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Persistent detection of alternatively spliced *BCR-ABL* variant results in a failure to achieve deep molecular response

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Treatment with tyrosine kinase inhibitors (TKI) may sequentially induce TKI-resistant *BCR-ABL* mutants in chronic myeloid leukemia (CML). Conventional PCR monitoring of *BCR-ABL* is an important indicator to determine therapeutic intervention for preventing disease progression. However, PCR cannot separately quantify amounts of *BCR-ABL* and its mutants, including alternatively spliced *BCR-ABL* with an insertion of 35 intronic nucleotides (*BCR-ABL*^{Ins35bp}) between *ABL* exons 8 and 9, which introduces the premature termination and loss of kinase activity. To assess the clinical impact of *BCR-ABL* mutants, we performed deep sequencing analysis of *BCR-ABL* transcripts of 409 samples from 37 patients with suboptimal response to frontline imatinib who were switched to nilotinib. At baseline, TKI-resistant mutations were documented in 3 patients, whereas *BCR-ABL*^{Ins35bp} was detected in all patients. After switching to nilotinib, both *BCR-ABL* and *BCR-ABL*^{Ins35bp} became undetectable in 3 patients who attained complete molecular response (CMR), whereas in the remaining all 34 patients, *BCR-ABL*^{Ins35bp} was persistently detected, and minimal residual disease (MRD) fluctuated at low but detectable levels. PCR monitoring underestimated molecular response in 5 patients whose *BCR-ABL*^{Ins35bp} was persisted, although *BCR-ABL*^{Ins35bp} does not definitively mark TKI resistance. Therefore, quantification of *BCR-ABL*^{Ins35bp} is useful for evaluating "functional" MRD and determining the effectiveness of TKI with accuracy.

Treatment with tyrosine kinase inhibitors (TKI) targeting *BCR-ABL* has dramatically improved outcomes in patients with chronic myeloid leukemia (CML) in the chronic phase.[1,2] Accumulating evidence indicates that CML patients who achieve earlier and deeper molecular response would favor improving of relapse free and overall survival.[2,3] Therefore, serial molecular monitoring of *BCR-ABL* using quantitative RT-PCR (qRT-PCR) is becoming increasingly important for assessing response to TKI therapy, which would allow timely therapeutic intervention for patients with a suboptimal response to or who experience failure of TKI treatment. Molecular monitoring is also important to ensure their eligibility criteria for discontinuation and early detection of molecular relapse after cessation of TKI treatment in patients with sustained deep molecular response (DMR) who are candidates for discontinuation of TKI treatment.[4]

Resistance to TKI occurs in approximately 10–20% of CML patients through several mechanisms, including point mutations in *BCR-ABL* kinase domain (KD), overexpression or alternative splicing of *BCR-ABL* transcripts,[5,6] low plasma concentration of TKI and abnormal drug efflux/influx.[7,8] Recent studies have shown that alternatively spliced *BCR-ABL* variants cause failure in achieving optimal molecular response to TKI, especially in patients under long-term TKI treatment.[5,9–11] Alternatively spliced *BCR-ABL* variants have been detected in approximately 20% of CML patients who have gained hematologic/cytogenetic response but have failed to achieve DMR through the conventional Sanger sequencing method.[12–14] A representative alternatively spliced *BCR-ABL*^{Ins35bp} variant, in which retention of 35 intronic nucleotides at the splice junction of *ABL* exons 8 and 9 introduces a stop codon, results in a frameshift leading to the addition of 10 intron-encoded residues and truncation of 653 residues (Fig. 1a). Premature termination at the *ABL* KD portion causes generation of "kinase-inactivated" *BCR-ABL*^{Ins35bp}.[9,15] This premature termination also induces a conformational change of BCR-ABL protein, which, in turn, hinders TKI to bind BCR-ABL, like BCR-ABL^{T315I} mutation.[6] Thus, BCR-ABL^{Ins35bp} is insensitive to TKI treatment, but does not induce activated tyrosine kinase signaling that is critical for malignant proliferation and survival for CML clones.

Importantly, internationally recognized conventional qRT-PCR of *BCR-ABL* is designed to amplify short length of approximately 150bp fusion gene,[16] by locating upstream and downstream primers at the junction spanning *BCR-ABL* fusion portion, to increase the sensitivity for detecting minimal residual disease (MRD). Therefore, the conventional qRT-PCR method cannot distinguish between functional *BCR-ABL* transcripts, *BCR-ABL* with KD mutations and abnormal splicing, including *BCR-ABL*^{Ins35bp}. Therefore, we wished to quantify functional *BCR-ABL* transcripts in patients with TKI treatment, to accurately evaluate active MRD status.

We have developed a highly sensitive system for MRD detection by combining a long-range PCR amplification of *BCR-ABL* and an ultra-deep sequencing (UDS), which enables us to qualitatively and quantitatively measure *BCR-ABL*, *BCR-ABL* with KD mutations, and splicing variants of *BCR-ABL*^{Ins35bp}. In the current study, we serially tracked *BCR-ABL* and *BCR-ABL*^{Ins35bp} in CML patients who had a suboptimal response after >18 months of frontline imatinib and switched to nilotinib, to investigate their molecular kinetics and clonal evolution in relation to treatment response.

Materials and Methods

Patients and samples. Forty-five patients were enrolled in a multi-center study, called "Study to Evaluate Nilotinib in CML Patients With Suboptimal Response (SENSOR; ClinicalTrials.gov identifier: NCT01043874)."[17] These patients had achieved a suboptimal MR but not a major MR (MMR or MR3.0, *BCR-ABL* to *ABL* ratio on the International Scale [IS] ≤0.1%), with frontline imatinib, and were switched to nilotinib 400 mg twice daily for 24 months to acquire further deep MR. MR was evaluated at baseline, monthly for months 1 to 3, and every 3 months thereafter through 24 months by a central laboratory (BML, Japan) through qRT-PCR testing of peripheral blood samples using the MolecularMD One-Step qRT-PCR BCR-ABL Kit (MolecularMD, MA, USA). The assay, using the International Scale with *ABL1* as a control gene, was sensitive to 4.5 0logs (*BCR-ABL/ABL* <0.0032%).[17] MR4.0 and MR4.5 were defined as *BCR-ABL/ABL* <0.01% and <0.0032%, respectively.[17] The remaining RNA samples were cryopreserved with strict quality control for the following long-range nested RT-PCR and UDS analyses described herein. Informed consent for further molecular analysis of *BCR-ABL* was obtained in 37 out of 45 patients enrolled in the SENSOR trial. This study was conducted in accordance with the latest Declaration of Helsinki, Good Clinical Practice and Good Post-Marketing Study Practice guidelines.

