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ORIGINAL ARTICLE

Quantitative monitoring by polymerase colony assay of known mutations resistant to ABL kinase inhibitors

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Resistance to molecularly targeted chemotherapy, and the development of novel agents that are active against resistant forms of target proteins create the need for a sensitive and quantitative assay to monitor drug-resistant mutations in patients to guide treatment and assess response. Here, we describe an application of the polymerase colony (polony) method to identify and quantify known point mutations in the *BCR-ABL* oncogene in patients with chronic myelogenous leukemia who evolve resistance to ABL kinase inhibitors. The assay can detect mutations with a sensitivity of 10^{-4}, quantify the burden of drug-resistant cells, and simultaneously monitor the dynamics of several coexisting mutations. As a proof of concept, we analysed blood samples from three patients undergoing therapy with ABL kinase inhibitors and found that the patients' response to therapy correlated with our molecular monitoring. We were also able to detect mutations emerging in patients long before clinical relapse. Therefore, the polony assay could be applied to a larger patient sample to assess the utility of early mutation detection in patient-specific treatment decisions. Finally, this methodology could be a valuable research tool to shed light on the natural behavior of mutations pre-existing kinase inhibitors therapy and either disappearing over time or slowly taking over.
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Introduction

Chronic myelogenous leukemia (CML), a myeloproliferative disorder that results from the malignant transformation of hematopoietic stem cells by the *BCR-ABL* oncogene (Daley *et al*., 1990), has served as a paradigm for understanding the molecular basis of transformation and for developing rationally designed chemotherapy. The great success of imatinib has stimulated the development of a host of targeted kinase inhibitors. Although virtually every chronic-phase CML patient attains a complete hematologic remission when treated with imatinib (Kantarjian *et al*., 2006), drug resistance occurs at a significant rate in chronic phase patients, and is frequent among patients who are diagnosed with advanced stage disease.

In most cases, resistance to imatinib is caused by amino-acid substitutions in the BCR-ABL protein that impede drug binding (Azam *et al*., 2003). Consequently, new kinase inhibitors are being developed that are active against imatinib-resistant mutants. Although these new inhibitors have a broad spectrum of activity against imatinib-resistant BCR-ABL variants, they remain ineffective against a handful of mutants and will elicit their own unique set of resistant mutations (Bradeen *et al*., 2006; Talpaz *et al*., 2006).

Although mutations to *BCR/ABL* have been described as the major mechanism of resistance to ABL kinase inhibitors, the clinical utility of mutation detection is still largely unknown. There is currently no consensus as to how and when patients should be screened for the presence of mutations or what sensitivity of detection is most beneficial (Hughes *et al*., 2006).

Therefore, it remains plausible that a sensitive and quantitative mutation detection method could provide early warning of the expansion of resistant mutant clones. We sought to develop a reliable method that would allow sensitive detection and quantification of known *BCR-ABL* mutations in peripheral blood (PB) samples. The polymerase colony (polony) technology is well suited for this purpose. Originally developed for detecting single-nucleotide polymorphisms (SNPs) and

combinatorial alternative splicing (Mitra and Church, 1999; Mitra *et al*., 2003; Zhu *et al*., 2003), the polony assay has been used for determining long-range haplotypes (Zhang *et al*., 2006), diagnosing loss of heterozygosity of p53 in tumor samples, and detecting *K-Ras* mutations in cancer cell lines (Butz *et al*., 2003).

The polony method entails an *in situ* PCR amplification of individual DNA molecules immobilized in an acrylamide matrix so that the amplification products of each DNA molecule form a PCR colony or 'polony'. Since one strand of each amplicon is covalently bound to the gel matrix, the unattached strand can be removed by denaturation. The sequence features (that is, the native or mutated nucleotide) of individual molecules can then be determined by single base extension (SBE) using primers whose 3′-end is juxtaposed to the polymorphic position of interest. Subsequently, the wild-type molecules are distinguished from mutated ones by differential incorporation of fluorescently labeled nucleotides. Afterwards, a conventional microarray scanner is used to acquire images of the polonies, enabling the identification of specific genotypes, alleles or mutations in a background of wild-type sequences (Figure 1).

