Comparison of allele specific oligonucleotide-polymerase chain reaction and direct sequencing for high throughput screening of ABL kinase domain mutations in chronic myeloid leukemia resistant to imatinib

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To identify a fast and sensitive method for screening for mutations in patients with imatinib-resistant chronic myeloid leukemia (CML), we compared allele specific oligonucleotide-polymerase chain reaction (ASO-PCR) assay with conventional direct sequencing. Among the 68 imatinib resistant CML patients studied, 18 amino acid substitutions were detected in 44 patients by two assays. The sensitivity of ASO-PCR was superior to that of direct sequencing as it could detect one mutant allele in 100~100,000 wild type sequences. The fastness, simplicity, and sensitivity of ASO-PCR assays will be useful for routine monitoring of mutations, especially for frequently identified mutations.

Key words: imatinib, CML, resistance, ASO-PCR, direct sequencing.

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Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder characterized by the *BCR-ABL* oncogenic fusion gene.[1,2] Treatment for patients with CML has been greatly improved by the use of imatinib, which is a selective inhibitor of ABL kinase.[3] However, most patients in advanced phase either exhibit primary refractoriness or relapse after an initial response to imatinib.[4] Recent studies have revealed that resistance to imatinib is mainly caused by mutations in the ABL kinase domain and, to a lesser extent, by amplification of the *BCR-ABL* gene.[4-12] In fact, mutations have been detected in up to 91% of patients who relapse after initial response[6] and they may pre-exist the onset of treatment.[11]

Several methods have been used to detect mutations in the ABL kinase domain. Direct sequencing of the kinase domain is most commonly used but has limited sensitivity.[6] Denaturing high-performance liquid chromatography allows for high throughput mutation screening.[13,14] Enhanced polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) showed excellent sensitivity by detecting one mutated cell from among 1,000 wild type cells.[15] Allele specific oligonucleotide (ASO)-PCR assays showed the best sensitivity by detecting mutations even after dilution in the 10,000-fold range.[11] Therefore, we established an ASO-PCR assay for the detection of 17 known mutations which were selected according to their frequency in imatinib-resistant CML patients.

Here we describe the ASO-PCR assay for detecting 17 known mutations and compare the results of mutation analysis by ASO-PCR with those of conventional direct sequencing.

Design and Methods

Patients

Four hundred and thirty *BCR-ABL*-positive CML patients were treated with imatinib at St. Mary's Hospital between May 2001 and February 2005. During this period 112 of the 430 patients had disease that was refractory to imatinib or relapsed after treatment with this agent. Sixty-eight patients, including 12 in chronic phase, 29 in accelerated phase, and 27 in blast crisis, were analyzed for the presence of mutations using both the ASO-PCR assay and direct sequencing. Informed consent was obtained from all patients involved in this study.

DNA and RNA extraction

Genomic DNA (gDNA) was extracted from 5×10^6 mononuclear cells (MNC) prepared from bone marrow using a High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany). The quantity and ratio of absorbance at 260nm and 280nm (A260/280) of purified gDNA was determined with Nanodrop (Nanodrop technologies, Delaware, USA). Total RNA was extracted from each sample using the Trizol reagent (Invitrogen, Carlsbad, CA, USA).

Cloning of PCR products

To determine the sensitivity of assays, PCR products containing a point mutation or wild type sequence were cloned. In detail, PCR amplifications of each mutation or wild type sequence were performed using gDNA from patient samples or K562 as template. Each amplified PCR product was directly cloned to pCR2.1 TA cloning vector (Invitrogen). The cloned PCR products were confirmed by *Eco*RI digestion analysis and direct sequencing.

ASO-PCR primers

We designed 17 different ASO-PCR primer sets according to the frequency of known *ABL* gene mutations. The sequences of forward and reverse primers used for ASO-PCR are shown in Table 1. The sequences of primers for T315 and M351 were adapted from a previously published article[11] and others were designed

Table 1. Summary of the ASO-PCR assay used in this study.

