Brief report

# The BCR-ABL$^{35INS}$ insertion/truncation mutant is kinase-inactive and does not contribute to tyrosine kinase inhibitor resistance in chronic myeloid leukemia

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**Chronic myeloid leukemia is effectively treated with imatinib, but reactivation of BCR-ABL frequently occurs through acquisition of kinase domain mutations. The additional approved ABL tyrosine kinase inhibitors (TKIs) nilotinib and dasatinib, along with investigational TKIs such as ponatinib (AP24534) and DCC-2036, support the possibility that mutation-mediated resistance in chronic myeloid leukemia can be fully controlled; however, the molecular events underlying resistance in patients lacking BCR-ABL point mutations are largely unknown. We previously reported on an insertion/truncation mutant, BCR-ABL$^{35INS}$, in which structural integrity of the kinase domain is compromised and all ABL sequence beyond the kinase domain is eliminated. Although we speculated that BCR-ABL$^{35INS}$ is kinase-inactive, recent reports propose this mutant contributes to ABL TKI resistance. We present cell-based and biochemical evidence establishing that BCR-ABL$^{35INS}$ is kinase-inactive and does not contribute to TKI resistance, and we find that detection of BCR-ABL$^{35INS}$ does not consistently track with or explain resistance in clinical samples from chronic myeloid leukemia patients. (*Blood*. 2011;118(19):5250-5254)**

## Introduction

Imatinib is an inhibitor of BCR-ABL, the tyrosine kinase that causes chronic myeloid leukemia (CML). Most newly diagnosed patients achieve durable remissions on imatinib therapy,[1,2] but 10%-15% fail to respond or relapse. The leading cause of imatinib resistance is reactivation of BCR-ABL because of kinase domain point mutations. Most BCR-ABL mutants are susceptible to alternative ABL tyrosine kinase inhibitor (TKI) therapies.[3-8] Sequencing of the BCR-ABL kinase domain in patients exhibiting signs of TKI treatment failure has also revealed the presence of alternatively spliced variants, including BCR-ABL$^{35INS}$, in which retention of 35 intronic nucleotides at the exon 8/9 splice junction introduces a stop codon after 10 intron-encoded residues.[9-13] The result is loss of the last 653 residues of BCR-ABL, including 22 native kinase domain residues.[10,12] Notably, the reported frequency of detection of the BCR-ABL$^{35INS}$ mutant in cases of imatinib resistance (including instances in which a point mutation is concurrently detected in the BCR-ABL kinase domain) as detected by direct sequencing is ~1%-2%,[10,14] although more sensitive quantitative assays have reported detection of very low levels of the mutant transcript at a considerably increased prevalence.[14]

Although BCR-ABL truncated immediately after the ABL kinase domain is fully transforming in a murine model of CML,[15] we predicted BCR-ABL$^{35INS}$ would lack kinase activity, because the mutation eliminates the last 2 helices of the ABL kinase domain and disrupts a complex set of interactions among noncontiguous residues.[10] By contrast, recent reports have suggested that BCR-ABL$^{35INS}$ confers TKI resistance in CML[9,12,14,16] and have proposed a BCR-ABL$^{35INS}$ tailored clinical trial,[16] but they have not addressed the mechanism for this or assessed BCR-ABL$^{35INS}$ catalytic activity. We provide cell-based and biochemical studies of BCR-ABL$^{35INS}$ and a retrospective analysis of its detection in the context of treatment and response in CML patients.

## Methods

### IL-3 withdrawal

Ba/F3 cells cultured in standard media (RPMI 1640 media, 10% FBS, L-glutamine, penicillin-streptomycin; Invitrogen) containing IL-3 from WEHI-conditioned media were infected with retrovirus expressing BCR-ABL, BCR-ABL$^{35INS}$, or BCR-ABL$^{K271P/35INS}$ (MSCV-IRES-GFP), and stable cell lines were sorted for GFP (FACSAria II; BD Biosciences). After IL-3 withdrawal, cells were counted daily.[17]