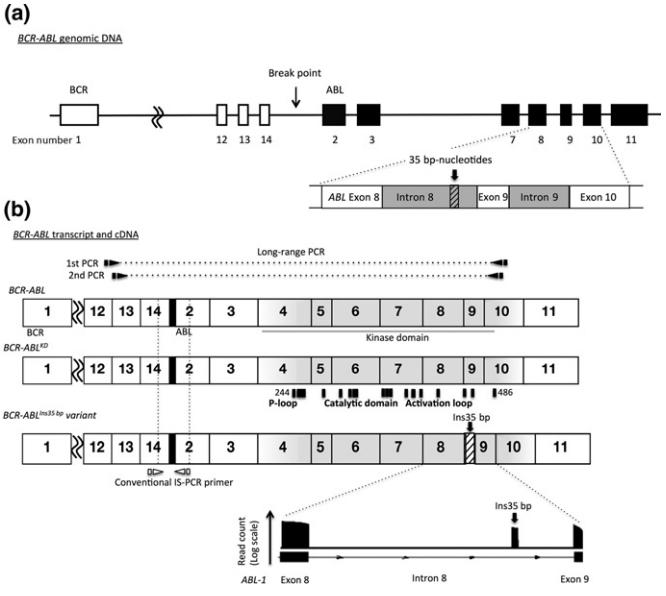

Fig. 1. Alternatively spliced *BCR-ABL*^{ins35bp} variant. (a) Schematic of *BCR-ABL*^{Ins35bp} showing 35 intronic nucleotides in *ABL* intron 8 that are not spliced out, but retained at the splice junction between exons 8 and 9. This results in a stop codon after 10 intron-encoded residues and generation of truncated *BCR-ABL* protein without tyrosine kinase activity (see the text). (b) Quantification of *BCR-ABL*^{Ins35bp} using long-range nested PCR and UDS. Conventional qRT-PCR amplifies a short length of approximately 150 bp spanning the breakpoint of *BCR* and *ABL* (open arrows), and, therefore, cannot distinguish between "non-mutated" and mutated *BCR-ABL* transcripts. PCR products amplified by long-range nested RT-PCR (filled arrows) contain mutation sites, such as *BCR-ABL*^{Ins35bp} and KD mutations. UDS analysis of PCR products provides the proportion of "non-mutated" *BCR-ABL*, *BCR-ABL*^{Ins35bp} and KD mutations, which enables estimation of the amount of *BCR-ABL*^{Ins35bp} (eINS35bp index) and KD mutations (eKD index) by multiplying their proportion by total IS *BCR-ABL*.

Long-range nested RT-PCR of *BCR-ABL* transcripts. Long-range nested RT-PCR analysis was performed to amplify *BCR-ABL* cDNA including all mutational sites in *BCR-ABL*^{Ins35bp} and *BCR-ABL* KD mutations categorized as being "resistant to TKI" or of "unknown sensitivity" by European LeukemiaNet.[18] For this purpose, the outer set of forward and reverse primers were designed on the border of *BCR* exons 12–13 (5′-GGAGCAGCAGAAGAAGTGTTTCAGA-3′) and *ABL* exon 10 (5′-GCTGTGAGTACCTTGCTGCAGGC-3′), respectively (Fig. 1b). The inner set of forward and reverse primers were also set on the border of *BCR* exon 13 (5′-GATGCTGACCAACTCGTGTG-3′) and a reverse primer on *ABL* exon 9 (5′-GAAATCCACCAAGCCTTTGA-3′). These primers would amplify both e13a2 and e14a2 *BCR-ABL* fusion genes. Amplification was performed with PrimeSTAR GXL DNA Polymerase (TAKARA-Bio, Japan)[19] using 20 cycles of denaturation (94°C, 30 s), annealing (54°C, 30 s) and extension (68°C, 2 min) for the first PCR, followed by 30 cycles of denaturation (94°C, 30 s), annealing (58°C, 30 s) and extension (68°C, 2 min) for the second PCR. Final amplicon length for e13a2 and e14a2 would be 1569 and 1545 bp, respectively. PCR products were electrophoresed on a 2% agarose gel to confirm successful PCR amplification. Nonspecific products and primer dimers were excluded by gel electrophoresis.

Deep sequencing of *BCR-ABL* transcripts. Amplicons were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). A sequencing library of *BCR-ABL* transcripts was prepared using Nextera technology (Illumina, San Diego, CA, USA), and was subjected to UDS using HiSeq1500 (Illumina) according to the manufacturer's recommendations. Sequencing results were aligned to the reference human genome (GRCh37/hg19; February 2009) using Bowtie[20] and TopHat2[21] software. Frequencies of AS *BCR-ABL*^{Ins35bp} and *BCR-ABL* KD mutants were determined using SAMtools software.[22,23] Sequence coverage of each exon and intron was calculated using Bedtools software.[24] ANNOVAR software was used to annotate the genetic variants[25] with information including gene annotations, amino acid change annotations, Single Nucleotids Polymorphism Database (dbSNP) identifiers and allele frequencies (based on the 1000 Genomes Project). Deep sequencing result from the sample in which average sequence coverage of ABL exon8 exceeded 1000 reads was considered to be reliable for further analysis.