Here, we demonstrate a successful application of a polony-based assay to the detection and quantification of mutations that confer resistance to imatinib and other BCR-ABL kinase inhibitors in clinical samples.

Results

A polony-based assay for detecting mutations in BCR-ABL

We have adapted a polony-based assay to detect imatinib-resistant mutations in the kinase domain of *BCR-ABL* and tested its sensitivity on mixtures of plasmids encoding either native BCR-ABL or a common imatinib-resistant mutant (G250E). The assay is highly sensitive, and able to detect, at a minimum, 1 mutant in a background of more than 10 000 native molecules (Figure 2a). The polony assay is more than 1000-fold more sensitive than the widely used direct sequencing assay. The sensitivity of the polony assay or any PCR-based approach is defined by the fidelity of the polymerase used to amplify the DNA templates, as well as by the amount of template available. Therefore, mutations detected below 10^{-4} may be false positive, since these events might be due to polymerase misincorporation. Indeed, such rare mutations may not be clinically significant.

Multiple mutation detection of the most common imatinib-resistant BCR-ABL mutations

A unique feature of the polony assay is that once polonies are generated each individual polony has a fixed 'address' on the polony slide. Thereafter, multiple rounds of SBEs can be performed sequentially with SBE primers for specific mutations, and the existence and abundance of several mutations can be reliably determined simultaneously. Nine mutations in the BCR-ABL kinase domain account for >90% of imatinib-resistant mutations found in patients (Hughes *et al*., 2006). The ability to simultaneously probe for all nine on the same polony slide provides a means for rapid identification of the predominant mutation. In addition, multiple mutations, rather than a single predominant one, have been described in many patients (Talpaz *et al*., 2006). A single polony slide can thus be used to follow dynamic changes in multiple coexisting mutations.

To demonstrate the ability to monitor multiple drug-resistant mutations simultaneously, we mixed cDNA samples from six available mouse cell lines engineered to express native BCR-ABL or the most common mutant forms (G250E, Q252H, Y253H, E255K or T315I (Figure 2b). A ~1 kb fragment of the *BCR-ABL* cDNA contains all kinase domain mutations, allowing us to sequentially screen for five mutations on a single slide.