### Ba/F3 immunoblotting

Ba/F3 parental cells and Ba/F3 cells expressing or coexpressing BCR-ABL, BCR-ABL$^{35INS}$, or BCR-ABL$^{K271P/35INS}$ were boiled for 10 minutes in SDS-PAGE loading buffer. Lysates were separated on 4%-15% Tris-HCl gels, transferred, and immunoblotted with antibodies for the BCR

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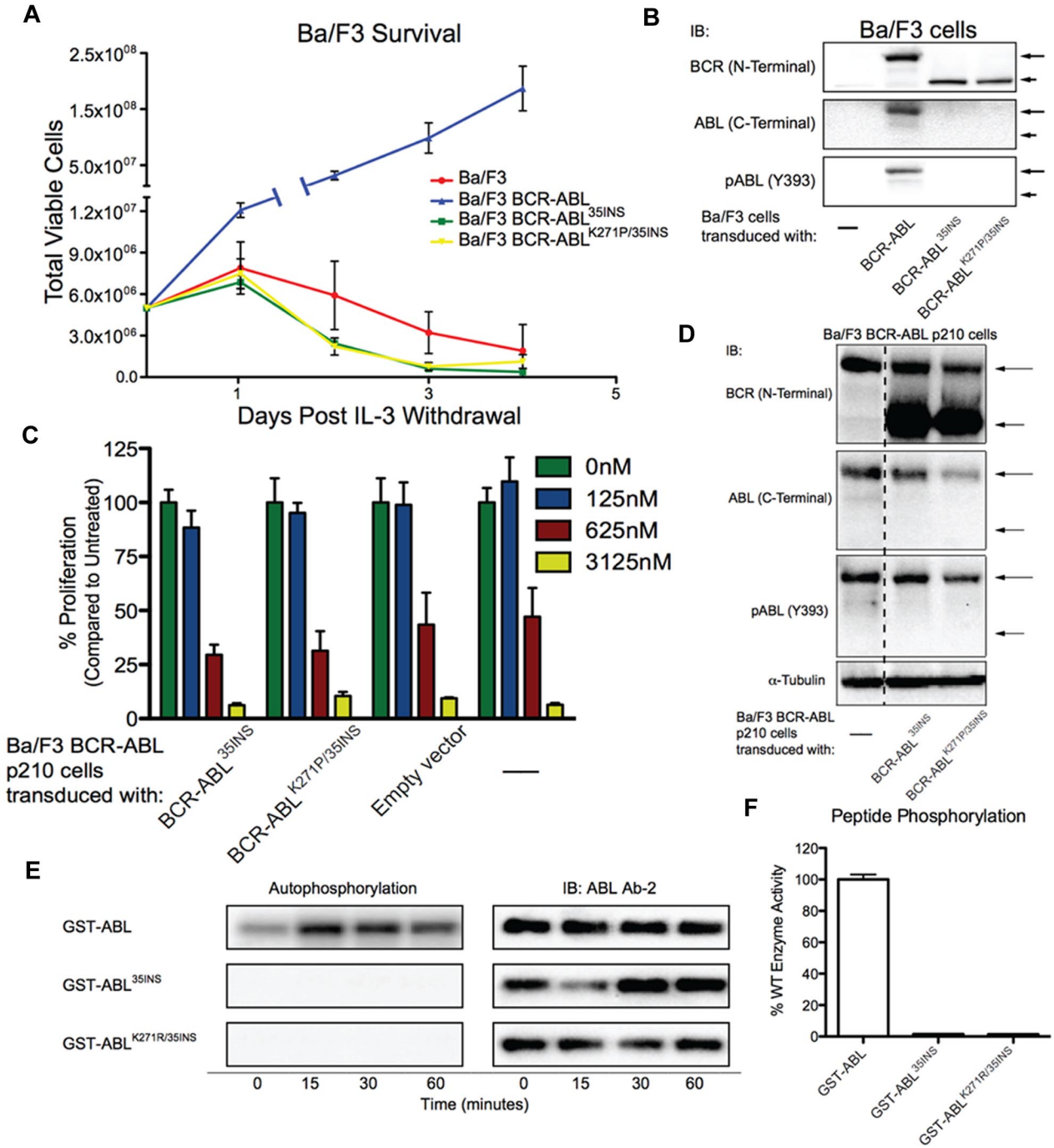