Titration assay of PCR amplification of *BCR-ABL* and *BCR-ABL*^{Ins35bp}. To evaluate whether *BCR-ABL* and *BCR-ABL*^{Ins35bp}

transcripts could be equally amplified under identical conditions, we performed long-range nested PCR using a mixture of plasmid DNA containing *BCR-ABL*^{Ins35bp} and *BCR-ABL*. *BCR-ABL*^{Ins35bp} was serially diluted into *BCR-ABL* at concentrations of 100, 50, 20, 10, 1 and 0% with a total plasmid amount of 100 copies. Long-range nested PCR followed by UDS analysis was performed in triplicate, to determine whether the number of *BCR-ABL*^{Ins35bp} transcripts detected corresponded to the dilution. These experiments established the linearity of the assay over three orders of magnitude with reproducibility (slope of calibration curves = 1.0043, $r = 0.99$: Fig. S1), and demonstrated that *BCR-ABL*^{Ins35bp} and *BCR-ABL* are amplified with equal efficiency by long-range nested PCR.

Quantification of *BCR-ABL* transcripts in samples. Using the established long-range nested PCR and UDS methods, we analyzed proportions of KD mutations, *BCR-ABL*^{Ins35bp} and *BCR-ABL* without these mutations (defined as "non-mutated" *BCR-ABL*) per total IS *BCR-ABL* in 422 frozen samples from 37 patients collected at 0 (baseline), 1, 2, 3, 6, 9, 12, 15, 18, 21 and 24 months after switching to nilotinib. In all of these samples, the absolute amount of residual IS *BCR-ABL* had been previously measured by qRT-PCR in the central laboratory, as mentioned above. An example demonstrating how quantification of the *BCR-ABL* mutants and "non-mutated" *BCR-ABL* was performed is shown below. For patient A at baseline analysis, IS *BCR-ABL* and proportion of *BCR-ABL*^{Ins35bp} to total *BCR-ABL* were 0.1100 and 1%, respectively; thereafter, at 12 months after switching to nilotinib, the sequential analyses demonstrated that his IS *BCR-ABL* and proportion of *BCR-ABL*^{Ins35bp} were 0.0100% and 15%, respectively. Based on this, a 1-log reduction from baseline in IS *BCR-ABL* at 12 months is obvious; however, whether the absolute amount of *BCR-ABL*^{Ins35bp} increased or decreased is less apparent. Therefore, we estimated the amount of *BCR-ABL*^{Ins35bp} at a given time point by multiplying its proportion of the total *BCR-ABL*, as determined by UDS, by the IS *BCR-ABL* at that time point. Thus, to make it clearly visualized, the estimated amount of *BCR-ABL*^{Ins35bp} in this example would be 0.001100% at baseline and 0.001500% at 12 months, indicating a slight increase in *BCR-ABL*^{Ins35bp} despite nilotinib treatment. In the present study, we substituted these estimated index values of *BCR-ABL*^{Ins35bp} (eINS35bp) and KD mutants (eKD) for their actual amounts, which enabled us to evaluate the abilities of residual *BCR-ABL*^{Ins35bp} and KD mutants to affect the achievement of DMR.

Statistical analysis. The statistical significance of differences in distributions, repeated measures and correlations were analyzed using two-sided non-parametric methods (e.g., Wilcoxon, Kruskal–Wallis rank tests), as appropriate. Comparisons between groups were made using the Mann–Whitney *U*-test with Bonferroni correction. *P*-values < 0.05 were considered statistically significant. The relationship between two paired groups was analyzed using a Pearson correlation coefficient.

Results

Mutational analyses at baseline. Patients are categorized based on the MRD status at 24 months after switching from imatinib to nilotinib. Among 37 patients enrolled in this study, 3 patients achieved MR4.5 (MR4.5 group) and 24 achieved MR3.0 (MR3.0 group), whereas the remaining 10 did not achieve MR3.0 (non-MR3.0 group) (Table 1). No significant difference in time since diagnosis of CML or duration of prior imatinib treatment was observed between the three groups (Table 1).

We first quantitatively and qualitatively assessed the mutational status of *BCR-ABL* at baseline in patients with suboptimal MR to frontline imatinib treatment. Surprisingly, our long-range nested PCR and UDS methods detected *BCR-ABL*^{Ins35bp}, in various amounts, in all of 37 patients (Table 2, Fig. S2). The mean proportion of *BCR-ABL*^{Ins35bp} per total *BCR-ABL* was 15.14% (range, 0.01 to 78.08%), and the mean eINS35bp index value, determined by multiplying the proportion of *BCR-ABL*^{Ins35bp} by IS BCR-ABL, was 0.040106% (range, 0.000039–0.873228%). There was a significant correlation between IS *BCR-ABL* and eINS35bp index among 37 patients at baseline ($r = 0.857$), suggesting the possible contribution of the high load of *BCR-ABL*^{Ins35bp} to the failure of acquiring MMR (IS *BCR-ABL* >0.1%) under imatinib treatment in these patients. Of note, when molecular response was evaluated focusing on the "non-mutated" *BCR-ABL* and KD mutations (i.e. excluding *BCR-ABL*^{Ins35bp}), 3 out of 37 (8%) patients (#3, #27 and #37: Fig. S2) presented less than 0.1% of IS *BCR-ABL* at baseline, indicating that these patients would have been categorized as having MMR if *BCR-ABL*^{Ins35bp} was regarded as "function-dead" and excluded from the value determining molecular response. In contrast, *BCR-ABL* KD mutations were simultaneously documented with *BCR-ABL*^{Ins35bp} in 5 of 37 patients at baseline. Three mutations were identified as nilotinib-resistant E255K, and imatinib-resistant M244V and E459K mutations (patients #4, #15 and #11, respectively), whereas the remaining two possessed KD mutations with unknown clinical significance (K415R, patient #3; N368K, patients #22) (Table 2; Fig. S2). Estimated KD mutation indices were 0.386621% with E255K, 0.109622% with M244V, and 0.161221% with E459K, which were relatively

Table 1. Comparison of the amount of IS BCR-ABL and eINS35bp index among MR4.5, MR3.0 and non-MR3.0 groups

