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Tyrosine kinase inhibitors induce alternative spliced *BCR-ABL*^{Ins35bp} variant via inhibition of RNA polymerase II on genomic BCR-ABL

Junichiro Yuda[1] | Jun Odawara[1] | Mariko Minami[1] | Tsuyoshi Muta[2] | Kentaro Kohno[3] | Kazuki Tanimoto[4] | Tetsuya Eto[5] | Takahiro Shima[1] | Yoshikane Kikushige[1] | Koji Kato[1] | Katsuto Takenaka[1] | Hiromi Iwasaki[1] | Yosuke Minami[6] | Yasuyuki Ohkawa[7] | Koichi Akashi[1] | Toshihiro Miyamoto[1]

[1]Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Science, Fukuoka, Japan

[2]Department of Hematology and Oncology, Japan Community Health Care Organization Kyushu Hospital, Fukuoka, Japan

[3]Department of Hematology and Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan

[4]Department of Haematology and Oncology, Japanese Red Cross Society Fukuoka Red Cross Hospital, Fukuoka, Japan

[5]Department of Hematology, Hamanomachi Hospital, Fukuoka, Japan

[6]Department of Hematology, National Cancer Center Hospital East, Kashiwa, Japan

[7]Department of Advanced Medical Initiatives, Kyushu University, Fukuoka, Japan

Correspondence
Toshihiro Miyamoto, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Email: toshmiya@intmed1.med.kyushu-u.ac.jp

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Abstract

To elucidate dynamic changes in native *BCR-ABL* and alternatively spliced tyrosine kinase inhibitor (TKI)-resistant but function-dead *BCR-ABL*^{Ins35bp} variant, following commencement or discontinuation of TKI therapy, each transcript was serially quantified in patients with chronic myeloid leukemia (CML) by deep sequencing. Because both transcripts were amplified together using conventional PCR system for measuring International Scale (IS), deep sequencing method was used for quantifying such *BCR-ABL* variants. At the initial diagnosis, 7 of 9 patients presented a small fraction of cells possessing *BCR-ABL*^{Ins35bp}, accounting for 0.8% of the total IS *BCR-ABL*, corresponding to actual *BCR-ABL*^{Ins35bp} value of 1.1539% IS. TKI rapidly decreased native *BCR-ABL* but not *BCR-ABL*^{Ins35bp}, leading to the initial increase in the proportion of *BCR-ABL*^{Ins35bp}. Thereafter, both native *BCR-ABL* and *BCR-ABL*^{Ins35bp} gradually decreased in the course of TKI treatment, whereas small populations positive for TKI-resistant *BCR-ABL*^{Ins35bp} continued fluctuating at low levels, possibly underestimating the molecular response (MR). Following TKI discontinuation, sequencing analysis of 54 patients revealed a rapid relapse, apparently derived from native *BCR-ABL*$^{+}$ clones. However, IS fluctuating at low levels around MR4.0 marked a predominant persistence of cells expressing function-dead *BCR-ABL*^{Ins35bp}, suggesting that TKI resumption was unnecessary. We clarified the possible mechanism underlying mis-splicing *BCR-ABL*^{Ins35bp}, occurring at the particular pseudo-splice site within intron8, which can be augmented by TKI treatment through inhibition of RNA polymerase II phosphorylation. No mutations were found in spliceosomal genes. Therefore, monitoring IS functional *BCR-ABL* extracting *BCR-ABL*^{Ins35bp} would lead us to a correct evaluation of MR status, thus determining the adequate therapeutic intervention.

KEYWORDS

alternative splicing, BCR-ABL, BCR-ABL^{Ins35bp}, chronic myeloid leukemia, MRD

1 | INTRODUCTION

Tyrosine kinase inhibitors (TKI) targeting *BCR-ABL* have resulted in a drastic paradigm shift in the treatment of patients with chronic myeloid leukemia (CML).[1,2] Achievement of a rapid deep molecular response (DMR) is desirable, as it improves long-term outcomes.[2,3] Moreover, cessation of TKI treatment has emerged as an ultimate goal of management for CML in the chronic phase (CML-CP).[4-7]

Several studies have detected alternatively spliced *BCR-ABL* variants in patients undergoing TKI treatment, among which the most frequently found has been *BCR-ABL*^{Ins35bp}.[8-10] This finding occurs in particular in patients who have failed to achieve DMR under relatively long-term TKI treatment and is rare in newly diagnosed patients.[11-17] *BCR-ABL*^{Ins35bp} is reproducibly generated by insertion of the specific 35 bp nucleotides derived from ABL intron 8 at the exon 8/9 splice junction[8-10] (Figure 1A). Retention of 35 bp nucleotides introduces a stop codon, resulting in a frame shift that leads to the addition of 10 intron-encoded residues and truncation of 653 residues. Prematurely terminated BCR-ABL protein lacks tyrosine kinase activity[9,12] and such premature termination induces a conformational change, hindering TKI from binding to the ATP binding site, in a similar manner to that observed in BCR-ABL^{T315I} mutations.[10] Therefore, cells harboring "TKI-resistant" but "function-dead" *BCR-ABL*^{Ins35bp} are not eradicated and can survive under TKI treatment, although they do not proliferate aggressively in a leukemic fashion. Because *BCR-ABL* and *BCR-ABL*^{Ins35bp} are amplified together by conventional PCR used for assessing International Scale (IS), IS should contain amounts of *BCR-ABL*^{Ins35bp} (Figure 1B). Therefore, a fraction of patients who fail to achieve DMR may have an underestimated MR status.

The aim of the present study was to clarify the mechanism underlying the reproducibility of spliced *BCR-ABL*^{Ins35bp} at the exact intronic 35-bp site of intron 8 under TKI treatment. In addition, to elucidate the clinical significance of *BCR-ABL*^{Ins35bp}, we serially traced amounts of *BCR-ABL* and *BCR-ABL*^{Ins35bp} during TKI treatment, in both newly diagnosed patients and those discontinuing TKI. This may help to accurately determine the necessity of therapeutic intervention in these patients.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

A total of 63 patients with CML-CP were enrolled in this study, including 9 newly diagnosed patients and 54 who had discontinued TKI. Among the newly diagnosed patients, 7 received dasatinib as the initial treatment, whereas 2 received nilotinib (Table 1). The median treatment period was 18 (12-18) months. The patients' characteristics are summarized in Table 1. Fifty-four patients discontinued TKI after sustained DMR for a median of 79.8 (38.9-189.8) months (Table 2). Patient characteristics are shown in Table 2. Blood samples were analyzed monthly during the first 6 months and every 2 months thereafter, to clarify the detailed kinetics of relapse or sustained DMR after TKI cessation. Relapse was defined as loss of complete MR (CMR, MR$^{4.5}$) for two consecutive time points. At the time of our NGS analysis, the median length of follow up was 18 months (range, 8-36) after discontinuation of TKI therapy. Out of 54 (54%) patients, 29 eventually relapsed at a median 4 months (range: 2-13 months) after TKI discontinuation. IS *BCR-ABL* levels were measured in a central laboratory (BML, Japan).[8,18]

This study was conducted in accordance with the Declaration of Helsinki and its amendments, and the Ethical Guidelines for Epidemiological Research by the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labour, and Welfare of Japan. The protocol was approved by the Ethics Committee of Kyushu University (approval nos. 24 033 and 25 132). All patients provided informed consent.

2.2 | Long-range PCR and deep sequencing of *BCR-ABL* transcripts

Long-range nested RT-PCR of *BCR-ABL* transcripts was performed to amplify approximately 1.6 kbp of *BCR-ABL* including all mutational sites in *BCR-ABL*^{Ins35bp} and *BCR-ABL* kinase domain (KD) mutations.[8,19] For this purpose, we performed long-range nested PCR of *BCR-ABL* transcript by using specific primers (Figure 1B, Table S1).[8] In our previous paper,[8] we confirmed that *BCR-ABL* and *BCR-ABL*^{Ins35bp} transcripts are equally amplified under identical conditions, by long-range nested PCR using a mixture of serially diluted plasmid DNAs containing *BCR-ABL*^{Ins35bp} and *BCR-ABL* followed by sequencing analysis.