**Figure 1. The BCR-ABL$^{35INS}$ mutant does not confer growth factor independence or imatinib resistance, and ABL$^{35INS}$ lacks kinase activity.** (A) Ba/F3 cells expressing the indicated BCR-ABL construct were cultured in standard media supplemented with IL-3. After IL-3 washout and replating in standard media, cell viability was monitored daily for 4 days with the Guava ViaCount assay (Millipore). Results are shown as the mean viable cells ± SEM from 3 independent experiments. (B) Cell lysates were subjected to SDS-PAGE followed by immunoblot (IB) analysis with the indicated antibodies. Results are representative of 2 independent experiments. The long and short arrows on the right side of the immunoblot indicate the expected positions of BCR-ABL and BCR-ABL$^{35INS}$, respectively. (C) Ba/F3 cells stably expressing BCR-ABL were transduced with the indicated BCR-ABL$^{35INS}$ mutant constructs, sorted for GFP, and cultured in standard media. Cells were plated in the presence of graded concentrations of imatinib (0-3125nM) and evaluated by methanethiosulfonate assay at 72 hours (CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay; Promega) with a Synergy 2 plate reader (BioTek). Results are normalized to those of untreated cells and expressed as the mean ± SEM of 3 independent experiments performed in quadruplicate. (D) Cell lysates were subjected to SDS-PAGE followed by immunoblot (IB) analysis with the indicated antibodies. The long and short arrows on the right side of the immunoblot are provided for reference and indicate the expected positions of BCR-ABL and BCR-ABL$^{35INS}$, respectively. The absence of signal corresponding to BCR-ABL$^{35INS}$ in the second and third panels signifies the lack of the ABL C-terminus and no phosphorylation on Y393, respectively. Lanes 1 and 2 represent nonadjacent lanes from the same gel (indicated by the vertical dashed line). (E) Equimolar amounts of purified, tyrosine-dephosphorylated GST-ABL kinase domain enzymes were incubated with [$\gamma$-$^{32}$P]-ATP. Samples collected at indicated times were subjected to SDS-PAGE, and signal intensity was measured by autoradiography. Comparable loading was confirmed by ABL immunoblot (IB) analysis with ABL antibody Ab-2 (Oncogene Science). Results are representative of 2 independent experiments. (F) Dephosphorylated GST-ABL enzymes were incubated with a biotinylated peptide substrate (biotin-GGEAIYAAPFKK-amide) and [$\gamma$-$^{32}$P]-ATP. At the end of the incubation period, reactions were terminated with 7M of guanidine hydrochloride solution, spotted in duplicate on SAM$^2$ Biotin Capture Membranes (Promega), washed according to the manufacturer's instructions, and subjected to liquid scintillation counting. GST-ABL reactions were quenched after 3.5 minutes, whereas GST-ABL$^{35INS}$ and GST-ABL$^{K271R/35INS}$ reactions were quenched after 1 hour because preliminary experiments at 3.5 minutes demonstrated no detectable activity. Results are shown as the mean enzymatic activity ± SEM normalized to that of GST-ABL. The autophosphorylation and peptide substrate phosphorylation activities of GST-ABL were inhibited by imatinib (data not shown). WT indicates wild-type.