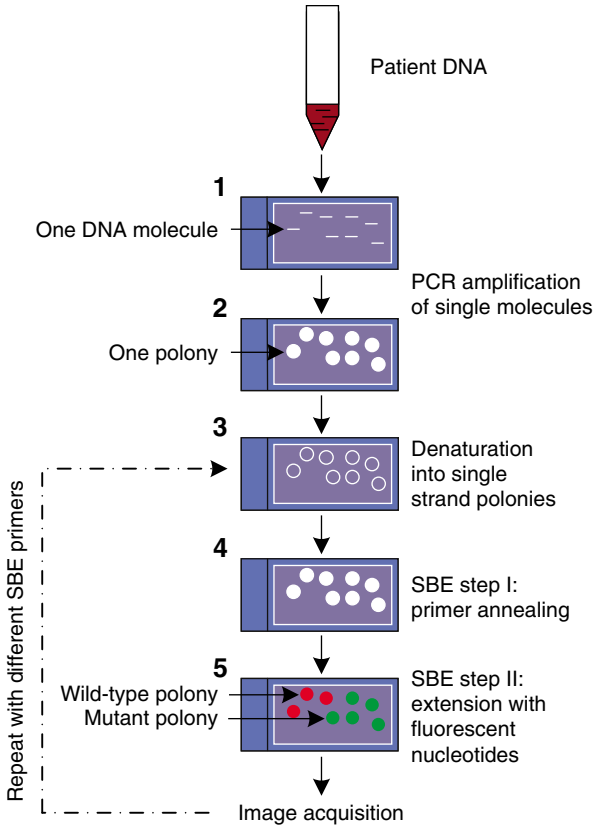

Figure 1 The polony assay. The main steps of polony assay mutation detection. (1) Either genomic DNA or cDNA are immobilized in an acrylamide gel poured on a microscope slide, together with an acrydite-modified primer that will adhere to the slide. (2) PCR is performed, resulting in parallel *in situ* amplification of single DNA molecules in the gel to produce PCR colonies or 'polonies'. (3) The gel is then denatured, reducing the DNA within polonies to single strand. (4) Primers are annealed next to the mutation of interest. (5) A SBE reaction is performed using two distinguishable fluorescently labeled nucleotides, one 'extending' the primer over the wild-type nucleotide, the other over the mutated base. A microarray scanner is then used to acquire images of the polonies. The slide can then be denatured and probed again for the next mutation of interest.