A sequencing library was prepared using Nextera technology (Illumina), and was subjected to deep sequencing using HiSeq1500 and Miseq (Illumina) according to the manufacturer's instructions. The frequencies of *BCR-ABL*^{Ins35bp} and KD mutants were calculated as previously reported.[8]

2.3 | Deep sequencing of *BCR-ABL* transcripts in samples

Using deep sequencing methods, we analyzed proportions of KD mutations, *BCR-ABL*^{Ins35bp} and *BCR-ABL* without these mutations (defined as native *BCR-ABL*) per total IS *BCR-ABL* in 725 frozen samples obtained from 63 patients. To determine the change in the absolute amount of *BCR-ABL*^{Ins35bp}, we estimated the amount of *BCR-ABL*^{Ins35bp} by multiplying IS *BCR-ABL* by its proportion identified by deep sequencing.[8] The estimated index values of *BCR-ABL*^{Ins35bp} (eINS35bp), native *BCR-ABL* (eNATIVE) and KD mutants (eKD) were replaced for their actual amounts,[8] which enabled us to evaluate the ability of residual *BCR-ABL*^{Ins35bp} and KD mutants to affect MR or relapse after TKI discontinuation.

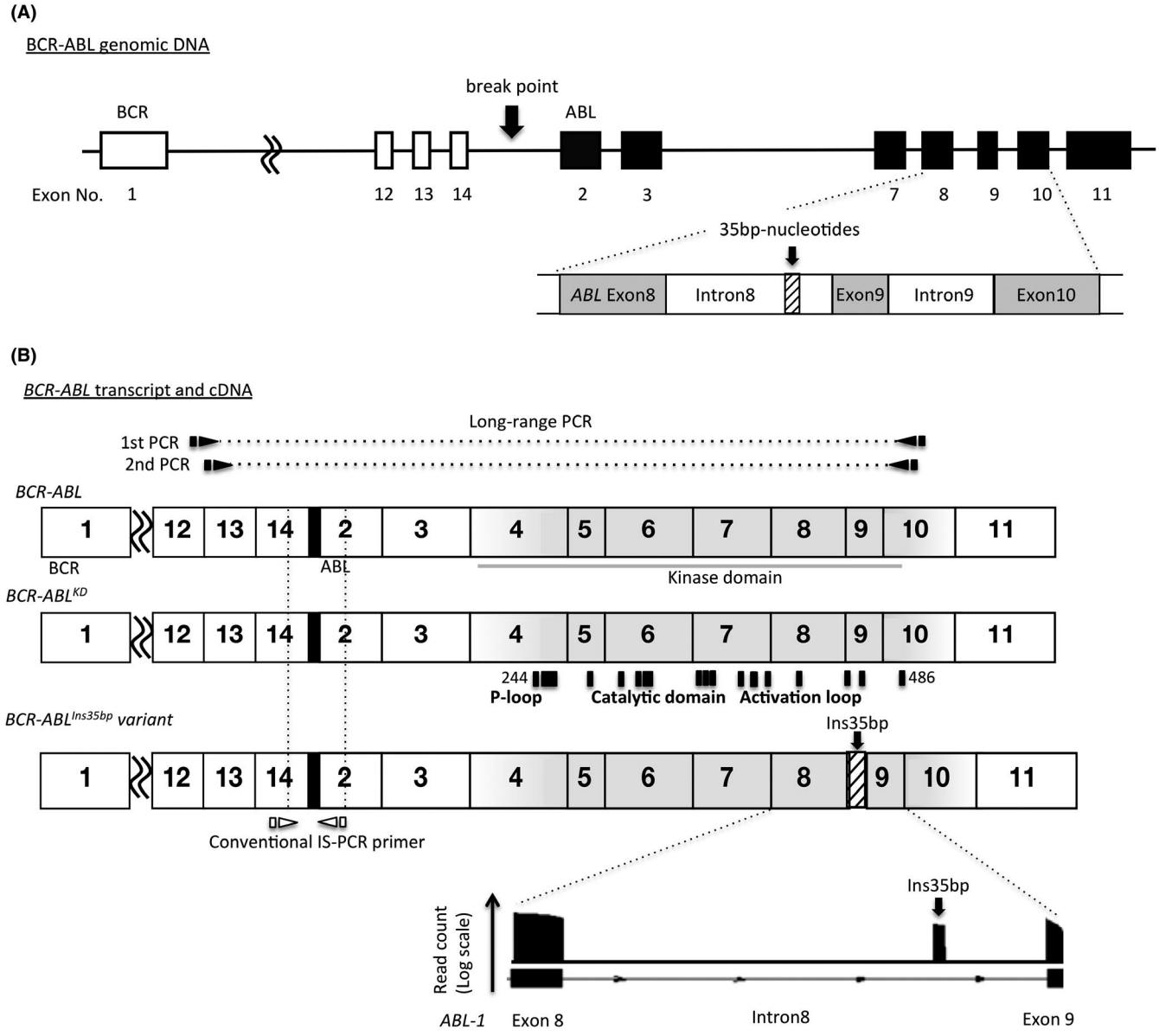

FIGURE 1 Alternatively spliced *BCR-ABL*^{ins35bp} variant. (A) Schematics of *BCR-ABL*^{Ins35bp} showing 35 intronic nucleotides in unspliced *ABL* intron 8, retained at the exon 8/9 splice junction. This results in a stop codon after 10 intron-encoded residues and in the generation of truncated *BCR-ABL* protein without tyrosine kinase activity (see the text). (B) Quantification of *BCR-ABL*^{Ins35bp} using combined long-range nested PCR and deep sequencing. Conventional quantitative RT-PCR amplifies a short length of ≈150 bp spanning the breakpoint of *BCR* and *ABL* (open arrows) and is, therefore, unable to distinguish between native 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 kinase domain (KD) mutations. Deep sequencing analysis provides the proportion of native *BCR-ABL*, *BCR-ABL*^{Ins35bp} and KD mutations, allowing us to estimate the amount of *BCR-ABL*^{Ins35bp} and KD mutations, by multiplying their proportion by total International Scale (IS) *BCR-ABL*

2.4 | Identifying spliceosome mutations

DNA sequence analysis of spliceosomal genes such as SF1, SF3A1, SF3B1, SRSF2, U2AF35, U2AF65 and ZRSR2 was performed in the patients with detection of *BCR-ABL*^{Ins35bp}. Ten patients were selected using the following criteria: (i) patients who failed to achieve MR4.5 for ≥18 months of TKI treatment; and (ii) patients with proportions of *BCR-ABL*^{Ins35bp} to total *BCR-ABL* ≥50%. DNA was isolated from the patients' peripheral blood, using a QIAamp DNA Mini Kit (Qiagen, Germany). DNA sequencing was performed using an ABI 3730 Genetic Analyzer (Applied Biosystems).

2.5 | Single-cell digital PCR

Single K562 cells were sorted into each well of 96-well PCR plates (FACSAria, BD Biosciences); after which, reverse-transcription was performed with a CellsDirect One-Step qRT-PCR Kit (Thermo). The

TABLE 1 Clinical characteristics of newly diagnosed patients

UPN	Age	Sex	Sokal Score	TKI dose (mg)	Observational period	Molecular response at 12 months		Molecular response at 18 months	
						IS (%)	IS-eINS35bp (%)	IS (%)	IS-eINS35bp (%)
1	62	M	Int	Das100 mg	18	MR3 (0.0820%)	→MR3 (0.082000%)	MR3 (0.0158%)	→MR3 (0.015480%)
2	60	M	Low	Das 100 mg	18	MR3 (0.0398%)	→MR3 (0.035422%)	MR3 (0.0150%)	→MR3 (0.013500%)
3	60	F	Low	Nil 600 mg	18	MR2 (0.6510%)	→MR2 (0.273420%)	MR2 (0.1481%)	**↑MR3 (0.044430%)**
4	58	M	Low	Das 100 mg	18	MR4.5 (<0.0032%)	NE	MR4.5 (<0.0032%)	NE
5	64	F	Low	Das 100 mg	18	MR4.5 (<0.0032%)	NE	MR4.5 (<0.0032%)	NE
6	52	M	Low	Das 100 mg	18	MR4.5 (<0.0032%)	NE	MR4.5 (<0.0032%)	NE
7	70	F	Int	Das 100 mg	12	MR3 (0.0391%)	→MR3 (0.038629%)	-	-
8	37	M	Int	Nil 600 mg	12	MR2 (0.1500%)	**↑MR3 (0.090000%)**	-	-
9	29	F	Int	Das 100 mg	12	MR2 (0.1790%)	→MR2 (0.168916%)	-	-

Note: The values in bold indicate the improvement in MR level when focusing on native *BCR-ABL*, which obtained by subtracting eINS35 from IS value.