A

| | No. of patients | Response status at time of 35INS detection | | | |
|---|---|---|---|---|---|
| | | Responding, n | Intolerance, n | Early resistance, n | Overt relapse, n |
| **Total patients** | **20** | 9 | 3 | 3 | 5 |
| **Component level of 35INS by direct sequencing** | | | | | |
| Minor (≤20%) | **18** | 7 | 3 | 3 | 5 |
| Major (>20%) | **2** | 2 | 0 | 0 | 0 |
| **Therapy on which 35INS first detected** | | | | | |
| 1st-line imatinib | **10** | 7 | 2 | 1 | 0 |
| 2nd- or 3rd-line TKI (nilotinib or dasatinib) | **9** | 1 | 1 | 2 | 5 |
| Consolidation chemotherapy | **1** | 1 | 0 | 0 | 0 |
| **Mutation(s) co-occurring with 35INS** | | | | | |
| 35INS mutation only | **16** | 8 ¶ | 3 * | 2 † | 3 § |
| Co-occurring point mutation(s) | **4** | 1 | 0 | 1 | 2 |

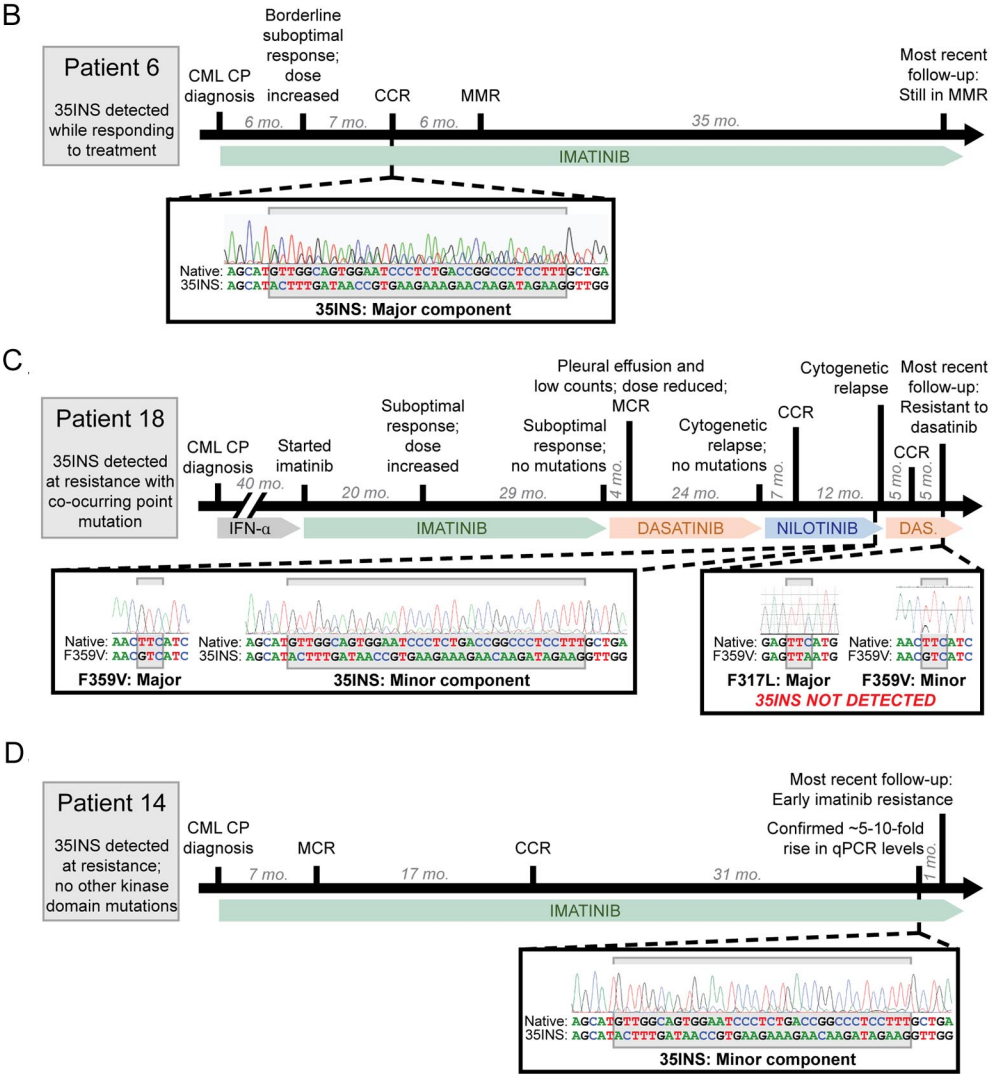


**Figure 2. Detection of the BCR-ABL$^{35INS}$ mutation in clinical samples does not consistently track with resistance to ABL TKI therapy.** Retrospective analysis of the detection of the BCR-ABL$^{35INS}$ mutation by direct sequencing was performed in the chronologic context of the treatment and response for 20 CML patients at our institution. (A) Summary of patient response status at the time of BCR-ABL$^{35INS}$ detection. Response status was categorized as follows: responding (improvement or maintenance of previous cytogenetic and molecular response level), intolerant (persistent grade 3 or higher toxicity leading to subsequent change of therapy), early resistance (confirmed increase in BCR-ABL transcripts and/or detection of a BCR-ABL point mutation without loss of level of cytogenetic response), or overt relapse (loss of previous cytogenetic response level). For comparison purposes, the approximate component level of the BCR-ABL$^{35INS}$ mutation by direct sequencing was designated as minor (≤ 20% of total electropherogram signal) or major (> 20%). Although not strictly quantitative, this illustrates that the majority of patients with evidence of BCR-ABL$^{35INS}$ by direct sequencing harbored the mutation at low levels that approached the sensitivity limits of this assay.[14] Sequencing primers used by the OHSU Knight Diagnostic Laboratories have been described previously[10]; primers used by MolecularMD remain proprietary. ¶An additional intron 4 insertion was detected along with 35INS in patient 7; both represented a minor

N-terminus (3902; Cell Signaling Technology), ABL C-terminus (24-11; Santa Cruz Biotechnology), phospho-ABL (Y412 [1b numbering] and Y393 [1a numbering]; Cell Signaling Technology), or α-tubulin (T6074; Sigma-Aldrich).

