here is capable of directly detecting expression of breast cancer susceptibility genes from mRNA extracts with high sensitivity and specificity without RT-PCR amplification. Gene expression differences less than onefold were successfully detected. The lowest detectable amount of a specific gene was found to be ~800 copies in 1.5 ng of mRNA. Integration of the sensor array into a fully automated microelectromechanical system, from tissue to nucleic acid isolation and quantification, may provide faster, less expensive, and simpler solutions for molecular diagnosis. Development of such an integrated system is being undertaken in our laboratory.

References


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Use of Denaturing HPLC for Detection of Mutations in the BCR-ABL Kinase Domain in Patients Resistant to Imatinib, Julie A.E. Irving,1 Stephen O’Brien,2 Anne L. Lennard,1,2 Lynne Minto,1 Feng Lin,2 and Andrew G. Hall1

1 Leukaemia Research Fund Molecular Pharmacology Laboratory and 2 Department of Haematology, School of Clinical and Laboratory Sciences, The Medical School, University of Newcastle, Newcastle, UK; * address correspondence to this author at: Northern Institute for Cancer Research, Cookson Building, Medical School, Newcastle NE2 4HH, UK; fax 44-191-222-7556, e-mail j.a.e.irving@ncl.ac.uk

The ABL tyrosine kinase inhibitor imatinib (STI571, Gleevec, Gleevec) has produced dramatic clinical responses in most patients with chronic myeloid leukemia (1–4). However, drug resistance has arisen as a result of increased expression of the BCR-ABL oncogene or the emergence of clones of cells harboring mutations in the ABL portion of the gene that reduce drug binding while retaining aberrant kinase activity (5). Original observations suggested that one specific substitution, T315I, was responsible (6). However, subsequent reports have demonstrated that this is not the case (7–13).

To monitor the emergence of drug resistance in patients treated with imatinib, there is a need to develop a reliable method for the screening of mutations in the BCR-ABL oncogene (5, 7). We report here the use of denaturing HPLC (DHPLC) as a method to screen for mutations in exons 4 and 6 of the ABL gene. Although originally described more than 6 years ago, the widespread application of this technique has only recently become possible with the introduction of commercially available specialized HPLC instrumentation dedicated to the performance of mutation analysis (14). Previous reports have demonstrated that the sensitivity and specificity of the technique are consistently high (15, 16).

Peripheral blood samples were obtained, after receipt of informed consent, from 22 patients with accelerated phase or blast crisis chronic myeloid leukemia. Genomic DNA from control samples was obtained from the Westlakes Research Institute at Cumbria after appropriate ethical consent. Genomic DNA was extracted by a standard phenol–chloroform method. PCR was performed with 50–100 ng of DNA, 2.5 mM MgCl2, 100 µM deoxynucleotide triphosphates, 0.2 µM each of forward and reverse primer, 1.25 U of Tag polymerase (AmpliTaq Gold; Perkin-Elmer), and 1× buffer. Primer pairs were designed with use of Omiga Software (Accelyrs Inc) to flank exons 4 and 6 of the ABL gene, based on criteria recommended by Transgenomic. The primer sequences were as follows: exon 4 forward, 5′-CTG TCT CTC TGG GCT GAA-3′, and exon 4 reverse, 5′-AAA CAC ACT CGG ACT ATG AGA A-3′; exon 6 forward, 5′-GAC TGA GCA GCA GAG TCA GA-3′ and exon 6 reverse 5′-GCC AGC ACT GAG TGT AGA A-3′. PCR conditions were as follows: 10 min of denaturation at 95 °C; a touchdown protocol of 20 s at 94 °C, 1 min at 66 °C (exon 4) or 63 °C (exon 6), and 1 min at 72 °C (−0.5 °C for 14 cycles); and 20 cycles of 20 s at 94 °C, 1 min at 59 °C (exon 4) or 56 °C (exon 6), and 1 min at 72 °C, with a final extension for 7 min at 72 °C. Heteroduplexes were formed in a thermal cycler by denaturation (95 °C for 5 min) and annealing/stabilization (starting at 94 °C for 2 min with a −1 °C touchdown until 4 °C). DHPLC was performed in a Trangenomic WAVE mutation detection apparatus. Samples (10 µL) were eluted from a DNAsel column by a gradient of 250 mM/L acetonitrile in 0.1 mol/L triethylammonium acetate (buffer B) against 0.1 mol/L triethylammonium acetate.
The gradients used for elution were as calculated by Wavemaker software, and temperatures of 62 °C (exon 6) and 64 °C (exon 4) were found to be optimal for analysis. For direct sequencing, PCR products were purified by use of a PCR Clean up reagent set (Qiagen) and sequenced by use of BigDye Terminators chemistry on an ABI automated DNA sequencer. To characterize minority PCR species, PCR products were subcloned into the Pgem-T-Easy plasmid according to the manufacturer’s instructions and inserted in *Escherichia coli* (JM109), and isolated plasmid DNA from positive transformants was then sequenced with M13 forward and reverse primers.