UPN, Unique Patient Number; TKI, tyrosine kinase inhibitor; IS, International Scale, Das, dasatinib; Int, intermediate; MR, molecular response, NE, not evaluated; Nil, nilotinib.

TABLE 2 Clinical characteristics of patients who had discontinued TKI

n = 54	
Age (range)	52 (26-75)
Sex	
Male	29
Female	25
Treatment prior to TKI discontinuation	
Imatinib → Dasatinib[a]	34 (63%)
Dasatinib	15 (28%)
Imatinib → Nilotinib[b]	3 (6%)
Nilotinib → Dasatinib[c]	2 (3%)
Sokal risk score	
Low	33 (61%)
Intermediate	18 (33%)
High	3 (6%)
Total duration of TKI therapy (months)	91.8 (46.9-158.4)

TKI, tyrosine kinase inhibitor.

[a]TKI was switched from frontline imatinib therapy to dasatinib.

[b]TKI was switched from frontline imatinib therapy to nilotinib.

[c]TKI was switched from frontline nilotinib therapy to dasatinib.

cDNA sample was then loaded onto the Fluidigm Dynamic Array Integrated Fluidic Chip and subjected to digital PCR (dPCR) using the BioMark system (Fluidigm).[20] The dPCR assay was performed according to the manufacturer's instructions (IFC Controller) and using statistical software (Biomark and EP1 Software). The primers used for dPCR are listed in Table S1. *BCR-ABL* and *BCR-ABL*^{Ins35bp} transcripts in individual K562 cells were quantitated pre–treatment and post–treatment with 100 nM imatinib mesylate (BioVision).

2.6 | Quantitative PCR

To test whether TKI can induce alternatively spliced insertion intronic 35-bp nucleotides at the same specific ABL exon 8/9 splice junction, the expression of *c-ABL*^{Ins35bp} in THP-1 and Jurkat cell lines was evaluated in triplicate using Power SYBR Green PCR Master Mix (Thermo) and the Mx3000P qPCR System (Agilent Technologies). Relative quantification of *c-ABL*^{Ins35bp} was performed using the comparative critical threshold (ΔΔCT) method after normalization by *GAPDH*. The expression level of *c-ABL*^{Ins35bp} at the steady state was defined as a control (1.0), and relative quantification of *c-ABL*^{Ins35bp} was performed after 2 hours of culture with 100 nM of imatinib. Primers for detecting *c-ABL*^{Ins35bp} are listed in Table S1.

2.7 | Apoptosis measurement

To test the effects of imatinib mesylate, flavopiridol (Sigma-Aldrich) and spliceostatin A (SSA) (AdooQ Bioscience) on the CML cell

survival rate, K562 cells were cultured with various concentrations of these agents in RPMI-1640 medium (Wako, Japan) containing 10% FBS (STEMCELL Technologies). Cultured K562 cells were then used for apoptosis measurement using annexin/propidium iodide (PI) staining.[21]

2.8 | ChIP

To analyze the status of second serine phosphorylation (S2P) in carboxyl-terminal domain (CTD) of RNA polymerase II (RNAPII), we assessed the RNAPII S2P level on genomic BCR-ABL (gBCR-ABL) of K562 cells, using ChIP-quantitative PCR (qPCR) analysis. ChIP assays were performed using rat monoclonal antibodies against RNAPII S2P (3E10).[22,23] The amount of gBCR-ABL in immunoprecipitated DNA with anti–RNAPII S2P antibody was quantified in triplicate using PowerSYBR Green PCR Master Mix (Thermo) and the Mx3000P qPCR System (Agilent Technologies). Relative amount of RNAPII S2P binding to gBCR-ABL was defined as the ratio of that in 1% of input (non–immunoprecipitated) genomic DNA.

2.9 | Statistical analysis

Differences in distributions, repeated measures and correlations were analyzed using two-sided non–parametric methods (eg, Wilcoxon and Kruskal-Wallis rank tests), as appropriate. Results are presented as mean ± standard error of the mean. Between-group comparisons were performed using Student's *t* test. A level of $P < 0.05$ was considered as statistically significant.

3 | RESULTS

3.1 | Relative increase in *BCR-ABL*^{Ins35bp} following tyrosine kinase inhibitor treatment for newly diagnosed chronic myeloid leukemia

The amounts of native *BCR-ABL*, *BCR-ABL*^{Ins35bp} and KD mutants were determined separately by deep sequence method in 9 patients with newly diagnosed CML-CP. Accordingly, we were able to evaluate values of native *BCR-ABL* and KD mutations, which are essentially responsible for the development of CML, excluding values of eINS35bp from IS *BCR-ABL*, because "function-dead" but TKI-resistant *BCR-ABL*^{Ins35bp} might underestimate the MR status. All samples from 9 patients were free from the KD mutation throughout their clinical courses.

At initial diagnosis, deep sequence analyses detected *BCR-ABL*^{Ins35bp} in 7 out of 9 patients (UPN#1, #2, #4, #5, #7, #8 and #9; Figure 2); the median proportion per total IS *BCR-ABL* was 0.8% (0-3%), and the median eINS35bp was 1.1539% (0-3.3836%; Figure 3). These results indicate the existence of a small but significant population of cells expressing *BCR-ABL*^{Ins35bp} in some CML patients prior to TKI treatment.

Following treatment initiation, CML cells naïve to TKI immediately responded to exposure of TKI, leading to an exponential decrease in IS *BCR-ABL* within the first 6 months (α-slope).[24] In our patients, IS *BCR-ABL* decreased rapidly, from 120.3040% (99.3452%-204.000%) to 9.4759% (0.0281-23.2524%) at 3 months and to 0.6339% (0.0092-1.9364%) at 6 months (Figure 3). In contrast, the proportion of *BCR-ABL*^{Ins35bp} was dramatically increased, up from 0.8% (0-3%) to 27.1% (17-60%) ($P < 0.01$) at 3 months, whereas its actual values did not change significantly (eINS35bp at 0 and 3 months was estimated as 1.1539% [0-3.3836%] and 1.8871% [0.0059-5.1992%], respectively $P = 0.28$]). These results varied according to each individual, as shown in Figure 2. Thereafter, IS *BCR-ABL* gradually decreased over 6 months, as characterized by the β-slope[24] (Figure 3). Similarly, eINS35bp gradually decreased during the course of TKI treatment. However, *BCR-ABL*^{Ins35bp} did not disappear, and its proportion continued to fluctuate at a low level (Figures 2 and 3). In patients presenting an early gain of MR4.5 (UPN #4, #5 and #6), both native *BCR-ABL* and *BCR-ABL*^{Ins35bp} were rapidly cleared following TKI treatment (Figure 2). In contrast, in patients without an MR4.5 gain (UPN #1, #2, #3, #7, #8 and #9), *BCR-ABL*^{Ins35bp} persisted at a low level under TKI treatment (Figure 2).

According to the ELN guidelines, treatment response is defined by IS *BCR-ABL* levels at each time point (ie, 3, 6 and 12 months after TKI treatment). As shown in Figure 2: the stars indicate the IS *BCR-ABL* value, defined as the optimal response at each time point (ie, 3, 6 and 12 months); the blue bars indicate the optimal response (IS *BCR-ABL* ≤ 10% at 3 months, <1% at 6 months, and ≤ 0.1% at 12 months); and the yellow bars indicate the warning (IS *BCR-ABL* > 10% at 3 months, 1-10% at 6 months, and 0.1-1% at 12 months). Failure did not occur for any patient at any time point. The optimal response was achieved in 5 of 9 patients (UPN#4, #5, #6, #8 and #9) at 3 months and 6 of 9 patients at both 6 (UPN#2, #4, #5, #6, #7 and #9) and 12 months (UPN#1, #2, #4, #5, #6 and #7; Figure 2 and Table 2).