### Imatinib dose response

Ba/F3 BCR-ABL cells were infected with retrovirus carrying BCR-ABL$^{35INS}$, BCR-ABL$^{K271P/35INS}$, or empty vector (MSCV-IRES-GFP), and cells were sorted by FACS for GFP. Resultant cell lines were plated in escalating concentrations of imatinib in quadruplicate, and proliferation was assessed after 72 hours. Analogous experiments were conducted with transfected, GFP-sorted K562 cells.

### ABL autophosphorylation and peptide-substrate assays

Autophosphorylation assays that used GST-ABL (residues 220-498), GST-ABL$^{35INS}$ (220-474, then YFDNREERTR-STOP),[10,12] and GST-ABL$^{K271R/35INS}$ were initiated with [γ-$^{32}$P]-ATP and quenched with SDS-PAGE loading buffer after 0-60 minutes, and proteins were separated on a 4%-15% Tris-HCl SDS-PAGE gel.[5] Gels were imaged with a storage phosphor screen (Typhoon 9400; GE Healthcare). Transferred gels were immunoblotted with ABL antibody Ab-2 (Oncogene Science) to assess protein loading.

Peptide-substrate phosphorylation assays that used GST-ABL, GST-ABL$^{35INS}$, and GST-ABL$^{K271R/35INS}$ and a peptide substrate (biotin-GGEAIYAAPFKK-amide; New England Peptides) were initiated with [γ-$^{32}$P]-ATP, quenched with guanidine hydrochloride (7M),[5] spotted onto duplicate SAM2 Biotin Capture membranes (Promega), washed according to the manufacturer's instructions, and counted. Enzyme concentrations were matched on the basis of Bradford analysis.

### Patients

Inclusion in the analysis required informed consent in accordance with the Declaration of Helsinki, a CML diagnosis, treatment with ABL TKIs, detection of BCR-ABL$^{35INS}$, and availability of clinical histories. All experiments with patient materials were approved by the Institutional Review Board of the Oregon Health and Science University (OHSU). Bone marrow or peripheral blood samples were collected at OHSU as clinically indicated during treatment. Direct BCR-ABL kinase domain sequencing was performed[10] and reported by the OHSU Knight Diagnostic Laboratories or MolecularMD Corporation.