	Molecular response at 24 months after switching to nilotinib			
	MR4.5 ($n = 3$)	MR3.0 ($n = 24$)	Non-MR3.0 ($n = 10$)	*P*-value (≥MR3.0 *vs* non-MR3.0)
Median time since diagnosis (range), months	20.4 (17.7–49.3)	31.1 (17.3–98.2)	20.4 (17.5–41.4)	$P = 0.854$
Median duration of prior IM use (range), months	20.0 (17.5–47.6)	28.11 (17.1–97.6)	20.6 (17.4–40.2)	$P = 0.177$
IS *BCR-ABL* at baseline (mean ± SD), %	0.2907 ± 0.1636	0.3336 ± 0.5283	1.2921 ± 1.1815	$P < 0.01$
eINS35bp index at baseline (mean ± SD), %	0.182240 ± 0.162842	0.05368 ± 0.108577	0.25939 ± 0.102610	$P < 0.01$
Cumulative amount of IS *BCR-ABL* 0-24 months (mean ± SD), %	0.3810 ± 0.0175	1.7805 ± 2.0436	7.0567 ± 15.0422	$P < 0.01$
Cumulative amount of eINS35bp index 0–24 months (mean ± SD), %	0.1441 ± 0.0521	0.2403 ± 0.5891	1.2871 ± 3.7042	$P < 0.01$

Table 2. Comparison of mutations detected by UDS among MR4.5, MR3.0, and non-MR3.0 groups

Molecular response at 24 months	At baseline			After baseline (Between 1–24 months of nilotinib treatment)		
	MR4.5 (n = 3)	MR3.0 (n = 24)	Non-MR3.0 (n = 10)	MR4.5 (n = 3)	MR3.0 (n = 24)	Non-MR3.0 (n = 10)
BCR-ABL^{Ins35bp}	3	24	10	3	24	10
BCR-ABL$^{KD\ mutations}$						
Nilotinib-resistant	0	1*[2]	0	0	1*[2]	0
Imatinib-resistant	0	2*[3]	0	0	2*[6]	0
Undetermined for clinical significant	1*[1]	1*[4]	0	2*[5]	13*[7]	1*[8]

Asterisks indicate the kinds of *BCR-ABL* kinase domain mutations as follows: *1: K415R, *2: E255K, *3: M244V, E459K, *4: N368K, *5: K378N, E409G *6: G250R, E459K, *7: L201F, P230L, Y232A, G249C, G251V, G259S, G259V, E308D, V335L H375R, F401L, T406A, L429P, S438F, S446F L452I, Stop gain, Synonymous, *8: K219N.

high compared to those in the 2 patients with KD mutations of unknown clinical significance (K415R, 0.25275%; N368K, 0.021706%).

Serial change in *BCR-ABL* transcripts after switching to nilotinib. We quantified the amounts of "non-mutated" *BCR-ABL*, *BCR-ABL*^{Ins35bp} and KD mutations during nilotinib treatment in 37 patients (Fig. S2). Representative examples are shown in Figure 2. In patient #2, who had been treated with imatinib for 47 months, his IS *BCR-ABL* at baseline was detected as 0.2381%. *BCR-ABL*^{Ins35bp} constituted 37% of IS *BCR-ABL*, and eINS35bp was counted as 0.088684% (Fig. 2a). The switch from imatinib into nilotinib rapidly reduced both "non-mutated" *BCR-ABL* and *BCR-ABL*^{Ins35bp} within the first 3 months. At 6 months after switching, three types of KD mutations, G251V, K378N and E409G, were detected at low levels (eKD mutation indices of 0.001148, 0.001210 and 0.001000%, respectively), although their clinical significance in terms of TKI sensitivity was undetermined. These KD mutations were detected only once, and never reappeared throughout the clinical course. Thereafter, the patient had successfully maintained MR4.5 in response to nilotinib (Fig. 2a).

In contrast, in patient #9, the amount of IS *BCR-ABL* decreased within the first 3 months after switching to nilotinib; however, *BCR-ABL*^{Ins35bp} was continuously detected thereafter, with the eINS35bp index fluctuating between 0.1% and >0.0032% (Fig. 2b). At 24 months of nilotinib treatment, eINS35bp was counted as 0.048656%, which accounted for approximately 58% of total IS *BCR-ABL* (0.0833%). The