Fig. 1. DHPLC chromatograms from patients showing the presence of mutations in the *BCR-ABL* oncogene in exons 6 (A) and 4 (B).

The results of serial samples are shown for two patients (patients 2 and 3) for exon 6, showing the emergence of a mutated clone of cells. The x axis is the elution time in min. Pt, patient.

The elution profiles for control samples for *ABL* exon 6 amplicons (n = 29) all showed a single peak representative of homoduplex DNA. Six of 22 patient samples had profiles containing one or more additional peaks, indicating the presence of a mismatch (Fig. 1A). Similarly, exon 4 amplicons gave elution profiles with a single homoduplex peak in 47 of 48 control samples, but with a distinct double peak in 1 case, whereas 5 patient samples also showed abnormal profiles (Fig. 1B). In two patient samples, abnormal elution profiles were detected in both exons 4 and 6.

The degree of resolution varied greatly from an obvious
extra peak (e.g., Fig. 1A, patient 4) to a more subtle
shouldering of the homoduplex peak (e.g., Fig. 1A, patient
1). However, these more subtle patterns were highly
reproducible in repeated analyses using fresh PCR prod-
ucts and were readily detectable when analyzed along-
side products from cases without mutations. In cases 2
and 3, serial samples were available from patients who
developed imatinib resistance. In these, the aberrant pat-
tern became more obvious with the evolution of resis-
tance, presumably as the proportion of the resistant clones
increased.

Direct sequencing of PCR products demonstrated a
heterozygote signal representative of the T315I mutation
in patient 4, the G250E mutation in patient 8, and the
E255K mutation in patient 9, but was not sufficiently
sensitive to conclusively identify mutations in the other
samples. However, sequencing of cloned PCR products
revealed mutations in a minority of clones in all of the
remaining cases (range, 5–47%; mean, 17%). Direct se-
quencing also identified the normal sample associated
with an altered DHPLC profile as being a K247R hetero-
zygote, which to our knowledge is not listed in any of the
major single-nucleotide polymorphism databases and is
presumably a rare polymorphism. Interestingly, three of
the nine patients had a mutation at position 359, one with
the substitution of phenylalanine for valine (F359V) and
two for cysteine (F359C), the latter not having been
reported previously. The detection of multiple indepen-
dent mutant clones in two patients has been observed in
other studies (7).

To date, 19 different amino acid mutations at 14 resi-
dues of the ABL protein have been described in clinical
samples (Table 1), including the new mutation reported
here. Kinase domain mutations can occur in the chronic
phase before the emergence of overt resistance (7), and
there is evidence that such mutations may even predate
the initiation of imatinib therapy (12,17,18). In vitro
studies have suggested that mutations outside the kinase
domain may also confer resistance (19) and that there
may be “super mutants” that can hyperactivate autophos-
phorylation of the BCR-ABL kinase (19,20). Studies of
clinical samples have not yet investigated these non-
kine domain mutants, but it seems likely that their
identification may well drive progress in drug develop-
ment and clinical management in the future. DHPLC
could be a useful technique to screen samples for such
mutations.

Mutational analysis may be performed with a variety of
gel-based techniques, but these are difficult to perform,
are not readily automated, and are prone to high false-
positive and -negative detection rates. Automated se-
quencing is frequently described as the “gold standard”
for this form of analysis, but mutations may readily be
missed in minority populations of cells unless multiple
PCR clones are analyzed. This point was emphasized in
the study by Shah et al. (7). The relatively low rate of
detection of ABL mutations in some other studies (13,21)
may be partly attributable to the use of direct sequencing
without cloning to assess samples. In our study, muta-
tions were detected in only a minority of PCR clones in
five of the nine patients studied. Other, more recently
described mutational screening methods, with sensitivity
superior to that of sequencing, include an endonuclease/
ligase-based technique and an enhanced PCR-restriction
fragment length polymorphism analysis method (22,23).
However, both techniques are multistep and not easily
amenable to routine screening of clinical samples.

