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Use of direct sequencing for detection of mutations in the BCR-ABL kinase domain in Slovak patients with chronic myeloid leukemia

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The presence of BCR-ABL oncogene mutations in patients with chronic myeloid leukemia (CML) may be responsible for the failure of tyrosine kinase inhibitor (TKI) treatment. The aim of the study was to evaluate the frequency of BCR-ABL gene mutations in patients with CML treated with tyrosine kinase inhibitors. Our lab received 64 samples (34 women, 30 men) from patients with CML who failed or had suboptimal response to TKI treatment. The mutation analysis was performed in 61 patients with CML, 3 patients could not be tested because of inadequate RNA quality. An 866 base pair fragment containing the ABL kinase domain was amplified in a seminested RT (reverse transcriptase)-PCR and then sequenced using Applied Biosystems BigDye Terminator chemistry with two pairs of primers. We analyzed 61 patients with CML, 11 mutations were detected in 13 (21%) patients and SNP (single nucleotide polymorphism) in 6 patients (10%). In addition to 9 point mutations (G250E / F317L, F359V, L387M, Y253H, M388L, M244V, T315I, D276G), 35 bp insertion between exons 8 and 9 and deletion exon 7 were detected. Our results demonstrate that direct sequencing is suitable for routine clinical monitoring patients with CML and may be useful for optimizing therapy.

Key words: chronic myeloid leukemia, mutation analysis, BCR-ABL, tyrosine kinase inhibitor

The Philadelphia chromosome, t(9;22)(q34;q11.2), has been observed in patients with CML and in some cases of ALL (acute lymphoblastic leukemia) [1]. This translocation fuses the ABL gene from chromosome 9 to the BCR gene on chromosome 22, resulting in an oncogene, which codes for a constitutively active BCR-ABL tyrosine kinase [2].

Modern treatment of CML relies upon tyrosine kinase inhibitors (TKIs) directed against BCR-ABL. Patients (pts) with CML are treated with imatinib as first line therapy [3]. Dasatinib and nilotinib are both next generation BCR-ABL tyrosine inhibitors that have been approved as second-line therapy for CML patients if imatinib therapy fails or is not tolerated [4]. But some of patients develop resistance to the drug over time. The most common mechanism for loss of response is acquired resistance due to the development of a point mutation in the ABL kinase domain of BCR-ABL [5]. Mutations have been detected over a range of 242 amino acids, which spans the entire kinase domain (KD) [6].

Patients with chronic phase CML who develop secondary resistance to imatinib, 30% to 50% will have one or more BCR-ABL kinase domain mutations detectable by direct DNA sequencing [7], whereas mutation frequencies are higher in those with accelerated or blast phases of disease, especially in lymphoid blast phases [8, 9]. These mutations affect amino acids involved in imatinib binding or in regulatory regions KD, resulting in decreased in vitro sensitivity to imatinib as assessed in assays of tyrosine phosphorylation and cellular proliferation [10-13]. More than 100 distinct resistance-conferring mutations have been detected, the majority fall within four regions of the kinase domain: the ATP-binding loop (P-loop) of the ABL kinase domain, the contact site, the SH2 binding site (activation loop), and the catalytic domain [14]. The most frequently mutated region of BCR-ABL is the P-loop, accounting for 36% to 48% of all mutations [15, 16].

Approximately 10% of resistant disease is associated with overproduction of BCR-ABL, typically through genomic amplification or the acquisition of additional cytogenetic abnormalities [17].

We perform the RT-PCR sequencing analysis to detect mutations in the KD of the BCR-ABL fusion gene associated with resistance to tyrosine kinase inhibitors (imatinib, nilotinib,

dasatinib). We apply direct Sanger sequencing using Applied Biosystems BigDye Terminator chemistry on the ABI 3500 genetic analyzer as described previously [18,19].

The sensitivity of the RT-PCR reaction is detection of one copy of the BCR-ABL transcript per 100,000 reference gene transcripts [20]. The sequencing assay is sensitive to detection of a mutation that is present in at least 20% of cells [8].

Materials and methods

Blood samples had been collected from 6 Slovak Hematology Departments in Bratislava, Košice, Banská Bystrica, Prešov, Martin. The mutation analysis was performed in 64 patients (34 women, 30 men) with CML (age range 25-78). All pts were treated with imatinib (Gleevec, Novartis) as first line therapy. 23 tested pts had received 6 or more months of imatinib therapy and were imatinib treated at the time of mutation analysis. Imatinib failure was defined according to European LeukemiaNet recommendations [21]. After imatinib failure 28 pts received a second TKI (dasatinib, n = 24, nilotinib, n = 4). 14 pts received a third TKI after relapse on second TKI (dasatinib, n = 2 nilotinib, n = 12).