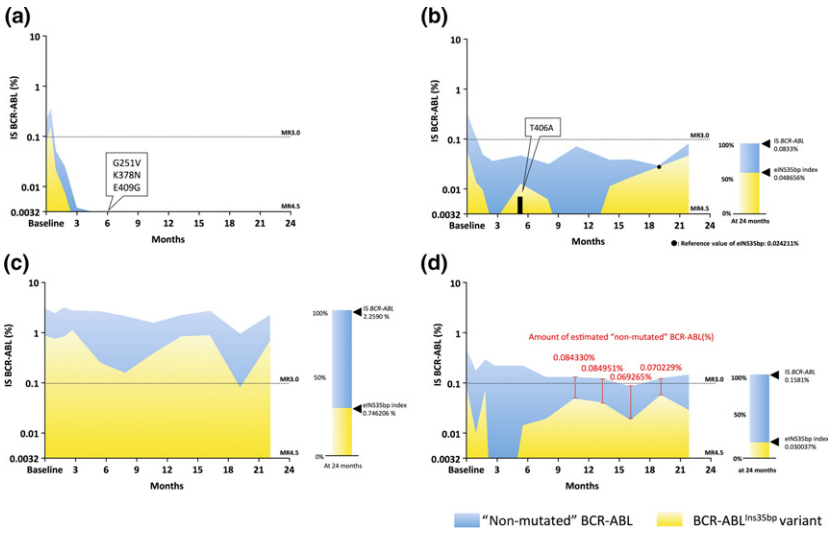

Fig. 2. Serial changes in *BCR-ABL* transcripts over time after the switch to nilotinib. Total amounts of IS *BCR-ABL* by quantitative RT-PCR, and proportions of *BCR-ABL*^{Ins35bp} and KD mutations by UDS were serially measured at 0, 1, 2, 3, 6, 9, 12, 15, 18, 21 and 24 months after the switch to nilotinib. Results are shown for 4 representative patients. Blue and yellow line graphs indicate IS *BCR-ABL* and eINS35bp index, respectively. Bar graphs at the right edge indicate amounts of estimated "non-mutated" *BCR-ABL* and eINS35bp index at 24 months of switchover to nilotinib, at which time molecular response levels in response to nilotinib were determined. (a) Achievement of MR4.5 (patient #2). At baseline, IS *BCR-ABL* was 0.2381%, of which *BCR-ABL*^{Ins35bp} constituted 37%. The switch to nilotinib quickly cleared both "non-mutated" *BCR-ABL* and *BCR-ABL*^{Ins35bp} within 6 months. *BCR-ABL* KD mutations with unknown TKI sensitivity were transiently detected but quickly disappeared. (b) Achievement of MR3.0 but not MR4.5 (patient #9). Nilotinib treatment decreased IS *BCR-ABL*, whereas the eINS35bp index was fluctuating at low levels between 0.1% and <0.0032% of IS. At 24 months, residual IS *BCR-ABL* was 0.0833%, of which BCR-ABL^{Ins35bp} constituted approximately 60%. (c) No achievement of MR3.0 (patient #34). Despite the switch to nilotinib, the amount of estimated "non-mutated" *BCR-ABL* and the eINS35bp index did not decrease below 0.1% by 24 months. (d) IS *BCR-ABL* exceeded >0.1% throughout the clinical course, except at 18 months, and *BCR-ABL*^{Ins35bp} was continuously detected (patient #31). Estimated "non-mutated" *BCR-ABL* levels, calculated by subtracting the eINS35bp index from IS *BCR-ABL*, was 0.084330% at 12 months, 0.0084951% at 15 months, 0.069265% at 18 months and 0.070229% at 21 months, indicating that the patient had attained MR3.0 at 4 measurement points if *BCR-ABL*^{Ins35bp} were excluded from MRD.

eINS35bp index solely exceeded 0.0032%; thus, this patient finally did not achieve MR4.5 and had molecular response that remained just below MR3.0 (Fig. 2b). At 6 months, *BCR-ABL*^{T406A}, which was also undetermined for clinical significance, was detected as 0.007052% of eKD mutation index, but its detection was transient and did not affect molecular response (Fig. 2b).

In contrast, in patient #31, both IS *BCR-ABL* and eINS35bp index, which were detected at relatively high levels (3.1138 and 0.873228%, respectively) at baseline, did not decrease below 0.1% throughout the duration of nilotinib treatment; this patient had not achieved even MR 3.0 (Fig. 2c). In another patient (#34) from the non-MR3.0 group, IS *BCR-ABL* did not decrease below 0.1% throughout the clinical course, except at 18 months (Fig. 2d). The eINS35bp index fluctuated around the relatively high levels between 0.1% and >0.0032%. In addition, KD mutations were not detected during this patient's clinical course. Thus, we estimated "non-mutated" *BCR-ABL* by subtracting from IS *BCR-ABL* to eINS35bp index at each measurement point. Amounts of "non-mutated" *BCR-ABL* were estimated as 0.084330% at 12 months, 0.084951% at 15 months, 0.069265% at 18 months and 0.070229% at 21 months, suggesting that this patient had attained MR3.0 at least at four measurement points, if *BCR-ABL*^{Ins35bp} were excluded from determination of the molecular response level due to being "function-dead." In total, 3 of 10 patients in the non-MR3.0 group (#34, #36 and #37: Fig. S2) exhibited less than 0.10% of the estimated "non-mutated" *BCR-ABL* level at more than 1 point throughout their clinical courses.

During nilotinib treatment, various types of KD mutations were detectable in 19 patients, including 1 with nilotinib-resistant E255K (#4), 2 with imatinib-resistant G250R (#25) and E459K (#11), and 16 with mutations of unknown clinical significance (Table 2 and Fig. S2). In contrast to *BCR-ABL*^{Ins35bp}, the vast majority of these KD mutations were transiently documented (at one or a few time points during time courses, Fig. S2) and did not affect the overall molecular responses.

Molecular response after switching to nilotinib. Based on the molecular response after 24 months of nilotinib treatment, 37 patients were divided into three groups to evaluate factors affecting acquisition of DMR: an MR4.5 group, $n = 3$; an MR3.0 group, $n = 27$ (including those who achieved MR4.5); and a non-MR3.0 group, $n = 10$. Figure 3a shows serial changes in IS *BCR-ABL* over time in each of the three groups. At baseline, mean levels of IS *BCR-ABL* were higher in the non-MR3.0 group ($1.2921 \pm 1.1815\%$ (mean $\pm$ SD)) than those in the MR4.5 ($0.2907 \pm 0.1636\%$) and MR3.0 ($0.3336 \pm 0.5283\%$) groups ($P < 0.01$; Table 1 and Fig. 3a), indicating that the non-MR3.0 group loaded higher leukemic burden at baseline. After switching to nilotinib, IS *BCR-ABL* were significantly decreased in MR4.5 and MR3.0 groups at 3, 6, 9, 12 and 24 months compared to that at baseline ($P < 0.01$). In contrast, in the non-MR3.0 group, switching to nilotinib gradually decreased IS *BCR-ABL*. After 24 months of nilotinib treatment, mean levels of IS *BCR-ABL* were decreased down to an undetectable level in the MR4.5 group, $0.0505 \pm 0.0278\%$ in the MR3.0 group ($P < 0.01$) and $0.4881 \pm 0.6536\%$ in the non-MR3.0 group ($P < 0.01$) compared to that at baseline. Reduction of IS *BCR-ABL* between 0 and 24 months of nilotinib treatment was documented in all three groups (Fig. 3c,d). The amount of decrease in IS *BCR-ABL* was the greatest in the non-MR3.0 group ($-0.8039 \pm 0.1636\%$), compared to that in the MR4.5 ($-0.2875 \pm 0.1636\%$) and MR3.0 ($-0.3014 \pm 0.5582\%$) groups ($P < 0.01$, $\geq$MR3.0 *vs* non-MR3.0; Fig. 3c), although the rate of reduction in IS *BCR-ABL* was comparable in these three groups ($-58.8 \pm 24.5\%$ in the non-MR3.0, $-75.1 \pm 16.3\%$ in the MR3.0 and $-98.7 \pm 0.7\%$ in the MR4.5 group; Fig. 3d).

