity test will provide some information for selecting one of the TKI at diagnosis from the viewpoint of drug sensitivity of leukemia cells.

One may also raise a question about the feasibility of this technique in a clinical laboratory. As described in Materials and Methods, we need only to isolate bone marrow mononuclear cells and to introduce the FRET-biosensor into CML cells according to the programmed protocol. As a result, the FRET-biosensor can be introduced into $CD34^+$ CML cells with transfection efficiency of 20%-30%.[21] This means that the FRET-based drug sensitivity test would be easy to apply for clinical purposes.

Our study indicated that the FRET-based drug sensitivity test could be a reliable prognostic marker at diagnosis for discriminating patients who will achieve an early and deep molecular response. Therefore, this method may add predictive information about the efficacy of a TKI before treatment.

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CONFLICT OF INTEREST

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ORCID

Takeshi Kondo http://orcid.org/0000-0001-7455-5824

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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