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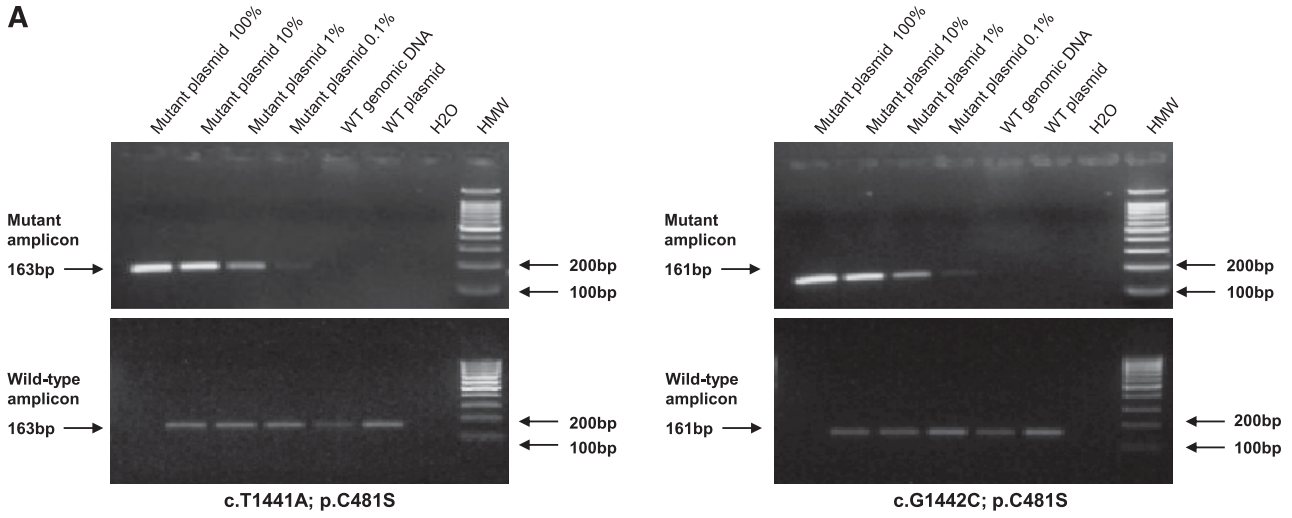
To the editor:

Ibrutinib-naïve chronic lymphocytic leukemia lacks Bruton tyrosine kinase mutations associated with treatment resistance

The Bruton tyrosine kinase (BTK) inhibitor ibrutinib blocks B-cell receptor signaling via covalent binding of the BTK C481 residue.[1] Although ibrutinib induces durable remissions in relapsed/refractory chronic lymphocytic leukemia (CLL), a fraction of patients treated with this targeted therapy still develop progressive disease after an initial response.[2] Genomic studies have disclosed mutations affecting the C481 codon of *BTK* in a sizeable fraction of ibrutinib-resistant CLL. These mutations interfere with the function of ibrutinib by blocking its covalent binding to BTK and have been observed in patients harboring prior poor-risk genetic lesions (ie, 17p deletion).[3,4]

In ibrutinib-treated CLL, resistant *BTK* mutations were not detectable at the baseline before drug exposure.[3,4] However, the identification of small numbers of *BTK* mutant CLL cells in the presence of large numbers of nonmutant CLL cells might be limited by the sensitivity of the methods used for the analysis, namely, Sanger sequencing (sensitivity of 10^{-1}) and low-depth next-generation sequencing (sensitivity of 10^{-2}).[3,4] Here we assessed the occurrence of small subclones harboring the C481S codon mutations (ie, c.T1441A; c.G1442C) of *BTK* in ibrutinib-naïve CLL patients using highly sensitive molecular methods.[5] Mutation analysis was performed by allele-specific polymerase chain reaction (AS-PCR) tailored at a sensitivity of 10^{-3} (ie, detection of 1 mutant allele per 1000 wild-type alleles), which is ~1 to 2 $\log_{10}$ higher than the sensitivity of previously used assays[3,4] (further details are available in the supplemental Appendix; see the *Blood* Web site). The study cohort comprised 553 newly presented CLL (151 with *TP53* abnormalities), 30 progressive and fludarabine refractory CLL (12 with *TP53* abnormalities), and 30 Richter syndrome (15 with *TP53* abnormalities) patients. In all cases, the fraction of tumor cells corresponded to 70% to 98% as assessed by flow cytometry or immunohistochemistry. All patients were ibrutinib naïve at the time of assessment. Patients provided informed consent in accordance with the Declaration of Helsinki. The study was approved by the Ethical Committee (Protocol Code 59/CE; Study Number CE 8/11).