We next analyzed serial changes in the eINS35bp index over time in three groups (Fig. 3b). At baseline, the mean level of the eINS35bp index was significantly higher in the non-MR3.0 group ($0.259390 \pm 0.301489\%$) than that in MR3.0 group ($0.053680 \pm 0.108577\%$, $P < 0.01$). After the switchover, similar to the decreased slope observed in IS *BCR-ABL*, the eINS35bp index was significantly decreased along with nilotinib treatment in the MR3.0 group, while the decrease of the eINS35bp index was more gradual in the

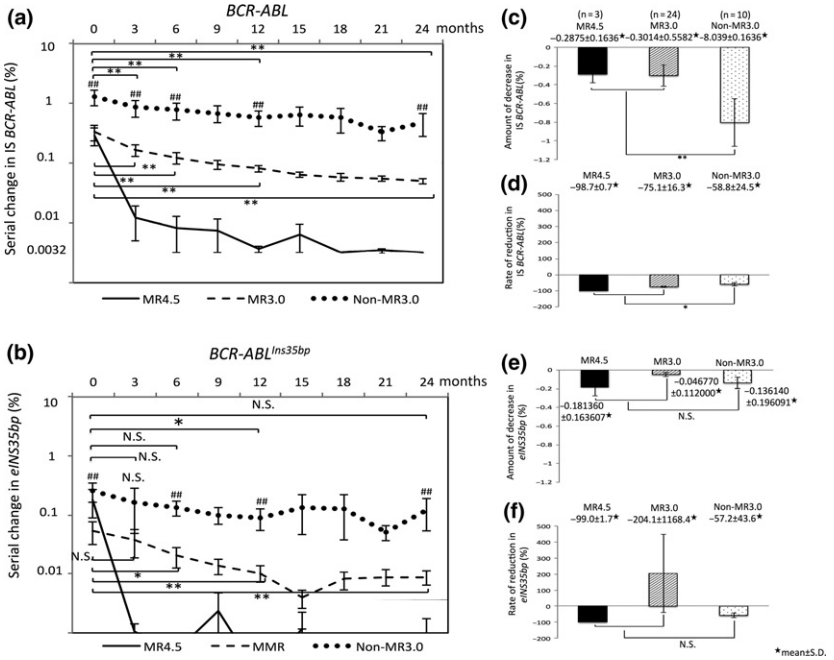

Fig. 3. Change in *BCR-ABL* transcripts. (a,b) Serial change in IS *BCR-ABL* (a) and the eINS35bp index (b) over time between 0 and 24 months of nilotinib treatment. Dotted, dashed and solid line graphs indicate IS *BCR-ABL* in MR4.5, MR3.0 and non-MR3.0 groups, respectively. A rapid reduction in the IS *BCR-ABL* and the INS35bp index was evident in the MR4.5 group. The IS *BCR-ABL* and the eINS35bp index were significantly decreased in the MR3.0 group, while their gradual decrease was documented in the non-MR3.0 group. *,**Significant decrease compared to level at baseline ($*P < 0.05$, $**P < 0.01$). #,##Significant difference between MR3.0 and non-MR3.0 group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). (c–f) Total amount of decrease and reduction rate of IS *BCR-ABL* (c,d) and the eINS35bp index (e,f) from baseline to 24 months of nilotinib treatment in each group. The amount of decrease in IS *BCR-ABL* was greater in the non-MR3.0 group, while no significant differences between groups were seen in the decrease of the eINS35bp index. For the reduction rate, no significant differences were observed between the three groups for both the *BCR-ABL* and the eINS35bp index. *indicates statistical significance.

non-MR3.0 group (Fig. 3b). In contrast, in the MR4.5 group, switching to nilotinib dramatically decreased the eINS35bp index to undetectable levels in the first 3 months, contributing to the acquisition of DMR in part (Fig. 3b). We found no significant differences between the three groups in the magnitude of decrease or the reduction rate of the eINS35bp index between 0 and 24 months of nilotinib treatment (Fig. 3e,f).

Reduction in *BCR-ABL* transcripts in individual patients. We analyzed decreases in IS *BCR-ABL* and eINS35bp indexes in each individual. IS *BCR-ABL* was reduced in all of the patients, irrespective of molecular response status after 24 months of nilotinib treatment (Fig. 4a,c). In contrast, lower decreases in eINS35bp index relative to *BCR-ABL* were seen ($P < 0.01$) (Fig. 4b,d). The amount of the eINS35bp index was reduced in all 3 patients in the MR4.5 group, in 19 of the 24 patients in the MR3.0 group, and in 8 of the 10 patients in non-MR3.0 group (Fig. 4d). The remaining 5 and 2 patients in the MR3.0 and non-MR3.0 groups, respectively, exhibited increases in the eINS35bp index after 24 months of nilotinib treatment, although the total amount of IS *BCR-ABL* was decreased in these patients (Fig. 4d). Collectively, these results suggested that persistence of $BCR\text{-}ABL^{Ins35bp}$ resulted in failure to achieve DMR.

Effect of cumulative amount of $BCR\text{-}ABL^{Ins35bp}$ on molecular response. We examined the relationship between molecular response and cumulative amounts of IS *BCR-ABL* as well as the eINS35bp index from baseline to 24 months. As expected, the mean cumulative amount of IS *BCR-ABL* was greatest in the non-MR3.0 group ($7.0567 \pm 15.0422\%$) compared to that in the MR4.5 group ($0.3810 \pm 0.0175\%$) and in the MR3.0 group ($1.7805 \pm 2.0436\%$), respectively ($P < 0.01$, Table 1). Similarly, the mean cumulative amount of the eINS35bp index was $0.1441 \pm 0.0521\%$, $0.2403 \pm 0.589\%$ and $1.2871 \pm 3.7042\%$ in the MR4.5, MR3.0 and non-MR3.0 groups, respectively ($P < 0.01$, Table 1). These results indicated that attainment of deeper MR was inversely correlated with the cumulative amount of *BCR-ABL* as well as $BCR\text{-}ABL^{Ins35b}$ under nilotinib treatment. In addition, a significant correlation was found between cumulative amounts of *BCR-ABL* and $BCR\text{-}ABL^{Ins35bp}$, suggesting that persistence of $BCR\text{-}ABL^{Ins35bp}$ reflected a failure of achievement of DMR ($P < 0.01$, $r = 0.837$, Fig. S3).