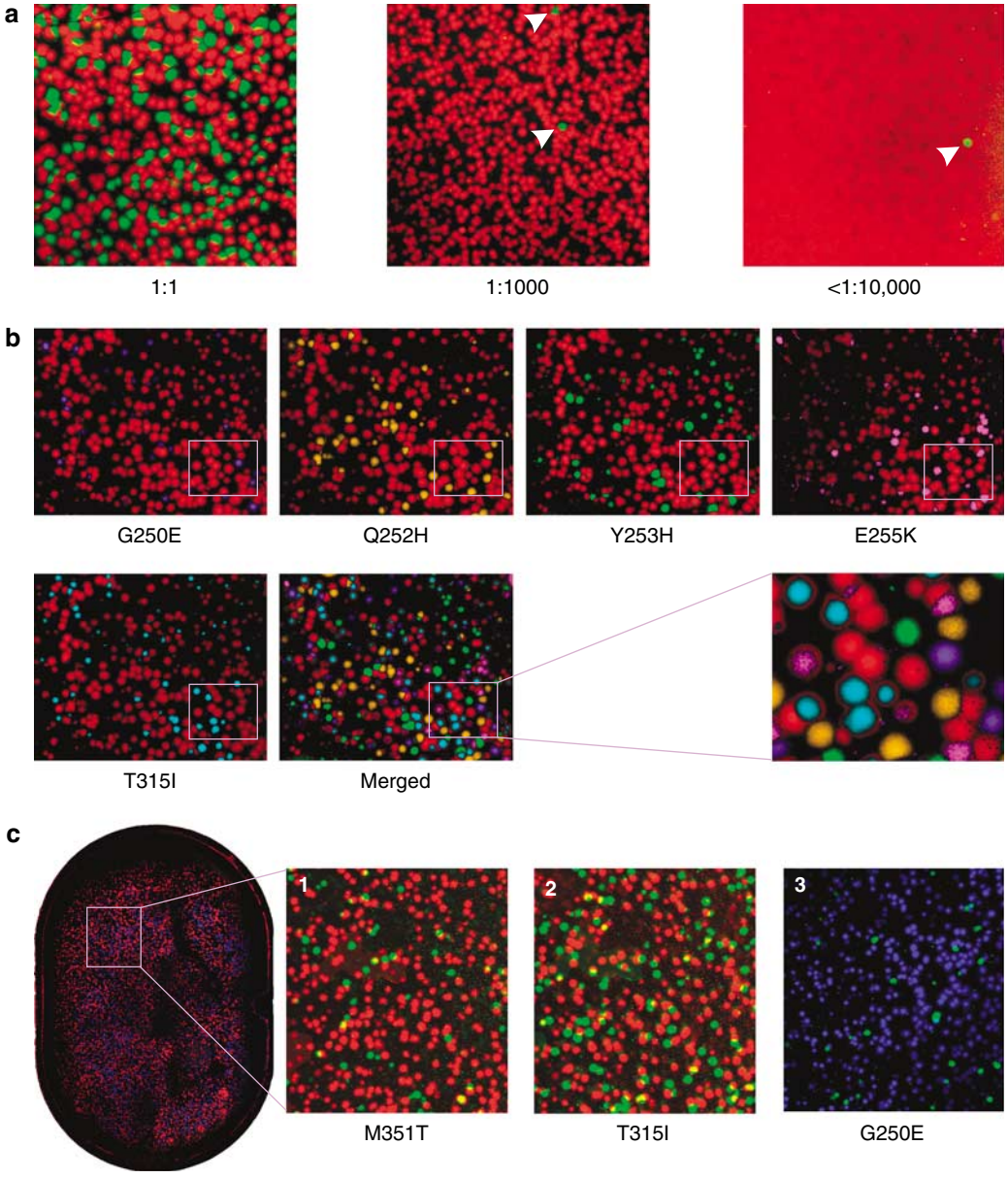

Figure 2 Application of a polony assay to detect mutant forms of *BCR-ABL*. (**a**) Determination of assay sensitivity. Polony detection was performed on cells carrying plasmids with the *BCR-ABL* G250E mutant mixed with cells carrying the native plasmid. Red, native *BCR-ABL*; green, G250E *BCR-ABL*. (**b**) Multiple mutation detection by the polony assay using cDNA templates. Five independent mutations can be identified and quantified on the same polony slide, as described in Materials and methods section. Green, BCR-ABL wt; red, mutated *BCR-ABL*. In the merged image: dark blue, G250E; yellow, Q252H; green, Y253H; light blue, T315I; magenta, E255K. (**c**) Multiple mutation detection by the polony assay using genomic DNA templates. Polonies were created by multiplex PCR with primers amplifying both exons 4 and 6 in discrete polonies. Left—the full polony slide. The rectangle is enlarged on the right to show the three mutations detected: 1—exon 6 M351T, 2—exon 6 T315I and 3—exon 4 G250E. Green—mutated *ABL*. Red—wild-type *ABL* exon 6. Blue—wild-type *ABL* exon 4.

As shown in the merged image (Figure 2b), we can unambiguously assign a mutation signature to each cDNA template and determine its relative frequency in the population. Although not showcased in our mixing experiment, coexistence of multiple mutations on the same molecule (Talpaz *et al*., 2006) can conceivably be determined, which could provide molecular insights into the complex mechanisms of drug resistance.

While polony assays with cDNA can be used to assess the frequency of mutant transcript in a given sample, genomic DNA can be used to assess the frequency of mutated cells. For that purpose, we developed a strategy to detect resistance mutations in genomic DNA samples. The most common imatinib-resistant *BCR-ABL* kinase domain mutations are encoded by exons 4 and 6, which are separated by 10 kb, precluding us from amplifying one genomic fragment that would encompass all nine common mutations. By using two sets of primers on the same slide, we were able to multiplex the assay, amplifying exons 4 (1130 bp) and 6 simultaneously

(1002 bp). Subsequently, we were able to screen for mutations falling on either exon. For this demonstration, we mixed genomic DNA from a CML patient carrying the T315I mutation and another carrying both the G250E and the M351T mutations, and successfully detected all three mutations (Figure 2c).

Quantitative monitoring of drug-resistant mutations in patients: correlation with clinical response to treatment with kinase inhibitors

To accurately quantify the load of drug-resistant leukemic cells—that is, the percentage of total blood cells that carry a mutant *BCR-ABL* gene—it is necessary to perform the polony assay using genomic DNA. Barring gene amplifications or deletions, the number of mutant polonies obtained when using genomic DNA will reflect one-half the number of mutated *BCR-ABL* cells in the sample. As CML cells typically contain single copies of the Philadelphia chromosome (and hence the *BCR-ABL* gene) and the wild-type *c-ABL*, the percentage of cells carrying a mutant *ABL* is two times the percentage of mutant polonies (for example, if 50% of the polonies appear mutated, then 100% of the cells carry the mutation). The ability to quantify the load of mutant cells is an important feature of the polony assay.