Mutant[1]	NT change[2]	Forward primer[3]	Sequence (5'→3')	Annealing temp. °C	ASO PCR[4]	Seq[5]	Sensitivity[6] (mutant/ wild type)
M244V	A1094G	244m	gaacgcacggacatcaccg	65.7	3	1	1/550
L248V	C1106G	248m	accatgaagcacaagg	55	0	0	1/1,000
G250E	G1113A	250m	gaagcacaagctgggcga	56	3	2	1/5,500
Q252H(a)	G1120C	252m1	agctgggcgggggccac	62	2	2	1/10,000
Q252H(b)	G1120T	252m2	agctgggcgggggccat	62	0	0	1/10,000
Y253H	T1121C	253m	gctgggcgggggccagc	62	6	3	1/1,000
Y253F	A1122T	253-2m	ctgggcgggggccagtt	55	3	1	1/100,000
E255K	G1127A	255m	gcgggggccagtacgggga	68	6	5	1/1,000
E255V	A1128T	255-2m	gcgggggccagtacggggt	58	4	4	1/ 10,000
T315I	C1308T	315m	gcccccgttctatatcatcat	63.4	13	10	1/1,000
F317L	C1315G	317m	ccgttctatatcatcactgagttg	54	0	0	1/10,000
M343T	T1392C	343m	gtggtgctgctgtacac	62	0	0	1/100
M351T	T1416C	351m	ccactcagatctcgtcagccac	70	1	2	1/100
E355G	A1428G	355m	gtcagccatggagtacctagg	56	1	0	1/5,500
F359V	T1439G	359m	gagtacctagagaagaaaaacg	50	3	2	1/10,000
H396R	A1551G	396m	ggacacctacacagcccg	62.5	0	0	1/100
F486S	T1821C	486m	tctgaccggccctcctc	62	0	0	1/1,000

[1]Substitutions of amino acids; positions according to GenBank no. AAB60394 for ABL type 1a; [2]Changes of nucleotide; positions according to GenBank no. M14752; [3]To amplify M244V, L248V, G250E, Q252H(a), Q252H(b), Y253H, Y253F, E255K, and E255V mutations, reverse primer 244r (gccaatgaagccctcggac) was used. The reverse primer 315r (ggatgaagtttttctttctccag) was used to detect T315I and F317L mutations. The 351r1 (gccctgagacctcctaggct) primer was used for M343T and M351T amplification, whereas the 351r2 (atgccaaagctggctttg) reverse primer was used for amplification of E355G and F359V. The 396r (ggacacctacacagcccg) and 486r (agctttctggtctcagga) reverse primers were used for amplification of H396R and F486S, respectively; [4]The numbers in the table indicate the times of mutations detected by ASO-PCR. [5]The numbers indicate the times of mutations detected by direct sequencing. [6]The sensitivity was measured by using two independent experiments.

specifically using BLAST search and Primer Premier 5 (PREMIER Biosoft international, Palo Alto, CA, USA).

Establishment of the ASO-PCR assay for 17 mutations in the ABL kinase domain

To determine the optimal conditions for ASO-PCR, we amplified 17 mutated sequences in a PCR reaction using 100 ng gDNA of K562 cells or patient samples and primers specific for wild type or mutated sequences. Cloned PCR products were used to determine the optimal PCR conditions. The ASO-PCR assay was done using 17 primer pairs specific for wild type or mutated sequences in a 30 μL reaction mixture using 0.12 pg of wild type or mutant plasmid, which correspond to approximately 30,000 cells, as a template. To establish the optimal annealing temperature for ASO-PCR, gradient PCR was performed by increasing the annealing temperature from 50°C to 70°C. The condition that amplified only a specific mutated sequence, but not a wild type sequence, was determined and used in the ASO-PCR assay.