Samples from 64 patients were available, but 3 patients could not be tested because of inadequate RNA quality. Peripheral blood samples were stabilized using RNA*later* Solution (Applied Biosystems, Foster City, California, USA) and were delivered promptly to the laboratory cooled to prevent RNA degradation. We also performed mutation analysis in samples from bone marrow (32 without mutation and 7 with mutation – F359V, L387M, Y253H, M388L, M244V, T315I, deletion exon 7). Only RNA of adequate quality was used to ensure accurate, reliable and reproducible mutation analysis. The quality of the RNA was assessed prior to sequencing by measurement of the level of BCR-ABL and ABL transcripts using 7300 Real Time PCR System (Applied Biosystems, Foster City, California, USA). The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) and at 280nm (A280) in a nanophotometer (Implen, München, Germany). The ratio A260/A280 was used for RNA purity check with acceptable range of 1.8 to 2.

RNA was isolated using RiboPure™-Blood Kit (Applied Biosystems, Foster City, California, USA). Isolated RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) according to manufacturer's instructions and amplified by the polymerase chain reaction using primers to the BCR-ABL kinase domain. The amount of cDNA added to the first-round PCR was 100 ng. The BCR-ABL allele was amplified using a forward primer that annealed in BCR exon b2 (5´- TGACCAACTCGTGTGTGAAACTC -3´) and a reverse primer that annealed at the junction of ABL exons 9 and 10 (5´- TTTTCCACTTCGTCTGAGATACTGG-3´). The following cycling conditions were used for first PCR: initial denaturation at 98°C for 30 seconds; 35 cycles: 98°C for 10 seconds, 70°C for 30 second, 72°C for 60 seconds, followed by one cycles of 72°C for 10 minutes.

An 866 base pair fragment containing the ABL kinase domain was amplified in a seminested PCR using forward primer in ABL exon 4 (5´- CGCAACAAGCCCACTGTCT-3´) and reverse primer was the same as in PCR 1. The following cycling conditions were used for seminested PCR: initial denaturation at 98°C for 30 seconds; 35 cycles: 98°C for 10 seconds, 67°C for 30 second, 72°C for 30 seconds, followed by one cycles of 72°C for 10 minutes. We used agarose gel electrophoresis to separate and identify PCR products, sometimes using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). The first-round PCR products prior to the seminested PCR and seminested PCR products were purified through QIAquick columns (Qiagen, Valencia, CA, USA) and subsequently sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, FosterCity, USA). Because of ABL kinase domain length, two forward and two reverse primers were used, are showed in Figure 1. The sequencing products were purified by DyeEx Spin Kit (Qiagen, Valencia, CA, USA), before running on an automated ABI 3500 genetic analyzer (Applied Biosystems, Foster City, California, USA) and sequences were analyzed using Sequencing Analysis software ver. 5.4. Sequences were compared with the ABL wild-type sequence (NCBI GenBank accession N. M14752). All mutations were confirmed by sequencing of forward and reverse strands. If a mutation is confirmed, this is indicated according to standard amino acid substitution nomenclature.

Results

Among 61 patients with CML, 11 mutations were detected in 13 (21%) patients and SNP (single nucleotide polymorphism) in 6 patients (10%) . The mutations, SNPs and number of patients are depicted in table 1. P-loop mutations predomi-

Table 1 Mutations, SNPs, nucleotide change and number patients.

Amino acid change	Nucleotide change	Number of patients
M244V	A/G	1
G250E	G/A	2*
Y253H	T/C	2
D276G	A/G	1
T315I	C/T	1
F317L	C/A	1*
F359V	T/G	2
L387M	T/A	1
M388L	A/T	1
Exon 7 deletion		1
35 bp insertion between exons 8/9		1
SNP		
L323L	C/A	1
E459K	G/A	1
E499K	A/G	4