To illustrate patient monitoring by polony assay, we surveyed the appearance and levels of known drug-resistant *ABL* variants in banked PB samples from a set of three patients, each of whom had developed imatinib resistance that prompted a change in their treatment regimen.

Patient 1 sustained a cytogenetic relapse while on imatinib, and was found to harbor the M244V mutation. A polony assay performed on a pretreatment genomic DNA sample taken in December 2000 lacked the M244V mutation (Figure 3a). However, in a sample from September 2002, when the patient experienced loss of cytogenetic and hematological response, the polony assay revealed the mutation in 18% of the cells. With only a single effective kinase inhibitor available in 2002, the favored treatment option was increased dose imatinib. An increase in imatinib dose from 400 to 800 mg/day led to a hematologic response. Consistently, the polony assay shows the subsequent reduction in the mutational load to 2.3 % of the cells (Figure 3a).

Patient 2 relapsed on imatinib and was found to harbor the T315I mutations (Figure 3b). Retrospective evaluation of banked blood samples showed that the T315I mutation was present at a low frequency in 2003 (0.1% of the cells) directly after the patient had relapsed on imatinib, and had undergone a bone marrow transplant, and that the mutation persisted without significant change in frequency until December 2005 (not shown). This mutation was not detectable by direct sequencing of *BCR-ABL* transcripts using cDNA isolated from the same samples. An increase in mutation frequency from 0.1 to 0.9% was noted by polony assay (but not sequencing) in March 2006, when the patient progressed to blast crisis and was switched to the alternative BCR-ABL kinase inhibitor dasatinib. Thereafter, a dramatic increase in the percentage of the T315I clone occurred between March and April 2006, when the polony assay shows 26% of genomic DNA molecules harboring this mutation. Assuming no gene amplification, this means that 52% of the patient's cells were carrying the T315I variant. The expansion of T315I mutant cells 1 month after dasatinib initiation raises the question whether the low frequency of T315I mutant cells detected by polony assay could have indicated the dramatic expansion of this dasatinib-resistant cell population.

Patient 3 was shown to have the E255K mutation by direct sequencing in 2006. In a genomic DNA polony-based survey of banked samples, the E255K mutation, undetectable upon diagnosis in April 2001 (Figure 3c), becomes detectable—exclusively by polony assay—at a low frequency (0.4%) in October 2002. At this time, the patient was in complete hematological remission without a corresponding cytogenetic response. The patient presented with accelerated disease in October 2005 and was enrolled in a clinical trial of the BCR-ABL inhibitor nilotinib. Polony assays done on a blood sample taken at that time reflect a higher frequency of the E255K mutation (25%), which, following 2 months of nilotinib treatment, had increased further by May 2006 (76%). In June 2006, the patient showed a white blood count of $>60\,000$ cells/mm^3 with numerous blasts. Resistance to nilotinib in patients with the E255K mutation was predicted by *in vitro* studies (Bradeen *et al*., 2006) and recently observed in patients (Quintas-Cardama *et al*., 2007). It resulted in rapid dramatic increase of mutated leukemic cells. Given the pattern of resistance revealed by the polony assay, nilotinib was discontinued and dasatinib began in July 2006. The patient showed a complete hematological response. Two months into treatment with dasatinib, the polony assay revealed a drop in mutation frequency to 36% of PB mononuclear cells, which continued to decrease over the next few months to a minimum of 29%, suggesting either additional mutations or alternative mechanisms of drug resistance.