## Results and discussion

Modeling studies[9,14] and clinical reports[12,14,16] have implicated BCR-ABL$^{35INS}$ as a potential mediator of resistance to ABL TKIs. However, critical mechanistic examination of BCR-ABL$^{35INS}$ in vitro and clinical analysis in the context of response status and time of resistance are lacking.

We tested the ability of Ba/F3 cells that expressed BCR-ABL or BCR-ABL$^{35INS}$ to grow without IL-3. As negative controls, we also tested parental Ba/F3 cells and Ba/F3 cells that expressed the BCR-ABL$^{35INS}$ mutant coupled with an additional point mutation of residue K271 that disrupts a critical salt bridge with E286 and precludes adoption of the active conformation of the kinase (BCR-ABL$^{K271P/35INS}$).[18,19] Only Ba/F3 BCR-ABL cells were rendered growth factor independent (Figure 1A). Immunoblotting confirmed the absence of the C-terminus of ABL in Ba/F3 BCR-ABL$^{35INS}$ cell lysate and further showed no phosphorylation of Y393, a critical activation loop residue that controls switching between active and inactive conformations[7,20-23] (Figure 1B). Lee et al[14] reported that imatinib resistance depends on BCR-ABL$^{35INS}$ expression level; however, we found that high-level coexpression of BCR-ABL$^{35INS}$ in Ba/F3 BCR-ABL cells did not influence imatinib sensitivity (Figure 1C-D); analogous results were obtained with K562 cells transfected with BCR-ABL$^{35INS}$ (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). In addition,Y393 of BCR-ABL$^{35INS}$ was not phosphorylated when coexpressed with native BCR-ABL, consistent with disruption of kinase domain architecture.

To evaluate catalytic competency, we compared GST-ABL, -ABL$^{35INS}$, and -ABL$^{K271R/35INS}$ in autophosphorylation (Figure 1E) and peptide-substrate phosphorylation assays (Figure 1F). No autophosphorylation was detectable for ABL$^{35INS}$ after 60 minutes, whereas full phosphorylation of ABL was reached by 15 minutes (Figure 1E). In the peptide-substrate assay, the extent of phosphorylation by ABL$^{35INS}$ was $< 2\%$ of the total phosphorylation by native ABL (Figure 1F).

As reported previously, we detect the BCR-ABL$^{35INS}$ mutant transcript by direct sequencing at our institution in ~1.7% of all cases of suspected imatinib resistance.[10] Analysis of 20 CML patients whose sequencing traces included evidence of BCR-ABL$^{35INS}$ at least once revealed that BCR-ABL$^{35INS}$ was demonstrably not the cause of disease progression or TKI resistance in 16 (80%) of 20 cases (Figure 2A; supplemental Table 1): 9 were responding to therapy at the time of BCR-ABL$^{35INS}$ detection (Figure 2B), 3 experienced treatment failure because of TKI intolerance, 1 lost response on dasatinib interruption (patient 16) but recaptured major molecular response on dasatinib resumption, and 3 (patients 15, 18, and 20) harbored a predominant, concurrent point mutation that adequately explained TKI resistance (Figure 2C). Thus, only 4 (20%) of 20 patients (patients 13, 14, 17, and 19) harboring BCR-ABL$^{35INS}$ at any point during therapy had this mutation exclusively detected at the time of resistance while undergoing ABL TKI therapy (Figure 2D).

It has been suggested that increased BCR-ABL$^{35INS}$ expression may lead to imatinib resistance.[14] However, we observed that BCR-ABL$^{35INS}$ was detected as a minor component ($\leq 20\%$ of total signal) by direct sequencing in 18 of 20 patients; both occurrences as a major component ($> 20\%$ of total signal) were in imatinib responders (Figure 2A). Furthermore, in patients for whom serial sequence samples were available, BCR-ABL$^{35INS}$ never emerged as a dominant clone (supplemental Table 1). Lastly, for all patients for whom sufficient material was available from