Next, analysis was focused on native *BCR-ABL*, excluding the eINS35bp values. Although UPN#8 was originally judged as a warning for IS *BCR-ABL* (0.1500%) at 12 months, the patient was re-evaluated as gain of optimal response, because the eNATIVE was 0.0900% (Table 1 and Figure 2). Moreover, when the response was evaluated in the same way, UPN#3 achieved MR3.0 at 18 months, because the eNATIVE was 0.0444% instead of IS *BCR-ABL* 0.1481% (Table 1 and Figure 2). Taken together, these results suggest that *BCR-ABL*^{Ins35bp} may affect the response definition in patients with a relative DMR gain during the latter, tumor shrinking phase, rather than during initial, high tumor burden phase.

3.2 | Dynamic changes in *BCR-ABL* and *BCR-ABL*^{Ins35bp} after tyrosine kinase inhibitor discontinuation

The dynamics of native *BCR-ABL* and *BCR-ABL*^{Ins35bp} was serially traced in patients who had discontinued TKI after long-term DMR,

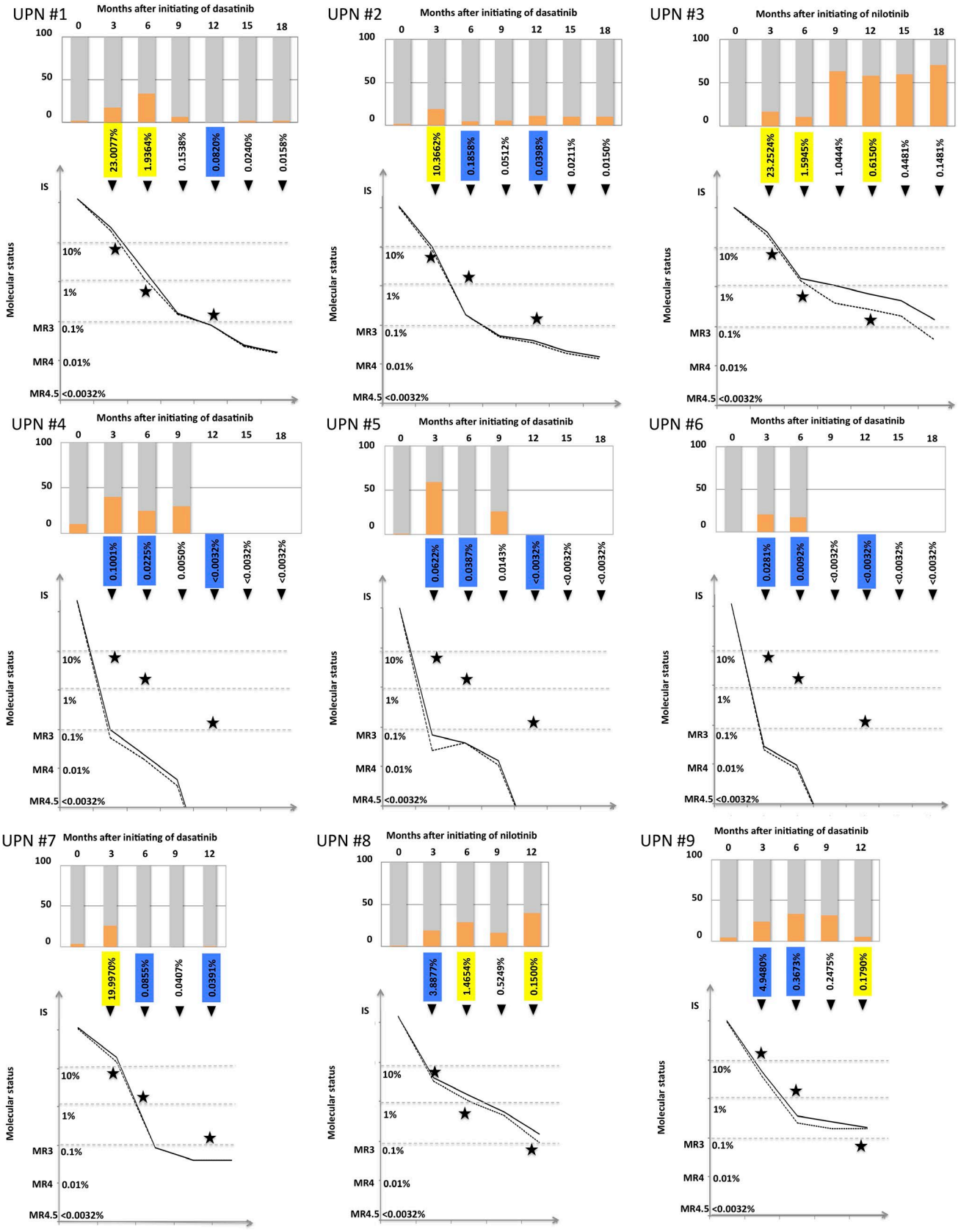
UPN #1
Months after initiating of dasatinib
0 3 6 9 12 15 18
100
50
0
23.0077% 1.9364% 0.1538% 0.0820% 0.0240% 0.0158%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #2
Months after initiating of dasatinib
0 3 6 9 12 15 18
100
50
0
10.3662% 0.1858% 0.0512% 0.0398% 0.0211% 0.0150%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #3
Months after initiating of nilotinib
0 3 6 9 12 15 18
100
50
0
23.2524% 1.5945% 1.0444% 0.6150% 0.4481% 0.1481%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #4
Months after initiating of dasatinib
0 3 6 9 12 15 18
100
50
0
0.1001% 0.0225% 0.0050% <0.0032% <0.0032% <0.0032%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #5
Months after initiating of dasatinib
0 3 6 9 12 15 18
100
50
0
0.0622% 0.0387% 0.0143% <0.0032% <0.0032% <0.0032%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #6
Months after initiating of dasatinib
0 3 6 9 12 15 18
100
50
0
0.0281% 0.0092% <0.0032% <0.0032% <0.0032% <0.0032%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #7
Months after initiating of dasatinib
0 3 6 9 12
100
50
0
19.9970% 0.0855% 0.0407% 0.0391%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #8
Months after initiating of nilotinib
0 3 6 9 12
100
50
0
3.8877% 1.4654% 0.5249% 0.1500%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
UPN #9
Months after initiating of dasatinib
0 3 6 9 12
100
50
0
4.9480% 0.3673% 0.2475% 0.1790%
IS
Molecular status
10%
1%
MR3 0.1%
MR4 0.01%
MR4.5 <0.0032%
Proportion to total BCR-ABL
native BCR-ABL
BCR-ABLIns35bp
Actual value
IS BCR-ABL
Native BCR-ABL
MR in ENL criteria
Optimal response
warning

FIGURE 2 Serial changes in *BCR-ABL* and *BCR-ABL*^{Ins35bp} in newly diagnosed chronic myeloid leukemia (CML) patients. The total amount of International Scale (IS) *BCR-ABL* and proportion of *BCR-ABL*^{Ins35bp} determined by deep sequencing were serially measured at 0, 3, 6, 9 and 12 months after tyrosine kinase inhibitor (TKI) initiation. The upper bar graphs indicate the proportions of native *BCR-ABL* (gray) and *BCR-ABL*^{Ins35bp} (orange) at each time point. The numerals in the middle portion indicate the values of IS *BCR-ABL*. The lower graphs represent the value of IS *BCR-ABL* (bold) and native *BCR-ABL* (dashed) at each time point. Stars show the response time points according to the ELN criteria: blue and yellow bars indicate an optimal response and a warning, respectively