Discussion

Sequencing of *BCR/ABL* has recently been recommended for patients lacking an objective drug response (Baccarani *et al*., 2006; Hughes *et al*., 2006). Although an increasing number of clinics now offer direct sequencing to patients, this method is only reliable at detecting mutations at a frequency greater than 20%. A more sensitive and quantitative method would allow one to test the hypothesis that only rising percentages of mutant leukemic cell populations represent an expansion of a drug-resistant clone. We have developed such a tool, and used it to retrospectively follow three patients as examples of its utility.

We have adapted the polony technique to clinical samples. Attributes of sensitivity, versatility, ease of use, and quantifiability make this technique particularly

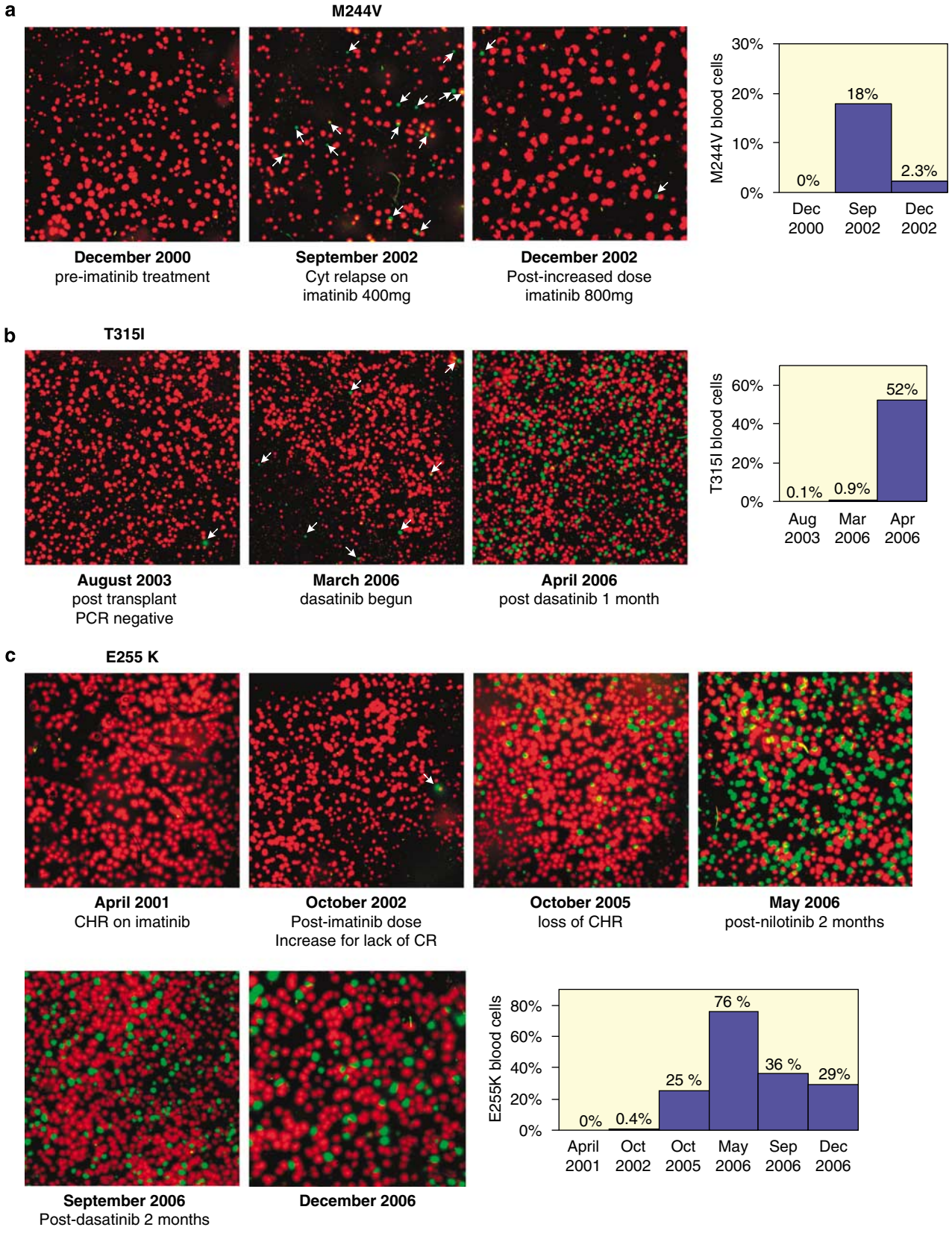

Figure 3 Determination of mutational load in patient samples by the polony assay. (**a**) Patient 1; (**b**) patient 2; (**c**) patient 3. Histograms show the frequency of cells carrying a mutation. Green, mutated nucleotide indicated by the white arrows; red, WT nucleotide. CHR, complete hematologic response. CR, cytogenetic response. We performed our assays on an average number of 2682 polonies (range 1728–4256; s.e.: 265).

valuable for monitoring CML patients particularly if coupled with a sensitive sequencing technology to detect new mutations. The polony method can be used for diagnostic screening and identification of known mutations, as well as for monitoring of multiple mutations in the same sample. One can readily determine when mutations occur on a single DNA strand because they will appear on the same polony. Indeed, the simultaneous occurrence of multiple cooperating mutations in the same protein has been observed following *in vitro* mutagenesis and selection (Azam *et al*., 2006) and is beginning to be observed in clinical practice (Talpaz *et al*., 2006). Polony technology also allows for the quantification of BCR-ABL to ABL ratio and mutation haplotyping (Supplementary Figure), another example of its versatility. The technique is easy to optimize and can be performed with a PCR machine with slide holder and a dual-wavelength microarray scanner. The approximate time from start to finish of the assay is about 18 h. Patients can be screened at costs comparable to Taqman PCR. Furthermore, when performed on genomic DNA, the polony assay can be used to quantify the load of cells bearing specific mutations and it requires very little input material ($\sim$100 ng genomic DNA), much less than emulsion-based platforms (Margulies *et al*., 2005; Thomas *et al*., 2006).