ASO-PCR assays

Mutated and wild-type alleles were specifically amplified in a 30 μL reaction mixture using 100 ng of gDNA as described previously.[11] Thermocycling conditions used were 5 minutes at 94°C followed by 30 cycles of denaturation at 94°C for 25 seconds, annealing at the indicated temperature for 25 seconds, extension at 72°C for 30 seconds, and a final extension for 5 minutes at 72°C (Table 1).

Direct sequencing analysis

The whole ABL kinase domain (amino acid n. 209 to 505) was directly sequenced after PCR amplification. In detail, 2 μg of total RNA were converted to cDNA using AMV reverse transcriptase (Roche, Penzberg, Germany) and oligo(dT) primers. A semi-nested PCR for *BCR-ABL* fusion transcript was carried out using a LA Taq (TaKaRa, Shiga, Japan) and the PCR products were analyzed using the ABI Prism 377 sequencer as described.[16]

Results and Discussion

Determination of sensitivity for the ASO-PCR assay and direct sequencing

The sensitivity of the ASO-PCR assay was measured for each mutation using samples containing mutant plasmid that was serially diluted in wild type gDNA. As shown in Table 1, the sensitivity varied for each mutation. Surprisingly, the ASO-PCR assay for Y253F mutation detected even one mutated sequence in the presence of 100,000 wild type DNA, showing the excellent sensitivity of this assay (Figure 1A). However, primers for M343T, M351T, and H396R exhibited poor sensitivity for detecting mutations when the percentage of mutant was more than 1% of the total (Table 1). For the determination of sensitivity for direct sequencing, we sequenced mutant plasmids serially diluted in wild type plasmids. The direct sequencing method detected mutated sequences of T315I when the proportion of mutant was 30% (Figure 1B).

Analysis of patients' samples with ASO-PCR and direct sequencing

Forty-four (65%) of the 68 patients had 53 mutations detected by the two assays. The ASO-PCR assay and direct sequencing detected 45 and 32 mutations, respectively (Table 2). Interestingly, the changes of three amino acids, Y253, E255, and T315, accounted for 71% of the total mutations identified by ASO-PCR and 59% by direct sequencing. As screening for 17 mutations with ASO-PCR is somewhat laborious, examining three amino acids, Y253, E255, and T315, followed by direct sequencing will be more fruitful. Besides the 17 mutations measured by ASO-PCR, seven mutations (F317L (C1315A), F359C, F359I, H396P, S417Y, E450K, and E459K) were additionally found by direct sequencing. Among these, E450K is a novel mutation. The two assays were performed to detect pre-existing mutations in bone marrow samples from 66 CML patients. While no mutation was detected by direct sequencing, ASO-PCR revealed mutations in two (5.2%) of 38 CML patients. Among these, one patient in blast crisis with T315I mutation failed to achieve either a hematologic or a cytogenetic response within 4 months of treatment. At that time, E255K, a newly developed mutation was detected weakly in addition to T315I by ASO-PCR, but direct sequencing only detected T315I. The other acute phase patient with an E255V mutation achieved both complete hematologic and cytogenetic responses at 6 months of treatment. However, this patient lost the cytogenetic response (100% Ph-positive) and the hematologic response at 12 and 15 months, respectively. At 15 months of treatment, the E255V mutation was detected by both