Discussion

Quantitative PCR targeting disease-specific molecules has provided a deep understanding of current patients' status in hematological malignancies.[26,27] In clinical practice, PCR detection of recurrent leukemia-specific chimeric genes (e.g. PML-RARA, AML1-ETO and CBFB-MYH11) in acute myelocytic leukemia (AML), or clonal immunoglobulin and T-cell receptor gene rearrangements in acute lymphocytic leukemia is closely associated with treatment response.[26–33] Therefore, MRD monitoring based on qPCR, with its increased sensitivity, plays a decisive role in treatment stratification in these leukemias as part of routine clinical use.[26,27,34,35] In CML, based on landmark analyses in large TKI trials, European LeukemiaNet (ELN) recommends stratified treatment in determining the effectiveness of TKI based on molecular IS *BCR-ABL* milestones at each time point, such as at 3, 6 and 12 months in clinical practice.[11,18] Currently, IS *BCR-ABL* monitoring by qPCR has become an indispensable indicator to guide early intervention for preventing relapse or disease progression in CML.[11,18] Nevertheless, the present study critically pointed out the possible pitfalls in interpreting MRD specifically in CML.

This study demonstrated that conventional qPCR amplifies both *BCR-ABL* and mutated *BCR-ABL* transcripts; consequently, IS *BCR-ABL* includes $BCR\text{-}ABL^{Ins35bp}$. Previous *in vitro* studies have suggested that alternatively spliced $BCR\text{-}ABL^{Ins35bp}$ results in premature termination in the *ABL* KD, leading to loss of leukemic proliferation as well as escape from TKI binding due to conformational change.[6,15] Accordingly, cells expressing $BCR\text{-}ABL^{Ins35bp}$ might survive TKI treatment but might not proliferate; thus persistence of $BCR\text{-}ABL^{Ins35bp}$ would not definitively reflect the disease activity of CML. Our findings indicate that the IS *BCR-ABL* measured by conventional qPCR includes non-functional *BCR-ABL* variants.

In the present study, $BCR\text{-}ABL^{Ins35bp}$ was detected at various levels in all of the patients with suboptimal response (IS *BCR-ABL* >0.1%) after ≥18 months of the frontline imatinib treatment. Therefore, if molecular responses were re-evaluated

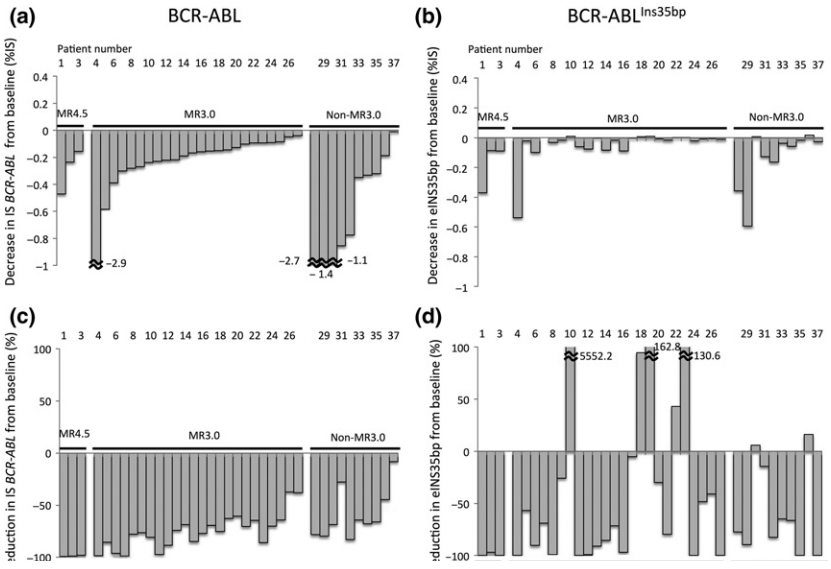

Fig. 4. Reduction in *BCR-ABL* transcript in individual. (a,c) Each bar indicates the amount of decrease (a) and reduction rate (c) for IS *BCR-ABL* between 0 and 24 months of nilotinib treatment in 37 individual patients. Reduction in IS *BCR-ABL* was observed in all patients irrespective of molecular response (a,c). In contrast, the decrease in the eINS35bp index was of lesser magnitude compared with IS *BCR-ABL* ($P < 0.01$; b,d). The decrease in the eINS35bp index was evident in 30 of 37 patients; in the remaining 7 patients, the eINS35bp index increased after 24 months of nilotinib treatment compared with that at baseline (d).

based on the "non-mutated" *BCR-ABL* levels, 3 of 37 (8%) patients would have IS "non-mutated" *BCR-ABL*<0.1% at baseline. These patients would then have been considered to have achieved MR3.0 and might not have needed to switch to nilotinib. According to ELN guidelines based on IS *BCR-ABL*,[11,18] patients with a relatively high burden of *BCR-ABL*^{Ins35bp} could potentially be improperly categorized as having lesser molecular response. Therefore, quantification of *BCR-ABL*^{Ins35bp} might be required in a fraction of patients who have not achieved DMR; otherwise these patients would be then given another treatments. It is also possible to extend candidates for TKI discontinuation in the patients whose IS *BCR-ABL* fluctuate around CMR but whose levels of "non-mutated" *BCR-ABL* have persistently remained below CMR. In addition, after TKI discontinuation, if the whole IS *BCR-ABL* level increases over the level defined as molecular relapse, it will not be required to restart TKI in the patients whose *BCR-ABL*^{Ins35bp} increases but "non-mutated" *BCR-ABL* does not. Thus, quantification of "function-dead" *BCR-ABL*^{Ins35bp} would be important to promote TKI discontinuation trial, because molecular relapse has been documented in 40–60% of the patients who had discontinued TKI.[36–39]