Denaturing-high-pressure liquid chromatography-based assays are a valuable tool for large-scale, semi-automated monitoring of *ABL* mutations (Soverini *et al*., 2004), although the sensitivity is only slightly higher than sequencing (5%) and the technique does not identify specific mutations. Once a mutant sample is found it must be sequenced to determine the specific mutation. Allele-specific oligonucleotide (ASO)-PCR can achieve high sensitivity, but can be highly variable between labs. Even within the same laboratory, the sensitivity of ASO-PCR can span 3 logs (from 1/100 to 1/100 000) depending on the primers used (Kang *et al*., 2006). In addition, ASO-PCR is not a reliable quantitative technique, and cannot be used to follow dynamic changes in mutation frequency.

The polony assay described here shares several attributes with other digital techniques, but also offers some key advantages. The Beads, Emulsion, Amplification and Magnetics (BEAM-ing) technique and the combination of bead-emulsion PCR with the high throughput application of pyrosequencing-by-synthesis are highly sensitive techniques that rely on the amplification of single DNA molecules attached to beads by emulsion PCR (Diehl *et al*., 2006; Li *et al*., 2006). The Beaming technique has a sensitivity in excess of 1 in 10^6 molecules, and it allows mutations to be scored by flow cytometry of the amplified fluorescently labeled beads. Although less sensitive (a detection limit of 2 in 10^3 molecules) a combination of pyrosequencing with bead emulsion PCR allows high throughput sequencing and thus is not limited to the detection of known mutations (a limitation common to both the polony and BEAM-ING assays; Margulies *et al*., 2005; Thomas *et al*., 2006). An important advantage of the polony assay over these alternative digital approaches is the large amplicon size that allows for sequential screening of multiple mutations on the same slide and the determination that mutations occur on the same DNA molecules. While other digital assays are limited to mutation detection in a short genomic fragment, a unique feature of the polony assay is the ability to simultaneously amplify distant genetic regions by PCR multiplexing (Mitra *et al*., 2003; Zhang *et al*., 2006).