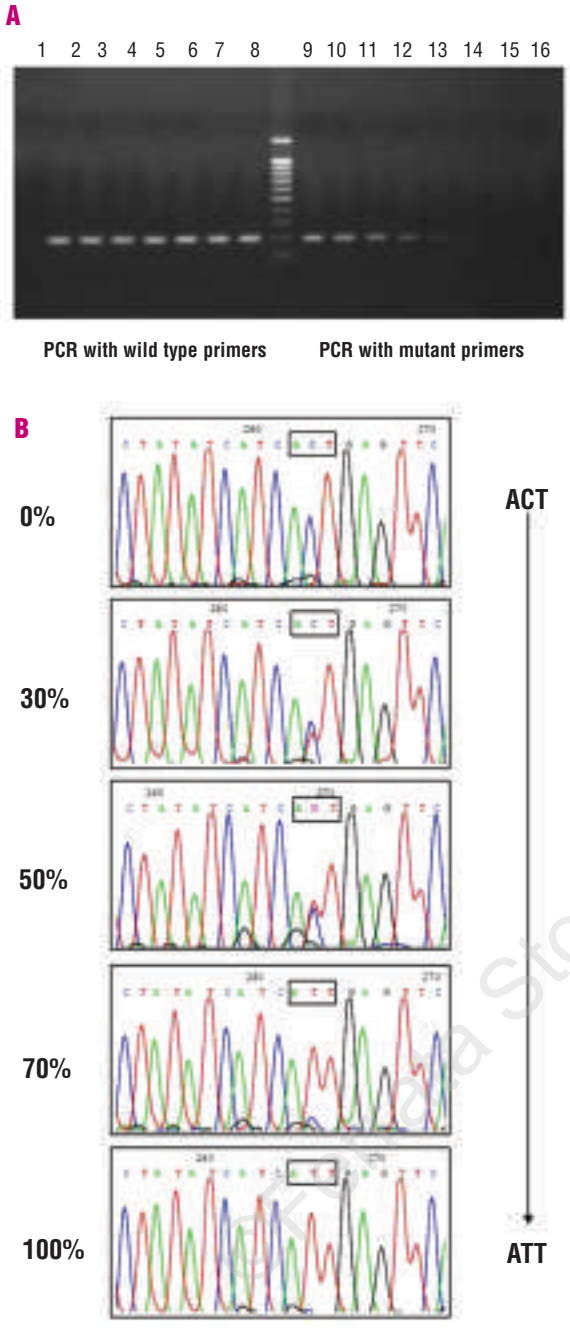

Figure 1. Determination of sensitivity for ASO-PCR and direct sequencing. A. Y253F mutant alleles were serially diluted in wild type sequences, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, respectively. ASO-PCR was done with wild type primers (lane 1-8) and mutant primers (lane 9-16). B. Mutant plasmid was diluted in wild type plasmid at concentrations of 0, 30, 50, 70 and 100%. Direct sequencing was performed with the mixed samples.

ASO-PCR and direct sequencing. To determine the most useful method in clinical practice, we developed an ASO-PCR assay using primer sets detecting common[17] mutational sites and compared the results of ASO-PCR with those of conventional direct sequencing. As expected, ASO-PCR showed excellent sensitivity. However, the sensitivity of ASO-PCR varied depending on the primers

Table 2. Comparison between ASO-PCR and direct sequencing for 17 mutations.

	ASO-PCR	*Sequencing*
No. of mutations detected	45	32
No. of mutation types detected	11	10
No. of patients with mutations	37	30
(Detected/Tested, %)	(37/68, 54%)	(30/68, 44%)
Sensitivity[a]	31/32 (97%)	31/45 (69%)
No. of patients with pre-existing mutation	2	0
Mutations detected only in one assay[b]	E355G	0
No. of patients harboring multiple mutations	7	1

a: sensitivity (%): (No. of mutations detected in one assay/No. of mutations detected in the other assay)×100; b: direct sequencing detected seven mutations, F317L, F359C, F359I, H396P, S417Y, E450K, and E459K, which were not tested in the ASO-PCR assay.

used. These results may be due to the different oligonucleotide composition of primer sets. The low solubility of gDNA in water might also affect the results because we diluted mutant plasmid in gDNA to measure sensitivity. More accurate measures will be obtained by using a cell-to-cell dilution method.[17] In addition to sensitivity, ASO-PCR assay has several advantages such as short turn-around time of analysis, lower costs, and a simple procedure.