**Figure 2. (continued)** component of the total signal. *Patient 11 subsequently relapsed while undergoing therapy with dasatinib, with a predominant co-occurring T315I point mutation, whereas 35INS was detected intermittently and always as a minor component. †In patient 13, 35INS was detected intermittently as a minor component while the patient was taking dasatinib but notably was not detected at the time of subsequent cytogenetic relapse. §35INS was detected as a minor component in patient 16 during interruption of dasatinib treatment because of pregnancy; major molecular response was recaptured rapidly after treatment resumed. Representative examples are shown of timing and level of detection of the BCR-ABL$^{35INS}$ mutation from each of 3 observed clinical treatment/response scenarios: (B) BCR-ABL$^{35INS}$ was detected while the patient was responding to treatment without signs of resistance; (C) BCR-ABL$^{35INS}$ was detected at the time of resistance, but with a co-occurring highly predominant BCR-ABL kinase domain point mutation that satisfactorily explained resistance; and (D) BCR-ABL$^{35INS}$ was the only kinase domain mutation detected at the time of resistance to therapy. Notably, for all patients who showed evidence of the BCR-ABL$^{35INS}$ mutation at the time of resistance (ie, scenarios C or D above), this mutation was always detected as a minor component by direct sequencing. For more detailed treatment, response, and sequencing information for all patients included in this analysis, see supplemental Table 1. CP indicates chronic phase; CCR, complete cytogenetic response; MMR, major molecular response; MCR, major cytogenetic response; and qPCR, quantitative PCR.

sequencing time points at which BCR-ABL$^{35INS}$ was detected, the same 35INS mutation was concurrently detected in native c-ABL (6/6 [100%]; supplemental Figure 2). Because of the retrospective nature of the present study and sample availability, this analysis could not be performed in the 2 patients who were imatinib responders and yet showed the BCR-ABL$^{35INS}$ mutation as a major component by direct sequencing (patients 5 and 6). Others have detected variant *ABL* transcripts at similar frequencies in CML patients and healthy individuals, which implies the existence of alternative splicing mechanisms of *ABL* unrelated to TKI resistance.[11,24-26]

In total, the results of the present study demonstrate that BCR-ABL$^{35INS}$ lacks the qualities of a functional tyrosine kinase at the cellular and biochemical level. An alternative possibility, that BCR-ABL$^{35INS}$ is catalytically inactive but sequesters TKIs, is untenable on stoichiometric grounds and excluded by our experiments that demonstrated that coexpression of BCR-ABL and BCR-ABL$^{35INS}$ in Ba/F3 cells does not mitigate imatinib sensitivity compared with Ba/F3 cells that express only BCR-ABL. Another possibility is that BCR-ABL$^{35INS}$ heterodimerizes with BCR-ABL and maintains it in an imatinib-inaccessible active conformation; however, the present results demonstrate that BCR-ABL$^{35INS}$ lacks the necessary kinase activity to maintain BCR-ABL in an activated state and that BCR-ABL$^{35INS}$ is not susceptible to Y393 phosphorylation when expressed alone or with BCR-ABL. On the basis of our biochemical and cellular data and retrospective clinical analysis, we conclude that BCR-ABL$^{35INS}$ is kinase-inactive, does not contribute to TKI resistance in vitro, does not explain or consistently track with time of resistance in CML patients, and should not be considered in treatment decisions.

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## Authorship

Contribution: T.O., M.S.Z., C.A.E., A.A., L.T.A., H.Y., A.S.C., F.Y., and J.T. performed research; T.O., M.S.Z., C.A.E., R.D.P., M.W.D., and B.J.D. designed research and analyzed data; R.D.P, V.M.R., and S.W. contributed vital reagents and analytical tools; T.O., M.S.Z., C.A.E., and B.J.D. wrote the paper; and all authors reviewed the manuscript.

Conflict-of-interest statement: V.M.R. is an employee of ARIAD Pharmaceuticals Inc; J.T. and S.W. are employees of MolecularMD Corporation; and OHSU and B.J.D. have a financial interest in MolecularMD. Technology used in this research has been licensed to MolecularMD; this potential conflict of interest has been reviewed and managed by the OHSU Conflict of Interest in Research Committee and the Integrity Program Oversight Council. The remaining authors declare no competing financial interests.

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