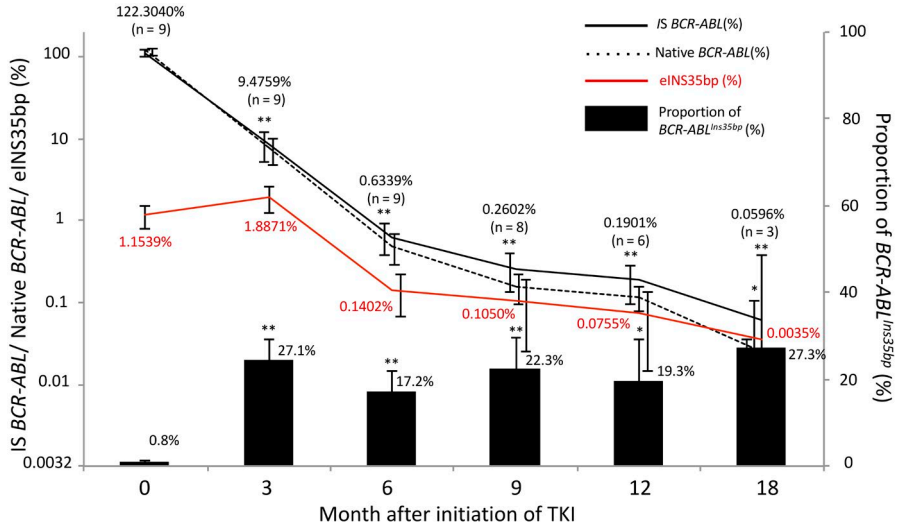

FIGURE 3 Tracking *BCR-ABL* and *BCR-ABL*^{Ins35bp} in newly diagnosed chronic myeloid leukemia (CML) patients. Black, dashed and red lines represent the mean values (%) of International Scale (IS) *BCR-ABL*, native *BCR-ABL* and *BCR-ABL*^{Ins35bp}, respectively, after tyrosine kinase inhibitor (TKI) therapy in 9 newly diagnosed CML patients. The black bars indicate the percentage of *BCR-ABL*^{Ins35bp} per total *BCR-ABL*. After initiating TKI treatment, IS *BCR-ABL* (black line) decreased exponentially within the initial 6 months, followed by a subsequent gradual decrease. In contrast, the proportion of *BCR-ABL*^{Ins35bp} (black box) increased dramatically at the initial 3 months, whereas its actual values (red line) did not change significantly. Thereafter, IS *BCR-ABL*^{Ins35bp} as well as native BCR-ABL (dashed line) gradually decreased. *Significant decrease compared with baseline level (P = < 0.05). **Significant decrease compared with baseline level (P = < 0.01)

which allowed us to clarify the detailed kinetics of relapse or sustained DMR after TKI cessation in 54 patients (Figure 4, Figure S1, Table 2).

Following TKI discontinuation, 26 patients sustained DMR with undetectable MRD (UMRD group), whereas 22 developed molecular relapse with loss of MMR (Relapse group). In contrast, the remaining 6 consistently exhibited fluctuation of IS *BCR-ABL* levels around MR4.0-4.5, and never experienced relapse, defined as loss of MR4.5 for 2 consecutive time points after TKI discontinuation (Fluctuation group).

Patients representing the Fluctuation group are shown in Figure 4A and B. In UPN#10, *BCR-ABL* were undetectable within the first 7 months after TKI discontinuation (Figure 4A), although MR4.5 was lost at 8 months. At this point, *IS BCR-ABL* was measured as 0.0044%, whereas *BCR-ABL*^{Ins35bp} constituted 93% of IS. Therefore, eNATIVE was determined as 0.0003%, indicating that upon exclusion of this function-dead *BCR-ABL*^{Ins35bp} in IS *BCR-ABL*, the patient had not relapsed. Thereafter, *BCR-ABL* spontaneously became undetectable, without restart of TKI, and the patient successfully maintained MR4.5. Similarly, UPN#11 had lost MR4.5 both at 5 and 10 months, although his eNATIVE was below 0.0032%, suggesting that MR4.5 had not been lost (Figure 4B).

Representative cases from the Relapse group are shown in Figure 4C and D. Following TKI discontinuation, UPN#12 consecutively lost MR4.5 and MR3.0 at 1 and 2 months, respectively (Figure 4C). Treatment restart with dasatinib induced a rapid decrease in *IS* level below MR4 at 4 months. In this patient, *BCR-ABL*^{Ins35bp} was not detected throughout his clinical course, suggesting that the rapid relapse clone could have derived from native *BCR-ABL*$^{+}$ cells. In contrast, UPN#13 lost MR3 at 2 months after discontinuation of dasatinib; thereafter, his IS *BCR-ABL* was rapidly increased up to 8.079% at 3 months (Figure 4D). At 3 months, IS *BCR-ABL* was mainly constituted of native *BCR-ABL* (74%); thus, restart with dasatinib resulted in a rapid decrease in IS and the patient eventually achieved MR4 at 7 months. These findings indicate that proliferation of cells expressing dominant native *BCR-ABL* might be responsible for the early relapse with rapid IS *BCR-ABL* increase following TKI discontinuation.

3.3 | Patients with the *BCR-ABL*^{Ins35bp} did not carry spliceosome gene mutations

We next investigated the mechanism for emergence of *BCR-ABL*^{Ins35bp}. Because spliceosome mutations can cause various types of

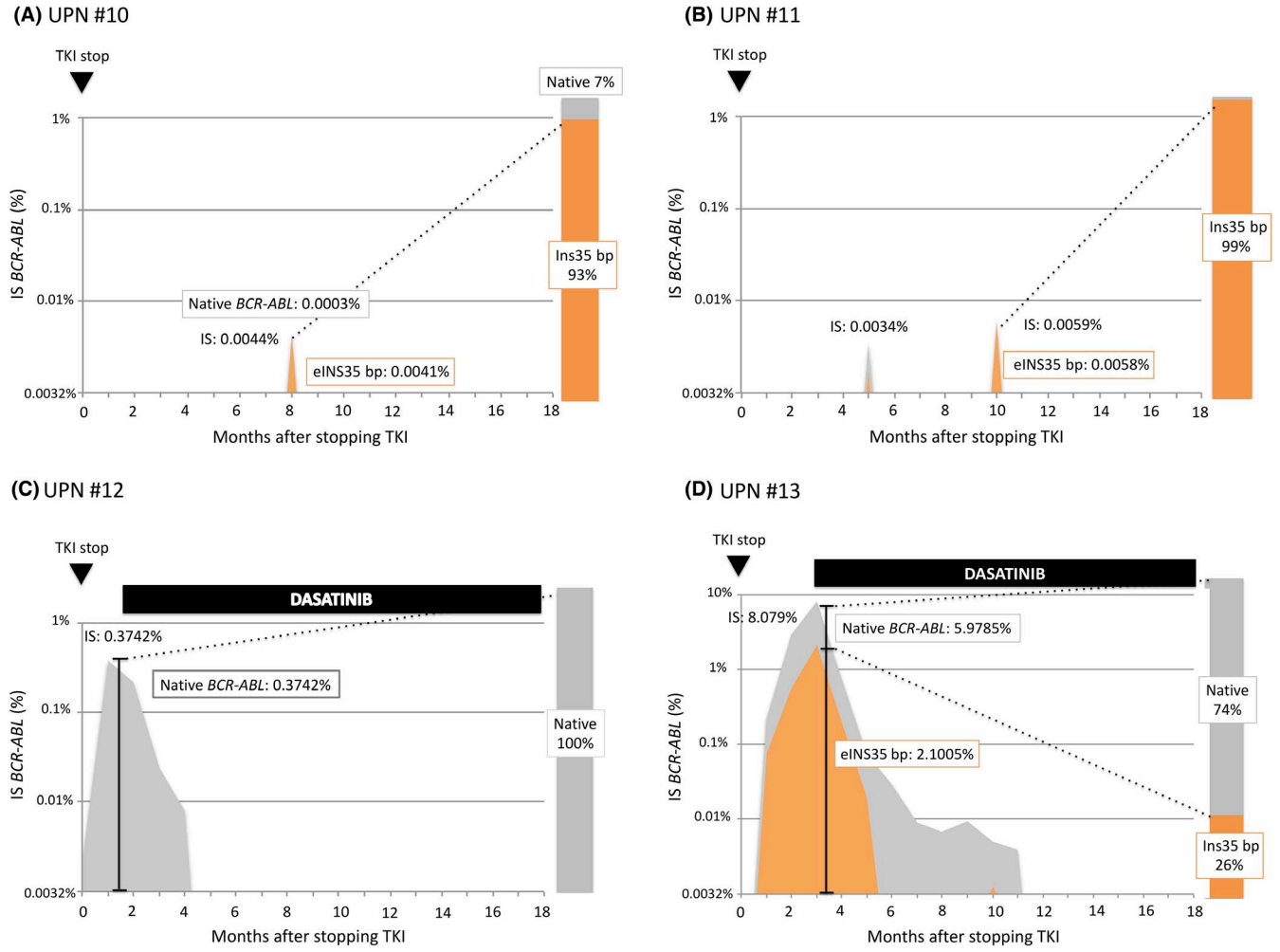

FIGURE 4 Serial change in *BCR-ABL* and *BCR-ABL*^{Ins35bp} after cessation of tyrosine kinase inhibitor (TKI) therapy. Results are shown separately for cases representing the patients who lost MR4.5 at a relatively late phase (A and B) and those who lost MR4.5 consecutively within the initial 3 months (C and D) after cessation of TKI. Cases #10 (A) and #12 (B) had lost MR4.5 once but regained it spontaneously without starting TKI. Their International Scale (IS) *BCR-ABL* at loss of MR4.5 were composed of 93% and 99% of *BCR-ABL*^{Ins35bp}, respectively. In contrast, cases #12 (C) and #13 (D) had lost MR4.5 early after TKI cessation, and their IS *BCR-ABL*, predominantly native *BCR-ABL* increased steeply, resulting in the resumption of treatment

