A COST-EFFECTIVENESS METHOD FOR DETECTION OF ABL MUTATIONS IN PATIENTS WHO DEVELOPED IMATINIB RESISTANCE

C. FIERRO¹, T. MUTO¹, M. TREMATERRA¹, F. MORANO¹, S. PUGLIESE², M. DI PAOLO², A. TROISI², G. CRESCENTE³

INTRODUCTION

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia chromosome (Ph+) resulting from a translocation between chromosomes 9 and 22. Ph chromosome creates the BCR-ABL fusion gene coding for a constitutive active tyrosine kinase ABL protein. Despite high response rate to specific tyrosine kinase inhibitor (TKI), primary and secondary resistance has been observed: upfront resistance is defined as lack of initial response and acquired resistance is defined as loss of an established response. BCR-ABL kinase domain (KD) mutations represent a well-established cause of resistance to tyrosine kinase inhibitors. Among different mutations identified the frequently observed T315I is of particular concern since it is not effectively targeted by the majority of TKIs so far available. The only drug showing activity against I315I positive CML is ponatinib.

Currently, the recommended method for BCR-ABL mutation detection is the sequencing of the KD of ABL gene from exon 4 to 10. This is time consuming and it allows reaching a maximum sensitivity of 10-15% of mutant DNA in a large excess amount of wild-type (wt) DNA.

The latter point represents a limit, as frequently mutated clones may be present at a lower percentage.
A relative new technique such the “ultra deep sequencing” allows to reach a very high level of sensitivity but it is far from being routinely applicable in world-wide laboratories. The availability of a simple, sensitive and quick assay, allowing a rapid detection of the T315I mutation is therefore crucial, as the detection of this mutation represents an important element in clinical decision for CML patients.

Since far time are used different techniques capable of identifying individual polymorphisms. Among these, some have received validation for clinical/diagnostic purposes [Single-Strand Conformational Polymorphism (SSCP) and Restriction Fragment Length Polymorphism (RFLP)] and others are still in an experimental phase of validation [Denaturing High Performance Liquid Chromatography (D-HPLC), High Resolution Melt (HRM) GENE-chip, MALDI-TOF, PNA Clamping PCR]. A good example of the comparative sensitivity and specificity of the method listed is summarized on (Table 1), which reporting the screening of point mutations in the ABL gene in patients with CML. Among all the techniques for the discrimination of point mutations, the PNA Clamping PCR is of great interest as it is capable of identifying minimal amounts of variant gene containing the mutation within a sample almost exclusively “wild-type” (for example, identification of small quantities of cancer cells bearing a mutation within a biopsy where there is an excess of normal tissue).

Oligo-PNA is a potent DNA mimic in terms of sequence specific with high hybridization affinity. The duplex PNA/DNA thermodynamic is more stable than DNA/DNA or DNA/RNA duplexes, but PNA sequences cannot be extended by DNA polymerase. As consequence, PNA/DNA duplex suppresses DNA PCR amplification. Furthermore, PNA/DNA hybridization shows a greater single-base-pair mismatch discrimination than the corresponding DNA/DNA duplex.

Based on this premise and previous data, we developed a novel and sensitive detection assay in order to quickly and easily identify T315I mutation in CML patients by PNA directed PCR clamping. The experimental design forecasts that both PNA and PCR primer target sites overlap, thus leading to a direct competition towards complementary DNA. When perfect matching occurs PNA/DNA template hybridization is favored more than primer-DNA template duplex and DNA amplification is suppressed. Conversely, a single mismatch destabilizes the PNA/DNA template duplex, favoring the hybridization between template and primer thus allowing template amplification. Competitor PNA sequence was designed to perfectly match wild-type (wt) template sequence. Therefore, when a single base pair mismatch occurs (like in the case of T315I) PNA-template stability is strongly impaired and DNA amplification favored.

In this report, we describe a new assay method based on 3’-Peptide nucleic acid (PNA) and modified reverse primer able to clump the amplification of wild-type (wt) DNA. This detection method is easy, sensitive, reproducible and cost-effective. This method should be perfect in terms of costs for small laboratory that processing few samples.

**MATERIALS AND METHODS**

**Positive controls**

Positive controls of the conventional PCR and sequencing are represented from the cellular line K562 in which the gene of BCR is present in fusion BCR/ABL p210 (exon 14, exon 2 of Abl) with b3a2. The rearrangement controls for the mutational screening of PNA Clamping PCR are represent by a plasmid of 2710 bases (bp) synthesized from the Gen company Script (The Biology Crow USA) in which it has been introduced the sequence of 643 bp of the ABL gene comprised between exon 4and exon the 7, that it contains the mutations that will come taken in consideration.