We used genomic DNA, rather than cDNA, to screen
for mutations. Although this allows retrospective analysis
of samples where mRNA may not be available, it will
decrease the sensitivity of the technique because alleles
from normal cells will be amplified along with normal
and chimeric ABL genes in Ph-positive cells. DHPLC
could be applied to cDNA samples reverse-transcribed
from BCR-ABL mRNA, although such amplicons would
have to be mixed with PCR products from nonmutated
cases to form heteroduplexes. We found no mutations in
patients demonstrating primary resistance, which is in

| Table 1. Summary of reported mutations in the BCR-ABL oncogene. |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Exon | n | M244 | G250 | Q252 | Y253 | E255 | P311 | T315 | F317 | M351 | E355 | F359 | V379 | L387 | H396 |
| Newcastle cohort | | | | | | | | | | | | | | | |
| This report | 9/22 | V | 2×E | H | K | I | 2×T | 2×C, V | NA | NA | NA | |
| Previous studies | | | | | | | | | | | | | | | |
| Gorre et al. (6) | 6/9 | | | | | | | | | | | | | | |
| Barth et al. (21) | 1/12 | K | | | | | | | | | | | | | |
| Hochhaus et al. (13) | 1/32 | V | | | | | | | | | | | | | |
| Branford et al. (9) | 12/18 | 2×E | H | 4×K | 3×I | L | T | | | | | | | | |
| von Bubnoff et al. (10) | 7/8 | 2×H | V, K | 2×I | | | | | | | | | | |
| Shah et al. (7) | 29/32 | V | 2×E | R, H | 2×H, 2×F | 10×K | 10×I | 3×L | 10×T | G | 2×V | I | M | R |
| Hochhaus et al. (11) | 23/66 | V | H | 4×H, F | 3×K, V | 6×I | 4×T | G | | | | | | |
| Roche-Lestienne et al. (12) | 5/24 | | | | | | | | | | | | | | |

* NA, not applicable.
keeping with most other studies, although the recent report by Shah et al. (7) demonstrates that this can occur.

In summary, we show that DHPLC can detect previously characterized and novel mutations associated with acquired resistance to imatinib. Each PCR product analysis is performed in less than 10 min in an automated instrumentation platform that has increased sensitivity over direct sequencing methods with considerably reduced labor and consumable costs.

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References

Detection and Monitoring of SARS Coronavirus in the Plasma and Peripheral Blood Lymphocytes of Patients with Severe Acute Respiratory Syndrome, Haibin Wang, Yuanli Mao, Liancai Ju, Jing Zhang, Zhiguo Liu, Xianzhi Zhou, Qinghong Li, Yuedong Wang, Sunghee Kim, and Lu-rong Zhang (Department of Laboratory Medicine, Beijing 302 Hospital, Beijing, People’s Republic of China; * address correspondence to H. Wang at: Laboratory Medicine, Beijing 302 Hospital, Beijing, People’s Republic of China; e-mail haibin_wang@sohu.com; or to L. Zhang at: University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY; e-mail lurong_zhang@urmc.rochester.edu)

Severe acute respiratory syndrome (SARS) is a newly emerged infectious disease, and a novel SARS-associated coronavirus (CoV) has been identified as a causative agent (1–3). Reliable and sensitive determination of the SARS CoV load would aid in the early identification of infected individuals, provide guidance for treatment (especially the use of steroid hormones and antiviral agents), and aid in monitoring of a patient’s clinical course and outcome.

Among the available tests, viral gene amplification by reverse transcription-PCR (RT-PCR) provides a relatively rapid and specific test for the diagnosis of individuals showing SARS-associated symptoms (4, 5). RT-PCR was successfully used to detect SARS CoV in nasopharyngeal aspirates, nasopharyngeal swabs, throat swabs, and bronchoalveolar lavage of SARS patients (6, 7). However, because the composition of these samples varies with time and among individuals, they are unlikely to serve as a standardized sample source for quantification, comparison, or monitoring of SARS CoV infection. To meet clinical needs, some improved RT-PCR methods have been developed, and plasma has been used as a sample source (8–10). The consistency of plasma composition makes it a good sample source for monitoring the CoV load.

CoV-enriched samples are critical for achieving a high detection rate. Current methods have a limited detection window, largely because they do not fully utilize the CoV viral RNA in the sample. Because of its high false-