hematological malignancies,[25-29] we performed the mutation analysis in spliceosome genes such as SF1, SF3A1, SF3B1, SRSF2, U2AF35, U2AF65 and ZRSR2 in 10 patients who had failed to achieve DMR. No mutations were detected in patients, indicating that spliceosomal mutations are not associated with emergence of *BCR-ABL*^{Ins35bp} (Table S2).

3.4 | Pseudo-splice sites surrounding 35bp-nucleotides within *ABL* intron8

We elucidated the mechanism underlying mis-splicing of *BCR-ABL*^{Ins35bp}, which occurs at the same specific 35 bp in intron 8. Generally, spliceosome complex recognizes splice sites (ss) through conserved sequences at the exon-intron junctions and cleave introns, after which exons are ligated together at their 5′ and 3′ ss. Thus, we investigated the presence of both 5′ and 3′ pseudo-ss, which possess sequences homologous to the normal ss (5′ ss: AGGURAGU, 3′ ss: Y_{10}NYAGR [R, Y and N represent A/G, C/U and any nucleotides]), spanning the specific 35 bp, with Human Splicing Finder software (HSF).[30] As shown in Figure 5A, pseudo-ss were identified in both ends of 35 bp: the sequence of 5′ pseudo-ss was AGGURAGU and 3′ pseudo-ss TTTCTTTTCATGAGA. Seven out of 8 (88%) nucleotides in 5′ pseudo-ss and 13 out of 15 (86%) nucleotides in 3′ pseudo-ss were consistent with the 5′ and 3′ ss consensus motif, respectively.

The HSF algorithm determines the similarity to ss consensus motif, which is expressed as a consensus value (CV) evaluating the strength of pseudo-ss. Therefore, we compared the CV of pseudo-ss in ABL intron 8 with that of conventional ss in *ABL* exons1-11 and 245 286 human exons.[30] As shown in Figure 5B, the CV of 5′ and 3′ pseudo-ss flanked by specific 35 bp were 95.7 and 90.3, respectively. In contrast, the mean CV of 5′ and 3′ ss in *ABL* exons1-11 were 88.4 ± 1.4 and 89.5 ± 1.9 (mean ± SD), respectively, whereas the mean CV of 5′ and 3′ ss in 245 286 human exons were 87.5 ± 8.3 and 86.8 ± 6.3 (mean ± SD).[30] Taken together, these results indicate that

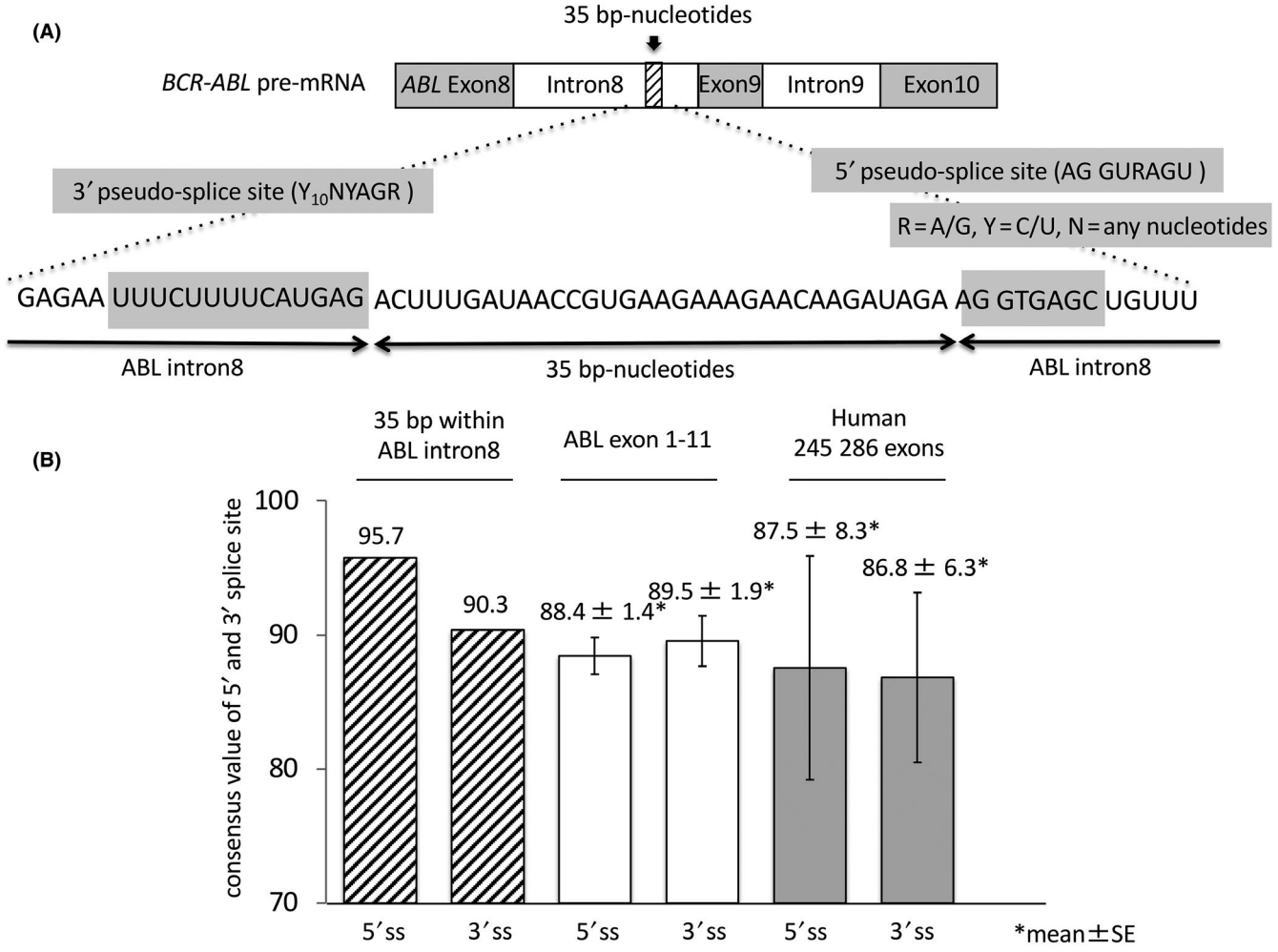

FIGURE 5 Pseudo-splice sites at both ends of 35 bp-nucleotides in intron 8. (A) Schematics of pseudo-splice sites (ss) at both ends of 35 bp nucleotides. ABL intron 8 possesses pseudo-ss with similar sequences to normal ss (5′ ss: AGGURAGU, 3′ ss: Y_{10}NYAGR [R, Y and N represent A/G, C/U and any nucleotides, respectively]). (B) The consensus value (CV) of 5′ and 3 ′ pseudo-ss was 95.7 and 90.3, respectively, which was quite high compared with the mean CV of ss in *ABL* exons 1-11 (mean ± SD; 88.4 ± 1.4 and 89.5 ± 1.9) and human 245, 286 exons (87.5 ± 8.3 and 86.8 ± 6.3)[30]

ABL intron 8 possesses highly conserved pseudo-ss at both ends of 35 bp, resulting in reproducible mis-splicing of specific 35 bp.