In this study two of the 66 CML patients had mutations before starting imatinib treatment. The two mutations found prior to therapy became dominant at the time of relapse, suggesting that a highly sensitive method can be used to detect early mutations. However, in a recent report, Willis *et al*. concluded that high sensitivity screening of mutations cannot be recommended because two mutations detected before therapy failed to expand.[17] From this unexpected observation, they suggested that the mutations may occur in cell clones that have limited self-renewal capacity and additional mechanisms, besides mutations, are required to confer a resistant phenotype. The discrepancy between the two results may be due to different detection limits. As our ASO-PCR assay was less sensitive than a fluorescent ASO-PCR, pre-existing mutations that occupy a relatively large proportion of the total might be detected in our study. However, the clinical meaning of the finding of low titer *ABL* mutations is still not clear. Further studies with more pre-existing mutations will be required.

DWK conceived the study. DWK and HYK designed the study. HYK, JYH, SHK and HGG performed the molecular analyses and contributed to writing the manuscript. DWK, HYK, and MSK interpreted the data together. DWK and HYK wrote the manuscript with contributions from the other authors. The final version of this manuscript was approved by all the authors. The authors declare that they have no potential conflicts of interest.

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References

1. Kurzrock R, Gutterman JU, Talpaz M. The molecular genetics of Philadelphia chromosome-positive leukemias. N Engl J Med 1988;319:990-8.
2. Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. Blood 1996; 88:2375-84.
3. Buchdunger E, Matter A, Druker BJ. Bcr-Abl inhibition as a modality of CML therapeutics. Biochim Biophys Acta 2001;1551:M11-M18.
4. Barthe C, Gharbi MJ, Lagarde V, Chollet C, Cony-Makhoul P, Reiffers J, et al. Mutations in the ATP-binding site of BCR-ABL in a patient with chronic myeloid leukemia with increasing resistance to STI571. Br J Haematol 2002;119:109-11.
5. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 2001;293:876-80.
6. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. Cancer Cell 2002;2:117-25.
7. Branford S. Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. Blood 2002;99:3472-5.
8. Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Szer J, et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. Blood 2003;102:276-83.
9. Hochhaus A, Kreil S, Corbin AS, La Rosee P, Muller MC, Lahaye T, et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 2002; 16: 2190-6.
10. von Bubnoff N. Schneller F, Peschel C, Duyster J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukemia to STI571: a prospective study. Lancet 2002;359:487-91.
11. Roche-Lestienne C, Soenen-Cornu V, Grardel-Duflos N, Lai JL, Philippe N, Facon T, et al. Several types of mutations of the abl gene can be found in chronic myeloid leukemia patients resistant to STI571, and they can pre-exist to the onset of treatment. Blood 2002;100:1014-8.
12. Al-Ali HK, Heinrich MC, Lange T, Krahl R, Mueller M, Mueller C, et al. High incidence of BCR-ABL kinase domain mutations and absence of mutations of the PDGFR and KIT activation loops in CML patients with secondary resistance to imatinib. Hematol J 2004;5:55-60.
13. Deininger MW, McGreevey L, Willis S, Bainbridge TM, Druker BJ, Heinrich MC. Detection of ABL kinase domain mutations with denaturing high-performance liquid chromatography. Leukemia 2004;18:864-71.
14. Irving JAE, O'Brien S, Lennard AL, Minto L, Lin F, Hall AG. Use of denaturing HPLC for detection of mutations in the BCR-ABL kinase domain in patients resistant to imatinib. Clin Chem 2004;50:1233-7.
15. Liu WH, Makrigiorgos GM. Sensitive and quantitative detection of mutations associated with clinical resistance to STI-571. Leuk Res 2003; 27: 979-82.
16. Ricci C, Scappini B, Divoky V, Gatto S, Onida F, Verstovsek S, et al. Mutations in the ATP-binding pocket of the ABL kinase domain in an STI571 resistant BCR/ABL-positive cell line. Cancer Cell 2002;62:5995-8.
17. Willis SG, Lange T, Demehri S, Otto S, Crossman L, Niederwieser D, et al. High-sensitivity detection of BCR-ABL kinase domain mutations in imatinib-naive patients: correlation with clonal cytogenetic evolution but not response to therapy. Blood 2005;106:2128-37.