<table>
<thead>
<tr>
<th>Method</th>
<th>Autor</th>
<th>Analytic sensibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA sequencing</td>
<td>Gorre et al</td>
<td>10-20%</td>
</tr>
<tr>
<td>Nested PCR+</td>
<td>Brandford et al</td>
<td>10%</td>
</tr>
<tr>
<td>Sequencing</td>
<td>Hochhaus et al</td>
<td>1%</td>
</tr>
<tr>
<td>D-HPLC</td>
<td>Soverini et al</td>
<td>1%</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>Ernst et al</td>
<td>1-5%</td>
</tr>
<tr>
<td>HRM</td>
<td>Poláková et al</td>
<td>1-5%</td>
</tr>
<tr>
<td>Double Gradient</td>
<td>Sorel et al</td>
<td>1%</td>
</tr>
<tr>
<td>Denaturing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrophoresis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Vivante et al</td>
<td>1%</td>
</tr>
<tr>
<td>Allele specific PCR</td>
<td>Roche-lestienne et al</td>
<td>1%</td>
</tr>
<tr>
<td>Scorpion Probe</td>
<td>Pelzarkermann et al</td>
<td>1%</td>
</tr>
<tr>
<td>PNA fluorescent-clamping-PCR</td>
<td>Kreuzer et al</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

**TABLE 1. Most important methodics applied for screening e identification of mutation in ABL.**
**Sampling**

White blood cells (WBC) from bone marrow and peripheral blood are obtained for erithrolisys using ipotonic solutions made up of ammonium chloride and potassium bicarbonate. The pellet cellular it comes washed in PBS (NaCl 137 mM, KCl 2.7 mM, NaHPO₄,7H₂O, 8.1 mM, KH₂PO₄, 1.9 mM) and quoted in 2 tubes for the extraction of nucleic acids. For the extraction of the DNA the pellet it comes frozen to -20°C, while for the extraction of the RNA, the share it comes mixture in phenol/guanidinic acid solution (TRIZOL Invitrogen, Milan, Italy) and frozen to -20°C.

**Isolation of RNA**

For the extraction of the RNA total, approximate-ly 10 million of WBC was combined to 1 mL of TRIZOL (Invitrogen, Milan, Italy), added 0.2 mL of Clarorhorme. After centrifugation to 12.000 g for 15 minute to 4°C was evident two phases: one organic (down) and one watery (upper). The water phase overhanging comes captured and added to one equal amount of isopropanol. The nucleic acid falls after centrifugate under shape of knows them. Eventual polluting agents are efficiently washing by successive 75%ethanol step. The pellet of RNA is resolved in 30 μL of RNAasi-free water. The quality of the extracted RNA has been tested with 2% agarose gel in which the ribosomal bands are visible 28S and 18S. The quantization is carried out on spectrophotometric reading to 260 and 280 nm. A ratio comprised between 1.7-2.0 is index of purity of the RNA total isolated.

**Reverse-transcription of the RNA (cDNA synthesis)**

To the extraction of the RNA is continuation on the reaction of inverse transcription with synthesis of complementary DNA (cDNA) beginning from RNA total and the activity of enzyme DNA employee polimerase RNA (Reverse Trascriptase).

The RNA of departure at first is denatured for 10 minut (min) to 70°C and then maintained on ice in order at least 10 min. The reverse-transcription has been realized in a final volume of 20 μL for reaction, containing 5x Buffer (Invitrogen, Milan, Italy), 5 nM of Random Examers (Applied Biosystems, Monza, Italy), 1 g of RNA total, 4 μL of dithiothreitol (DTT), 200 nM of every deoxyinucleotidetriphosphate (dNTPs), 40U RNAasi inibitor (Life Technologies, Milan, Italy) and 200U of Reverse Transcriptase “Super Script III” (Life Technologies, Milan, Italy). The reaction was incubated for 60 minutes to 42°C and subsequently for 15 minutes to 75°C.

**Selective amplification of transcribed fusion gene BCR/ABL**

For the mutational screening uses a amplicon obtained from a conventional PCR that uses a primer that it recognizes the gene in the region of the Major-Breakpoint Cluster Region (M-BCR), post on exon the 12 of BCR and a designed other on exon 7 of ABL. The Forward BCR exon 12 primer sequence is 5’-TCC GCT GAC CAT CAA TAA GG-3’ and ABL Reverse 5’-CCA GAC GTC GGA CTT GAT GG-3’.