3.5 | Dynamic kinetics of *BCR-ABL* and *BCR-ABL*^{Ins35bp} transcripts in single-cells during tyrosine kinase inhibitor treatment

To track the kinetics of both *BCR-ABL* and *BCR-ABL*^{Ins35bp} during TKI treatment, we quantified the transcription levels of single K562 cells using a dPCR system. Notably, prior to TKI treatment, both transcripts were detected in all individual K562 cells, where the medians of *BCR-ABL* and *BCR-ABL*^{Ins35bp} transcripts were 656 copies (range, 593-747) and 11 copies (range, 5-21), respectively (Figure 6). Thus, *BCR-ABL*^{Ins35bp} accounts for 1.7% of the total *BCR-ABL* (range, 0.7-3.1%).

We investigated the effects of imatinib on the levels of *BCR-ABL* and *BCR-ABL*^{Ins35bp} transcripts of individual CML cells. To determine the optimal concentration of imatinib, K562 cells were cultured for 2 hours with various concentrations, after which apoptosis was measured through Annexin/PI staining. No significant difference was found between the control and imatinib concentrations up to 100 nM for the proportion of live cells (data not shown). As shown in Figure 6, the level of *BCR-ABL* transcripts in individual cells was decreased by approximately 2-fold, from 656 copies (range, 593-712) to 348 copies (range, 65-638), compared with the control ($P < 0.01$; Figure 6). In contrast, the amount of *BCR-ABL*^{Ins35bp} transcripts increased approximately 2-fold from 11 (range, 5-16) to 27 copies (range, 14-39) after culture with imatinib, compared with control ($P = < 0.01$; Figure 6). Relative ratio of *BCR-ABL*^{Ins35bp} to *BCR-ABL* transcripts within each cell was increased up to 8.6% (2.1%-35.4%) from 1.7% (0.7%-3.1%) after treatment with imatinib. This indicates that, in vitro, imatinib increases *BCR-ABL*^{Ins35bp} transcripts while decreasing *BCR-ABL* transcripts in individual CML cell lines.

3.6 | Imatinib increases *c-ABL*^{Ins35bp} in *BCR-ABL*-negative cell lines

To clarify whether TKI can induce *c-ABL*^{Ins35bp}, we evaluated the transcription levels of *c-ABL*^{Ins35bp} in *BCR-ABL*-negative cell lines by qPCR analysis, because levels of both *c-ABL* and *c-ABL*^{Ins35bp} in patients' samples were too low to be quantified in patients' samples (data not shown). The amount of *c-ABL*^{Ins35bp} in THP-1 and Jurkat cell lines before culture with imatinib was defined as a control (1.0). After culture with imatinib, the transcription levels of *c-ABL*^{Ins35bp} exhibited 1.52 ± 0.13 and 2.12 ± 0.05-fold increase in THP-1 and Jurkat cell lines, respectively (P = < 0.05). These results indicate that TKI can induce the alternative spliced *c-ABL*^{Ins35bp} by its off–target effect.

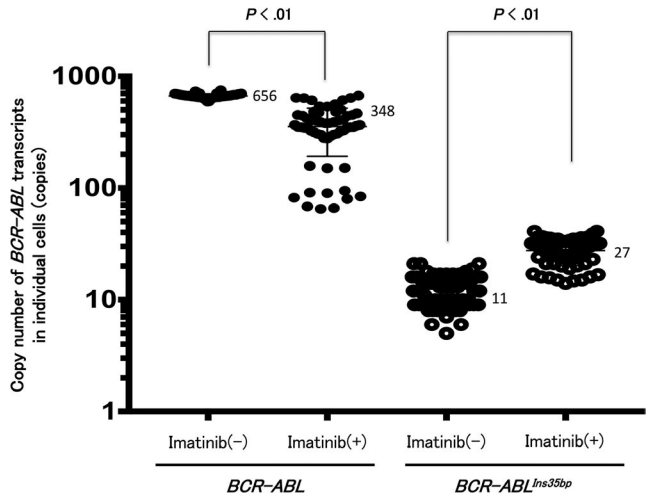

FIGURE 6 *BCR-ABL* and *BCR-ABL*^{Ins35bp} transcripts in single K562 cells. Closed and open circles indicate the number of *BCR-ABL* and *BCR-ABL*^{Ins35bp} transcript copies in untreated single K562 cells, respectively (left panel). Treatment with imatinib significantly decreased levels of *BCR-ABL* (P = < 0.01), while increasing the copy number of *BCR-ABL*^{Ins35bp} transcript (P = < 0.01) (right panel)

3.7 | Imatinib inhibits the RNA polymerase II complex binding to gBCR-ABL

RNA polymerase II (RNAPII) is an enzyme complex in which the second serine phosphorylation (S2P) of the CTD promotes both transcription and pre–mRNA splicing.[31,32] Therefore, RNAPII S2P might be responsible for regulating transcription of *BCR-ABL* and *BCR-ABL*^{Ins35bp} via a change in the phosphorylation status of CTD. To test whether imatinib alters the phosphorylation status of RNAPII CTD, we assessed the RNAPII S2P level on gBCR-ABL by ChIP-qPCR analysis. As shown in Figure 7A, ChIP-qPCR analysis demonstrated that the level of RNAPII S2P on gBCR-ABL was decreased dramatically after 2 hours of culture with 100 nM imatinib (P = < 0.01).

Next, we tested whether direct inhibition of RNAPII S2P by flavopiridol[33] or inhibition of SF3B1, the major component of spliceosome, by spliceostatin A[34] could increase *BCR-ABL*^{Ins35bp} transcripts. We first confirmed that there was no significant difference in the proportion of live K562 cells in the presence of 100 nM imatinib, 100 nM flavopiridol or 100nM SSA and control (data not shown). As shown in Figure 7B, after 2 hours of culture, the level of *BCR-ABL*^{Ins35bp} increased 7-fold, 6-fold and 7-fold (P = < 0.01), respectively, as compared with the control (Figure 7B). These results indicate that imatinib inhibits RNAPII S2P binding to gBCR-ABL, then impairs splicing of *BCR-ABL*, leading to the emergence of *BCR-ABL*^{Ins35bp}.

4 | DISCUSSION

The present study demonstrates that, prior to TKI treatment, most newly diagnosed CML patients carry a small population of cells harboring the *BCR-ABL*^{Ins35bp}. Our highly sensitive NGS analysis revealed that, at the initial diagnosis, *BCR-ABL*^{Ins35bp} constitute 0.8% of the total *BCR-ABL*, and following conversion to IS, its amount is estimated as up to 1.1539%. Because such low levels of *BCR-ABL*^{Ins35bp}

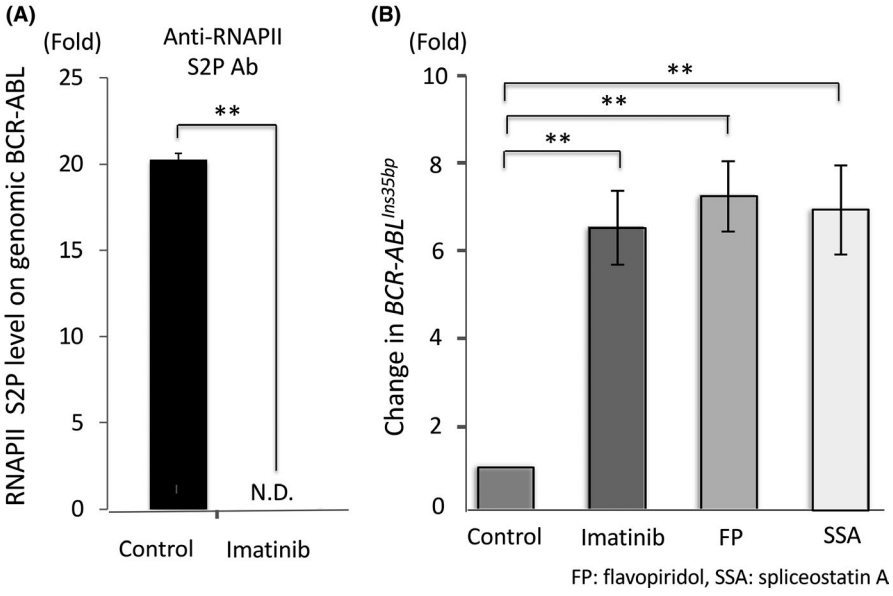

FIGURE 7 Imatinib induces *BCR-ABL*^{Ins35bp} through inhibition of RNA polymerase II serine phosphorylation on BCR-ABL. A, Imatinib significantly decreased the levels of RNA polymerase II second serine phosphorylation (RNAPII S2P) on gBCR-ABL. RNAPII S2P level on gBCR-ABL was quantified by ChIP-quantitative PCR analysis with rat monoclonal antibodies in K562 cells. B, Significant increase of *BCR-ABL*^{Ins35bp} through inhibition of RNAPII S2P by imatinib, flavopiridol (FP) and spliceostatin A (SSA). **Significant decrease compared with the baseline level (P = < 0.01)

