Brief report

The BCR-ABL\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{35INS} is kinase-inactive and does not contribute to TKI resistance, and we find that detection of BCR-ABL\textsuperscript{35INS} does not consistently track with or explain resistance in clinical samples from chronic myeloid leukemia patients. (Blood. 2011;118(19):5250-5254)

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\textsuperscript{35INS}, or BCR-ABL\textsuperscript{K271P/35INS} (MSCV-IRE-GFP), and stable cell lines were sorted for GFP (FACSAnn II; BD Biosciences). After IL-3 withdrawal, cells were counted daily.

Ba/F3 immunoblotting

Ba/F3 parental cells and Ba/F3 cells expressing or coexpressing BCR-ABL, BCR-ABL\textsuperscript{35INS}, or BCR-ABL\textsuperscript{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-ABL kinase.
Figure 1. The BCR-ABL35INS mutant does not confer growth factor independence or imatinib resistance, and ABL35INS 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-ABL35INS, respectively. (C) Ba/F3 cells stably expressing BCR-ABL were transduced with the indicated BCR-ABL35INS 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-assay, 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-ABL35INS, respectively. The absence of signal corresponding to BCR-ABL35INS 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 [γ-32P]-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 [γ-32P]-ATP. At the end of the incubation period, reactions were terminated with 7M of guanidine hydrochloride solution, spotted in duplicate on SAMP 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-ABL35INS and GST-ABL271R/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.
Figure 2. Detection of the BCR-ABL35INS mutation in clinical samples does not consistently track with resistance to ABL TKI therapy. Retrospective analysis of the detection of the BCR-ABL35INS 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-ABL35INS 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-ABL35INS 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 previously10; primers used by MolecularMD remain proprietary. ¶An additional intron 4 insertion was detected along with 35INS in patient 7; both represented a minor

![Figure 2](image-url)
N-terminals (3902; Cell Signaling Technology), ABL C-terminals (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-ABL35INS, BCR-ABL271R35INS, or empty vector (MSCV-IRE-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-ABL35INS (220-474, then YFDNREER-STOP),10,12 and GST-ABL271R/35INS were initiated with [γ-32P]-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.7 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-ABL35INS, and GST-ABL271R/35INS and a peptide substrate (biotin-GGEAIYAAPFKK-amide; New England Peptides) were initiated with [γ-32P]-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, marrow or peripheral blood samples were collected at OHSU as clinically indicated during treatment. Direct BCR-ABL kinase domain sequencing was performed10 and reported by the OHSU Knight Diagnostic Laboratories or MolecularMD Corporation.

**Results and discussion**

Modeling studies8,9,14 and clinical reports12,14,16 have implicated BCR-ABL35INS as a potential mediator of resistance to ABL TKIs. However, critical mechanistic examination of BCR-ABL35INS 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-ABL35INS to grow without IL-3. As negative controls, we also tested parental Ba/F3 cells and Ba/F3 cells that expressed the BCR-ABL35INS 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-ABL17,35INS).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-ABL35INS cell lysate and further showed no phosphorylation of Y393, a critical activation loop residue that controls switching between active and inactive conformations2,20-23 (Figure 1B). Lee et al14 reported that imatinib resistance depends on BCR-ABL35INS expression level; however, we found that high-level coexpression of BCR-ABL35INS in Ba/F3 BCR-ABL cells did not influence imatinib sensitivity (Figure 1C-D); analogous results were obtained with K562 cells transfected with BCR-ABL35INS (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-K562 was not phosphorylated when coexpressed with native BCR-ABL, consistent with disruption of kinase domain architecture.

To evaluate catalytic competency, we compared GST-ABL, -ABL35INS, and -ABL271R/35INS in autophosphorylation (Figure 1E) and peptide-substrate phosphorylation assays (Figure 1F). No autophosphorylation was detectable for ABL35INS 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 ABL35INS was < 2% of the total phosphorylation by native ABL (Figure 1F).

As reported previously, we detect the BCR-ABL35INS 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-ABL35INS at least once revealed that BCR-ABL35INS 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-ABL35INS detection, and 3 (patients 15, 18, and 20) harbored a predominant, concurrent point mutation that adequately explained TKI resistance (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-ABL35INS at any point during therapy harbored this mutation exclusively detected at the time of resistance while undergoing ABL TKI therapy (Figure 2D).

It has been suggested that increased BCR-ABL35INS expression may lead to imatinib resistance.14 However, we observed that BCR-ABL35INS was detected as a minor component (≤ 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-ABL35INS never emerged as a dominant clone (supplemental Table 1). Lastly, for all patients for whom sufficient material was available from
sequencing time points at which BCR-ABL35INS 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-ABL35INS 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-ABL35INS lacks the qualities of a functional tyrosine kinase at the cellular and biochemical level. An alternative possibility, that BCR-ABL35INS 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-ABL35INS in Ba/F3 cells does not mitigate imatinib sensitivity compared with Ba/F3 cells that express only BCR-ABL. Another possibility is that BCR-ABL35INS heterodimerizes with BCR-ABL; however, we have discovered that BCR-ABL35INS in Ba/F3 cells does not mitigate imatinib sensitivity on the basis of our biochemical and cellular data and retrospective clinical analysis, we conclude that BCR-ABL35INS 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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References


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