In the current study, after switching into nilotinib, *BCR-ABL* and *BCR-ABL*^{Ins35bp} rapidly decreased to below detectable levels by qPCR in the 3 responders (MR4.5 group); these patients finally achieved MR4.5 after 24 months. In contrast, 34 of 37 patients did not gain MR4.5 after switching to nilotinib, although all had reduced IS *BCR-ABL* (Fig. 4a,c). During nilotinib treatment in these patients, their *BCR-ABL*^{Ins35bp} was continuously detected and MRD fluctuated at low but detectable levels throughout their treatment courses. However, none of these patients lost hematological response or progressed to full relapse during the 24 months of nilotinib treatment. These clinical results also supported the concepts that CML stem cells expressing *BCR-ABL*^{Ins35bp} are not eradicated by TKI, can survive the long-term treatment with TKI,[40–42] and continue to proliferate very slowly, like the manner as quiescent slowly-cycling hematopoietic stem cells with stemness properties. As a result, persistent detection of *BCR-ABL*^{Ins35bp} might hamper attainment of DMR. In addition, it is crucial to clarify whether individual CML cells express both "non-mutated" *BCR-ABL* and *BCR-ABL*^{Ins35bp} or either one. We have extended experiments of expression of these transcripts in individual K562 cell lines (manuscript in preparation).

Three patients in the MR4.5 group might have harbored CML cells expressing both these transcripts at a relatively high rate, which were rapidly cleared in response to nilotinib. In contrast, the patients from the MR3.0 group may have carried a heavier load of cells expressing exclusively *BCR-ABL*^{Ins35bp} during 24 months of nilotinib treatment, because there was no significant difference in the total amounts of IS *BCR-ABL* and *BCR-ABL*^{Ins35bp} (eINS35bp) between the MR4.5 and MR3.0 groups at baseline (Fig. 3a,b). Further studies are needed to clarify whether the expression levels of *BCR-ABL* and *BCR-ABL*^{Ins35bp} differ among individual stem cells and mature cells, and whether these differences may influence MRD in response to TKI treatment.

It has remained unclear how alternatively spliced *BCR-ABL*^{Ins35bp} variants can be acquired in CML cells under TKI treatment, and why mis-splicing reproducibly occurs at the specific sites of intronic 35 bp in *ABL* intron 8. Several reports have demonstrated that *BCR-ABL*^{Ins35bp} is detected mainly in patients who have achieved hematological/cytogenetic response, but not DMR, despite long-term TKI treatment.[6,12,15,43] In contrast, extremely low amounts of *BCR-ABL*^{Ins35bp} were detected in patients with newly-diagnosed, previously untreated CML (data not shown). We found that TKI treatment inhibits recruitment of RNA polymerase α toward genomic BCR-ABL, which positively regulates both transcription and pre-mRNA intron splicing, resulting in a relative increase in the amount of *BCR-ABL*^{Ins35bp} with a decrease of total *BCR-ABL* transcript level. In addition, 35 bp-nucleotides within *ABL* intron 8 are flanked by pseudo-splice sites, suggesting that mis-splicing of specific 35-bp nucleotides reproducibly occurs specific at the *ABL* exon8/9 junction (manuscript in preparation). These findings clearly indicate that TKI might induce the alternatively spliced *BCR-ABL*^{Ins35bp} variant, although acquisition of this variant does not inevitably mark the warning mutation responsible for full relapse. It is possible that TKI cause spliceosomal errors by inducing spliceosomal mutations or inhibiting epigenetic regulation in pre-mRNA splicing.[44–46] Further basic studies are required to elucidate the mechanisms responsible for mis-splicing of *BCR-ABL* pre-mRNA as well as the precise regulation of *BCR-ABL* mRNA splicing under TKI treatment.

In conclusion, using long-range PCR and UDS methods, we demonstrated that TKI treatment might induce *BCR-ABL*^{Ins35bp}, which persists and fluctuates at low levels in patients who have not achieved DMR. Persistence of *BCR-ABL*^{Ins35bp} might result in underestimating the molecular response status in a fraction of these patients, who might have been switched to another TKI treatment. Therefore, it would be helpful for determining the effectiveness of TKI to separately quantify "function-dead" *BCR-ABL*^{Ins35bp} and *BCR-ABL* and KD mutations that contribute to TKI resistance. Further studies are warranted to clarify the clinical significance of *BCR-ABL*^{Ins35bp} and the precise mechanism of its emergence.

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Disclosure Statement

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Titration assay of PCR amplification of *BCR-ABL* and *BCR-ABL*^{Ins35bp}. Serial dilutions of pUC19 plasmid DNA containing *BCR-ABL*^{Ins35bp} into that containing *BCR-ABL* were mixed as 100, 50, 20, 10, 1 and 0% with a total number as 100 copies. Long-range nested PCR

followed by UDS analysis was performed to see if the number of *BCR-ABL*^{Ins35bp} corresponded to the dilution. These results established the linearity of the assay over three orders of magnitude (slope of calibration curves = 1.0043, correlation coefficient = 0.99).

Fig. S2. Serial changes in BCR-ABL transcripts over time in 37 individual patients receiving nilotinib treatment. *BCR-ABL*, *BCR-ABL*^{Ins35bp} and KD mutations were serially quantified by NGS analysis at each measure points in 37 patients after the switch to nilotinib. Blue and yellow line graphs indicate the amount of the IS *BCR-ABL* and the eINS35bp index, respectively. The bar graph at the right edge indicates the estimated "non-mutated" *BCR-ABL* and eINS35bp index at 24 months after the switch to nilotinib: 3 patients achieved MR4.5 (patients #1–3), 24 patients achieved MR3.0 (patients #4–27), while 10 patients did not achieve MR3.0 (patients #28–37).

Fig. S3. Relationship between cumulative amount of IS *BCR-ABL*^{Ins35bp} and eINS35bp index. The *x*-axis and *y*-axis indicate tge cumulative amounts of eINS35bp index and IS *BCR-ABL*, respectively. Circles, triangles and squares indicate individual patients in the MR4.5 group, the MR3.0 group and the non-MR3.0 group, respectively. There was a significant relationship among cumulative amounts of IS *BCR-ABL* and eINS35bp index ($r = 0.837$; $P < 0.01$).