In this pilot study, we explored the possibility of quantitatively monitoring imatinib-resistant mutations in response to treatment with imatinib and the novel BCR-ABL kinase inhibitors dasatinib and nilotinib. In three highly illustrative cases, the load of leukemic cells harboring *BCR-ABL* drug-resistant mutations correlated tightly with the clinical response of the patients to treatment. Moreover, we could identify the early appearance and dynamic increase of mutated cells before the resistant clones became dominant.

The study of additional patients by polony assay is warranted to shed light on the prognostic value of the persistence of a mutant clone in low frequency over time, and to determine whether the presence of such a cell may be a risk factor for treatment relapse.

Given that several additional kinase inhibitors are now available for treatment of CML patients, a prospective clinical survey is needed to demonstrate directly the means by which molecular monitoring by polony assay can be used to guide treatment decisions and influence patient outcomes.

Materials and methods

Samples from patients

We received human PB, cell pellets or DNA harvested and banked from CML patients treated at Dana Farber Cancer Institute, MD Anderson Medical Center, or Oregon Health and Science University according to the Institutional Review Board's approved protocols. All patients had previously consented to blood donation for research purposes in those institutions.

Template preparation and direct sequencing

Genomic DNA and/or RNA from patient samples or from the murine BaF3 cell lines engineered to express either wild-type *BCR-ABL* or imatinib-resistant mutated *BCR-ABL* (Azam *et al*., 2003) were purified using DNeasy (no. 69506, Qiagen, Valencia, CA, USA) and RNeasy (no. 74104, Qiagen). Reverse transcription of RNA was performed using Superscript III reverse transcriptase (no. 180080-051, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Nested PCRs were performed to isolate and sequence a *BCR-ABL* fragment spanning the kinase domain using (iProof GC 2 $\times$ Master Mix (no. 172-5320, Bio-Rad, Hercules, CA, USA) and published primers (Willis *et al*., 2005; Supplementary material).

Polony slide preparation

Polony amplification, denaturation, SBE-primer annealing and SBE was performed as described previously (Mitra and Church, 1999; Mitra *et al*., 2003; Zhu *et al*., 2003;

Supplementary material). For specific primers and SBE nucleotides used see Supplementary Tables 1 and 2.

Image acquisition
Gel images were acquired with a GenePix 4000B microarray scanner (Molecular Devices Corporation, Sunnyvale, CA, USA) with 10 μm/pixel resolution, using either a 635 nm (Cy5 detection) or a 532 nm (Cy3 detection) laser.

Sensitivity of the polony assay
To assess the mutation detection sensitivity of the polony assay by controlled dilution experiments, we performed the polony assay on mixtures of native *BCR-ABL* plasmid and plasmids carrying the *BCR-ABL*-G250E mutant (Azam *et al*., 2003). We serially diluted the wild-type plasmid from 10^7 molecules to 10^3 molecules, leaving the mutant plasmid at 10^3 molecules.

'Sequential screen' experiments
Up to approximately 10 SBE extension sets can be done on one polony slide. To demonstrate this application, we grew 3 million BaF3 cells transformed with either native BCR-ABL or five different mutated *BCR-ABL* plasmids (G250E, Q252H, Y253H, E255K, T315I (Azam *et al*., 2003)), harvested their RNA, obtained cDNA from 2 μg of RNA/cell line in a 20 μl reaction, and mixed 2 μl of each cell line cDNA. Each time before performing an SBE, we scanned the denatured slide to verify that we extinguished the background fluorescence from the previous SBE cycle. For the sequential multiplexing screen using patients' genomic DNA, we mixed genomic DNA that we harvested from two patients with known *BCR-ABL* point mutations located on different *ABL* exons. To do so, we incorporated two primer pairs at the same time (one of each pair was acrydite modified), one specific for exon 4 and one specific for exon 6.

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