By AS-PCR, neither newly presented CLL nor progressive fludarabine refractory CLL or Richter syndrome harbored *BTK* C481S–mutated clones above the sensitivity threshold of the assay (ie, >1/1000 tumor cells) (Figure 1). In order to validate this observation with an independent platform, 24 ibrutinib-naïve CLL patients harboring *TP53* disruption, who seem to be at higher risk of developing *BTK* variants,[3] were also investigated by ultradeep next-generation sequencing of the *BTK* mutation hot spot using the 454 chemistry.[5] The *BTK* region of interest was covered by sequence-specific primer pairs, each flanked by tagged sequences to bar code the samples. In each experiment, 12 amplicons were amplified from genomic DNA by using a high-fidelity Taq polymerase (FastStart



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Diagnosis	No of cases analyzed	c.T1441A p.C481S	c.G1442C p.C481S
Newly diagnosed CLL			
***TP53* wild type**	402	0	0
***TP53* disrupted**	151	0	0
Fludarabine-refractory CLL	30	0	0
Richters syndrome	30	0	0

Figure 1. AS-PCR assay for the detection of ibrutinib-resistant *BTK* mutations. (A) Conventional agarose-gel electrophoresis of the AS-PCR products documenting the sensitivity (10^{-3}) and specificity of the AS-PCR assay. After AS-PCR for the mutant allele, a mutation-specific band is amplified from the mutated plasmid DNA (positive control) and its serial dilutions into wild-type plasmid DNA down to 0.1%. No bands are amplified from the wild-type plasmid DNA and the wild-type genomic DNA from a healthy donor (negative controls), thus confirming the specificity of the assay. (B) Results of the AS-PCR screening of the *BTK* c.T1441A; p.C481S and c.G1442C; p.C481S mutations in ibrutinib-naïve CLL patients representative of different phases of the disease.

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High fidelity PCR System; Roche Diagnostics) and subjected to ultradeep next-generation sequencing on a Genome Sequencer Junior (454 Life Sciences). The target coverage was ~10 000× per amplicon to obtain a sensitivity of 10^{-3} (ie, detection of 1 mutant allele per 1000 wild-type alleles; further details are available in the supplemental Appendix). This approach confirmed that none of the 24 *TP53*-disrupted CLLs harbored *BTK* C481S–mutated clones.

Overall, these data indicate that, among CLLs that have not been exposed to ibrutinib, the *BTK* C481S variant conferring resistance to this drug is absent or limited to a subtle fraction of the clone that cannot be resolved with the current approaches. In this respect, CLL differs from chronic myeloid leukemia (CML) and Ph+ acute lymphoblastic leukemia (ALL), which represent other models of mutation-driven resistance to tyrosine kinase inhibitors (TKIs). In CML and Ph+ ALL, resistant mutations of *ABL* can be identified even in the early phase of the disease in a small fraction of the tumor clone before exposure to the selective pressure of TKI.[6-8] TKI-resistant mutations of *ABL* target different amino acids involved in TKI binding or in regulatory regions of the ABL kinase domain, resulting in decreased sensitivity to TKI while retaining aberrant kinase activity.[9] At variance with *ABL* mutations of CML and Ph+ ALL, ibrutinib-resistant *BTK* mutations in CLL (1) are selected to affect 1 single codon to which ibrutinib covalently binds and (2) do not occur in the absence of selective pressures imposed by ibrutinib.

From a diagnostic standpoint, our AS-PCR may serve as a new tool for the monitoring and the early identification of treatment-emergent CLL clones harboring the ibrutinib-resistant *BTK* mutation, which is one of the genetic causes of ibrutinib resistance in CLL.

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