This PCR product is used as template for the next PCR-screening for one requirement:
1. To increase the sensibility of amplification
2. To select the allele BCR-ABL rearrangement in the neoplastic cells excluding the wild-type ABL (that it major increases the specificity of the analysis).

The amplification was performed with 1 μl of cDNA in a mixture of 25 μL of 10 x Buffer (Roche, Milan, Italy) in which is contained 1.5mm MgCl₂, 200 nM of both primers, 200 nM dNTPs, 0.5U “Accura” High Fidelity Taq DNA polymerase (TEMA research, Italy).

Process begins them step of denaturation of 2 minutes to the temperature of 95°C has been followed from the amplification that happens in 35 cycles, everyone of which constituted by dena-turation for 30 sec to 95°C; annealing for 30 sec to 60°C; extension for 1 min and 20 sec to 72°C.

To the end of the 35 cycles of amplification final phase of extension for 5 minutes to 72°C is added one. The amplicon turning out it has a length of 1310 bp (in the event of rearrangment between exon the 14 of BCR and exon the 2 of ABL) b3a2 or the 1235 bp (in the event breach has on exon the 13 of BCR and exon the 2 of ABL) b2a2.

**Analytical optimization of PNA amount and PCR mediated PNA-clamping**

The validation assay of the PNA clamping PCR has been obtained like follows: amount of PNA required for the clamping of cDNA the wil-de-type, has been recognized on the amplification of increasing amounts of PNA (5-30 μM), in presence of cDNA of K562 (50 ng/μL).
type from those with T315I mutations responsible for resistance to TKIs. This method was applied for the identification of mutation T315 by the use of one specific oligo-PNA that interacts with the respective wild-type DNA region. The T315I mutation is very relevant because it is present in the binding site of the drug with the protein in which is lost Phenilalaninic bond between drug component and the substrate. This turns out to be one of the most important mutations as even the second-generation drugs such as dasatinib and nilotinib fail to have meaningful interaction with the substrate, and leave intact the link with the ATP.

Clamping of wt for T315

The clamping PCR was obtained using 25 μM PNA wt T315. The test for evaluating the amount of PNA needs for Clamping PCR was performed by amplifying 50 ng/μL of K562 cDNA in increasing concentrations from magnitude 5 to 25 μM as shown in Figure 1. The primers used in this reaction are ABL ex6 Fw and ABL ex6 rev to amplified amplicon of 145 bp. The PNA and primers sequences are listed in Table 2.

Sequencing

Confirmation of the mutations found by the PNA Clamping PCR assay screening, was assessed by sequencing of the BCR/ABL amplicon produced by the selective PCR. Sequencing assay was performed using primer inner 640 ABLexon 4 Forward 5'-CTC CAT TAT CCA GCC CCA AA-3' to the concentration of 2 pmol/μL. The sequencing has been carried out using a sequencer automatic rifle ABI PRISM 377 DNA Analyzer (Life Technologies, Monza, Italy) and the “Big Dye Terminator Cycle Sequencing Kit” (Life Technologies, Monza, Italy).

In our studies, the sequence obtained for every samples has been confronted with the sequence wild-type of ABL (Genbank Accession number NM_005157), using program BLAST (Basic Local Alignment Search Tool) of the European Bioinformatic Institute.

RESULTS

The study by PNA Clamping PCR was conducted to discriminate the fusion gene BCR/ABL wild-
CONCLUSION AND FUTURE OUTLOOK

PNA mediated clamping PCR can be performed in any laboratory with basic PCR equipment. It is very cost-effective and can easily be adapted to detect hot spot mutations in other genes. Our results confirm that it is possible to reduce the number of samples to be sequenced, to samples in which the mutation is present in order to identify the nucleotide replaced. In the next future the oncologists will have new means based on the individual genetic information, to plan the personal treatment for their patients in order to maximize benefits and minimize toxicity. Therefore, it is fundamental that pharmaceutical and biotechnology companies join, in order to develop an extensive study on the standardization method to validate tests suitable for routine diagnostics in pharmacogenomics.

CONFLICT OF INTERESTS:
The Authors declare that they have no conflict of interests.

REFERENCES


5. QUINTAS-CARDAMA A, GIBBONS DL, KANTARjian H, TALPAZ M, DONATO N, CORTES J. Sequencing of subcloned PCR products facilitates earlier detection of BCR-ABL1 (T315I) and other mutants compared to direct sequencing of the ABL1 kinase domain. Leukemia 2008; 22: 885-888.


24. ROCHE-LESTIER C, SOENEN-CORNU V, GRARDEL-DUPLOS N, LAI JL, PHILIPPEN, FACON T, FENAUX P, PREUDOMME C. 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: 1014-1018.