*** dual mutation in 1 patient**

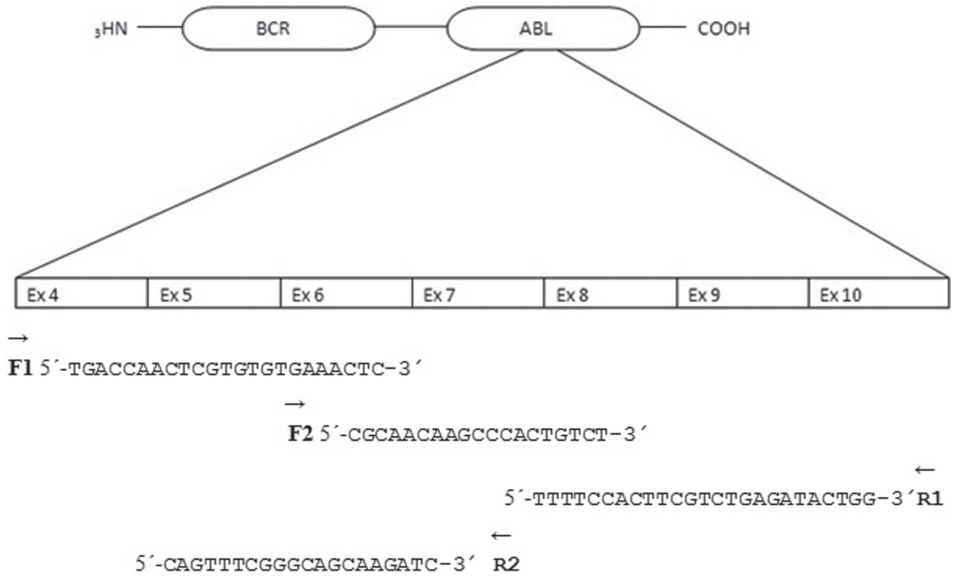

Figure 1. Two pairs of primers were used in sequencing analysis ABL kinase domain. Primers F1 in exon 4 and primer F2 in exon 6 for forward direction and primers R1 at the junction of exons 9 and 10 and R2 in exon 7 for reverse direction.

nated with an overall frequency of 30% (4 of 13 patients), the contact site mutation T315I was identified in 1 patient (7,7%), in the activating loop were identified in 2 patients (15%), and mutations in the catalytic domain were detected in 3 patients (23%). Two mutations (D276G) and 35 bp insertion mutation were localized outside this region, in SH3 contact site and in C-terminal lobe. A dual mutation was identified in 1 patient, G250E/F317L. All 11 mutations are depicted in Figure 2.

Within the BCR-ABL kinase domain, the following SNPs rs1064156 (E459K) and rs2227985 (E499E) were detected (NCBI, Ensembl). The E499E change was the SNPs within the BCR-ABL kinase domain of CML patients most frequently observed polymorphism with frequency of 4/61 (6,5%). In one patient a single-nucleotide polymorphism (L323L) was detected (not described in Ensembl). In addition to point mutations, 35 bp insertion between exons 8 and 9 and deletion exon 7 were detected.

Comparison of mutation analysis in peripheral blood and bone marrow was performed in 39 pts. There was no difference between results obtained from both types of sample, all 7 mutations in 7 pts and no mutation in 32 pts were confirmed.

Indications for BCR-ABL kinase domain mutational analysis include a) inadequate initial response to TKIs or evidence of loss of response and b) advanced phase CML or progression to accelerated or blast phase CML. All 61 patients were treated with imatinib as first line therapy. 14 patients who did not respond or relapsed on second TKI switched to a third TKI. Five of those patients had BCR-ABL KD mutations.

In the time of mutation analysis the majority of CML patients (23 pts) were treated with imatinib. 14 patients were treated with dasatinib and the same number with nilotinib. In 10 patients we didn´t know treatment.

Three patients with mutations G250E/F317L, deletion exon 7, F359V were in group treated with imatinib, 6 patients with L387M, Y253H, M388L, G250E, M244V, F359V were treated with dasatinib as second or third TKI and 3 patients with T315I, D276G, Y253H were treated with nilotinib as second or third TKI. 35 bp insertion between exons 8 and 9 were detected in 1 patient, in whom we didn´t know treatment.

We compared these results mentioned above with results obtained from analysis of first delivered samples to our laboratory. No mutations were found.

Discussion

The aim of the present study was to investigate the characteristics of BCR-ABL KD mutations in Slovak pts with CML.

It is recommended that CML patients are monitored with quantitative RT-PCR and that patients with increase in quantitative BCR-ABL levels are assayed for the presence of mutations. Depending on the exact mutation present, clinical intervention such as increasing dosage of imatinib mesylate or adding another kinase inhibitor may be effective in controlling the levels of BCR-ABL.