do not affect disease staging or treatment choice, no attention has been paid to its presence at diagnosis. Following initiation of TKI treatment, native *BCR-ABL* was exponentially decreased by 2-3-log reduction within the first 3 months, which corresponded to the rapid initial decrease in cycling mature cells or progenitors (α-slope phase).[24] Thereafter, native *BCR-ABL* gradually decreased, corresponding to the slow reduction in non–cycling cells such as CML stem cells (β-slope phase).[24] In contrast, the total amount of *BCR-ABL*^{Ins35bp} did not change significantly within the first 3 months, whereas its proportion per total IS increased to approximately 24%, because native *BCR-ABL* was declining in response to the first exposure to TKI. Thereafter, the amount of *BCR-ABL*^{Ins35bp} gradually decreased, whereas its proportion relative to IS *BCR-ABL* fluctuated around 15-30% after IS *BCR-ABL* has decreased below 1%. These results indicate that even a small population of *BCR-ABL*^{Ins35bp} can affect the attainment of MR such as MR3.0 or MR4.5 at critical time points. In fact, within our patient series, UPN#3 and #7 were classified as not reaching MMR at 18 and 12 months, respectively. However, upon reevaluation of MR based on the native *BCR-ABL* levels, by subtracting *BCR-ABL*^{Ins35bp}, both patients would have eNATIVE < 0.1% and be classified as having an optimal response instead of a warning. Thus, it is much more important to evaluate *BCR-ABL*^{Ins35bp} quantitatively in the patients with low tumor burden during the late phase of TKI treatment rather than with high tumor burden before or in the early phase of TKI treatment.

Quantitative analyses of *BCR-ABL*^{Ins35bp} have also provided new insights for tracking molecular dynamics in patients who have discontinued TKI after long-term maintenance of DMR. Mahon et al reported in the STIM trial that most cases of relapse would occur early, within 6 months after TKI cessation, whereas few patients would relapse at a later stage of TKI discontinuation.[4] We demonstrated that patients who relapsed early presented a rapid IS increase after TKI discontinuation, where IS comprised the vast majority of native *BCR-ABL* and none or few *BCR-ABL*^{Ins35bp}. These results suggest that the *BCR-ABL*$^{+}$ cells responsible for early relapse might derive from the residual native *BCR-ABL* addicted clones.[35] In contrast, some patients transiently lost DMR during a relatively late phase after TKI discontinuation and thereafter regained DMR spontaneously without TKI resumption. This finding indicates that IS *BCR-ABL* fluctuates around the lower threshold detectable by PCR. These transiently increased IS comprised a mixture with *BCR-ABL*^{Ins35bp} and native *BCR-ABL* in various proportions in each individual. Therefore, a transient, small IS rise was detected occasionally, when synchronized with the physiologic, periodic self-renewal of HSC possessing *BCR-ABL*^{Ins35bp}, and not in a leukemic proliferative fashion. Similarly, if IS *BCR-ABL* were reevaluated on the basis of native *BCR-ABL* levels, in patients who lost DMR, a small but significant fraction would have been regarded as not having lost DMR, thus not restarting TKI. For example, although the original STIM trial[4,36] defined the criteria as loss of MR5.0 by a second successive analysis, the A-STIM trial alleviated the criterion for relapse and resuming therapy as a loss of MR3.0 at two consecutive time points. As a result, the A-STIM trial revealed that incidence of relapse decreased to 35% (28 of 80 patients), and IS *BCR-ABL* fluctuation below MR3.0 threshold was documented in 31% of the patients following imatinib discontinuation. These findings suggest that quantification of *BCR-ABL*^{Ins35bp} in patients who relapse slowly at the late stages of TKI discontinuation is crucial to avoid unnecessary TKI resumption. In addition, quantification of IS *BCR-ABL* and *BCR-ABL*^{Ins35bp} would expand the candidate patients for TKI discontinuation to those whose IS *BCR-ABL* have been fluctuating around the level of DMR but have not reached the criteria for cessation of TKI.

Our study investigated the mechanism underlying *BCR-ABL*^{Ins35bp} mis-splicing occurring at the same specific 35 bp in ABL intron 8. First, we looked into spliceosomal mutations that might be associated with the development of hematological malignancies.[25-29] However, any spliceosomal mutations contributing to the mis-splicing of *BCR-ABL*^{Ins35bp} were not found. Next, we found that pseudo-ss sharing sequences with over 90% similarity to the consensus sequences of ss are located at both 5′ and 3′ ends of that particular 35 bp in ABL intron 8, which might lead to mis-splicing exactly the same 35 bp at high reproducibility. In addition, we demonstrated that TKI impair the recruitment of the splicing complex by inhibiting RNAPII S2P through its off–target effect, which, in turn, dysregulates pre–mRNA splicing (Figure S2). We also showed in vitro that imatinib decreases the amount of native *BCR-ABL* while increasing the amount of *BCR-ABL*^{Ins35bp} 2-fold in single-cell lines. In addition, imatinib can increase the amount of *c-ABL*^{Ins35bp} by 1.5 and 2.1-fold in *BCR-ABL*-negative THP-1 and Jurkat cell lines, respectively. Collectively, these results indicate that TKI-induced dysregulation of splicing machinery enhances the mis-splicing of *BCR-ABL*^{Ins35bp} and *c-ABL*^{Ins35bp}.

As shown by our PCR analysis, some CML cells may possess native *BCR-ABL* and *BCR-ABL*^{Ins35bp} in various proportions. Once TKI treatment starts, cycling mature CML cells are sensitive to TKI; therefore, cells expressing native *BCR-ABL* alone or both native *BCR-ABL* and *BCR-ABL*^{Ins35bp} rapidly disappear. In contrast, TKI concurrently increases the amount of *BCR-ABL*^{Ins35bp} by inducing mis-splicing through inhibition of RNAPII S2P. Therefore, during the early phase of TKI treatment, the total amount of *BCR-ABL*^{Ins35bp} would not change, whereas the ratio of *BCR-ABL*^{Ins35bp} per IS would increase. Thereafter, immature CML cells expressing either both transcripts or native *BCR-ABL* alone gradually decrease in response to TKI. The total amount of the *BCR-ABL*^{Ins35bp} gradually decreases, whereas the ratio of *BCR-ABL*^{Ins35bp} per IS remains stable and fluctuating. Because CML cells co–expressing both transcripts might be less sensitive to TKI, maximizing cell eradication in the early phase would be crucial to reach DMR.

In conclusion, we demonstrated that *BCR-ABL*^{Ins35bp} are produced by mis-splicing at the pseudo-ss of the intron 8, which can be augmented by TKI treatment through its inhibition of RNAPII S2P. However, cells expressing *BCR-ABL*^{Ins35bp} are neither totally eradicated by TKI nor do they proliferate in a leukemic fashion. Rather, they persist and fluctuate around the deep MR level, affecting treatment response in some patients. Therefore, monitoring function-dead *BCR-ABL*^{Ins35bp} would be beneficial for an accurate evaluation of TKI efficacy and the need for treatment reinitiation.

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DISCLOSURE

The authors declare no competing financial interests.

ORCID

Junichiro Yuda https://orcid.org/0000-0002-4908-1636
Toshihiro Miyamoto https://orcid.org/0000-0002-6533-1594

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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