Mutational analysis may be performed with a variety of techniques. Direct sequencing is frequently described as the "gold standard"for this form of analysis. Other screening methods for BCR-ABL KD mutations that have been reported include denaturing high performance liquid chromatography, targeted microarrays, liquid bead arrays and PCR-based pyrosequencing [22]. Because the detection of low levels of mutant clones may not be clinically significant, direct sequencing of the BCR-ABL transcript by the Sanger method is currently the

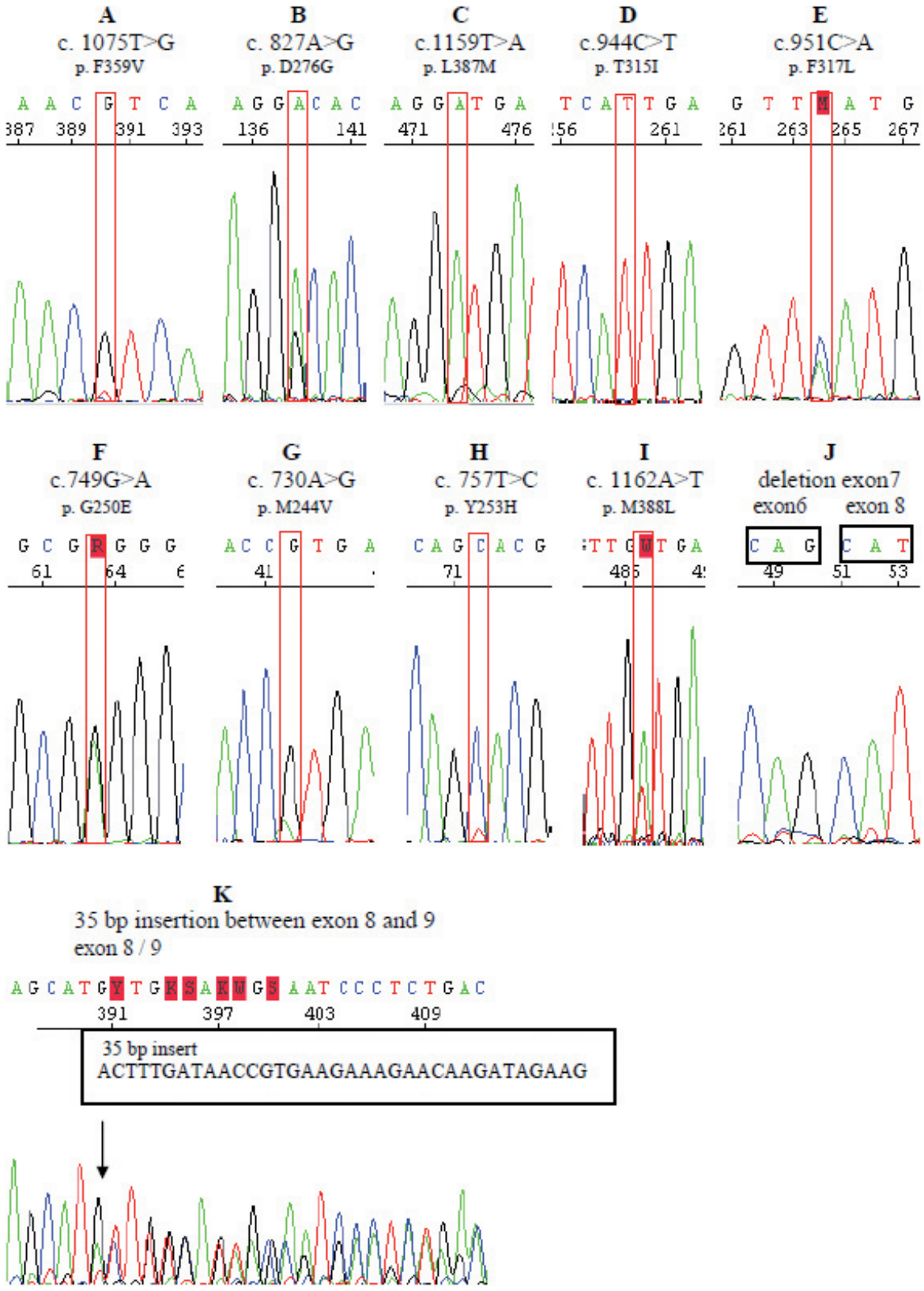

Figure 2. A-K Sequencing chromatogams (forward sequence) represented mutations found in ABL kinase domain. A p. F359V, B p. D276G, C p. L387M, D p. T315I, E p. F317L, F p. G250E, G p. M244V, H p. Y253H, I p. M388L, J deletion exon 7, K 35 bp insertion between exon 8 and 9.

most appropriate screening test, and was recommended by an international consensus panel [23].

Our lab performed bidirectional sequencing and we reported positive results only when detecting a mutation in both (forward and reverse) strands. Only mutations located in the analyzed exons of ABL kinase domain are detected. Other mechanisms of drug resistance such as amplification of the BCR-ABL fusion gene are not detected by this assay.

A negative result does not preclude the presence of BCR/ABL mutations in transcripts below the detection limit of this test. More than 100 different point mutations were identified in the kinase domain of BCR-ABL in patients, but some of them show a higher frequency than others [14].

Most of the clinically relevant mutations develop at just a few residues in the P-loop (G250E, Y253F/H, and E255K/V), contact site (T315I), and catalytic domain (M351T and F359V [9].

We identified 11 mutations in 13 patients (21%). It is too low in comparison with other publication data. Low incidence of BCR-ABL mutations in our group might be related to the characteristics of the study patients: predominance of patients treated up-front with imatinib, and a relatively short follow-up. Other reason of low frequency of BCR-ABL mutations in CML patients can be the sensitivity of sequencing. From the practical point of view, low sensitivity of direct sequencing detect only the "driver" mutation(s) responsible for the disease progression or TKI resistance.

Of 1,043 patients who underwent mutational analysis, 39% had a BCR-ABL mutation prior to dasatinib and 48% of 805 patients with either imatinib resistance or a poor response to imatinib had a BCR-ABL gene mutation. Sixty-three different BCR-ABL mutations were detected, with G250, M351, M244, and F359 most frequently found [24]. In one patient treated with nilotinib we detected mutation T315I. Patient died. He was 71 years old, so we don´t know if he died because of his age or resistance to treatment.

The T315I mutation is reported to correlate with resistance to most second generation tyrosine kinase inhibitor therapies including imatinib [8]. In 6 patients (10%) the mutation analysis revealed nucleotide changes within the BCR-ABL kinase domain indicating SNPs. We detected three SNPs E459K, E499E and L323L. E459K has been described as a acquired mutation in imatinib resistance, but it is not our case. [25]. Because SNPs must be distinguished from acquired mutations because they cannot contribute to secondary resistance to tyrosine kinase inhibitors. In addition to point mutations, 35 bp insertion between exons 8 and 9 and deletion exon 7 were detected, but only in one patient each.

Gaillard and colleagues [26] screened 63 resistant and detected 34 pts with deletion exon 7 (54%). They thought that deletion seems to be the result of an alternative splicing mechanism and to be independent from the occurrence of resistance. Such deletion and proteins arising from alternatively spliced transcripts may act as dominant-negative inhibitors of the full-length BCR-ABL [27].

We detected 35-bp intronic insertion, which occured at the exon 8/9 junction, after amino acid 474 kinase domain of BCR-ABL. Translation of this mutant transcript produce a BCR-ABL protein with an insertion of 10 amino acids followed by a stop codon.

Berman and colleagues (2010) identified 40 pts who had 35 bp between exons 8 and 9, first described in 2008. All 40 patients began initial therapy with imatinib, in 9 pts, the mutation was detected during retrospective analysis and two pts had the mutation detected at the time of diagnosis [28, 29].

According to our results we recommend to perform mutational analysis in pts not only with failure, suboptimal response to TKI or transcript elevation, but in every patient, who is long treated with TKI in half year interval. We suppose, it is sufficient to perform mutation analysis from peripheral blood only, bone marrow analysis is not needed. Sampling from peripheral blood is less painful, more time and cost effective.

We didn´t detect any mutation in pts at the time of first mutation analysis, so we assume, that all 11 mutations (G250E, F317L, F359V, L387M, Y253H, M388L, M244V, T315I, D276G, 35 bp insertion between exons 8 and 9, deletion exon 7) are somatic variations.

Identification of BCR-ABL mutations as a source of protein tyrosine kinase inhibitor resistance may be a useful research tool in understanding of CML. Although certain BCR-ABL mutations may be associated with protein tyrosine kinase inhibitor resistance, the significance of many other mutations is unknown. To our knowledge this is the first study in Slovak Republic in which BCR-ABL mutations were identified.

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