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

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Abstract – *Background:* *Despite high response rate to specific tyrosine kinase inhibitors (TKI), resistances have been observed in patients in treatment with Imatinib because acquired mutations into* BCR-ABL1 *kinase domain (KD). This can prove a challenge and underlines the importance of utilizing a detection method that is easy, sensitive, reproducible and cost-effective.*

Materials and Methods: *We developed a high specific and sensitive detection assay in order to quickly and easily identify T315I mutation in gene ABL patients by Peptide Nucleic Acid (PNA) directed PCR clamping.*

Results: *The experimental design forecasts that both PNA and PCR primer mutant target sites overlap, thus leading to a direct competition towards complementary DNA (cDNA). 25 μM of oligo-PNA was enough to perfect matching PNA/cDNA duplex template in wild-type ABL sequence and PCR amplification is suppressed. Contemporary, in the mutant DNA (I315I) the PNA/cDNA duplex hybridization fail and PCR can be performed.*

Conclusions: *This detection method is easy, sensitive, reproducible and very cheap. Sequencing method could be restricted as confirmation method only in doubtful mutant samples. Finally, this platform should be ideal for small laboratory that processing few samples.*

KEYWORDS: *PNA Clamping PCR, BCR-Abl, T315I, Imatinib, Sequencing, Chronic myeloid leukemia, Philadelphia chromosome.*

INTRODUCTION

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia chromosome (Ph+) resulting from a translocation between chromosomes 9 and 22[1]. 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[2]. 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[3]. The only drug showing activity against I315I positive CML is ponatinib[4].

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[5].

The latter point represents a limit, as frequently mutated clones may be present at a lower percentage[6].

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A relative new technique such the "ultra deep sequencing" allows to reach a very high level of sensitivity but it is far from been routinely applicable in world-wide laboratories[7].

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)[8].

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[9], but PNA sequences cannot be extended by DNA polymerase[10]. 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[11]. 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.

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

Method	*Autor*	*Analitic sensibility*
DNA sequencing	Gorre et al[12] Shah et al[13]	10-20%
Nested PCR+ Sequencing	Brandford et al[14] Hochhaus et al[15]	10%
D-HPLC	Soverini et al[16] Irving et al[17] Deiniger et al[18] Ernst et al[19]	1-5 %
Pyrosequencing	Khorashad et al[20]	1-5%
HRM	Polàkovà et al[21]	1-5%
Double Gradient Denaturing Electrophoresis	Sorel et al[22]	
MALDI-TOF	Vivante et al[23]	
Allele specific PCR	Roche-lestienne et al[24] Gruber et al[25] Pelzackermann et al[26]	1%
Scorpion Probe	Khorashad et al[27]	0.1 %
PNA fluorescent-clamping-PCR	Kreuzer et al[28]	0.1%

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.

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, $Na_2HPO_4.7H_2O$, 8.1 mM, KH_2PO_4 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, approximately 10 million of WBC was combined to 1 mL of TRIZOL (Invitrogen, Milan, Italy), added 0.2 mL of Clorophorme. 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 centrifuge 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 ribosomial 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 $MgCl_2$, 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° has been followed from the amplification that happens in 35 cycles, everyone of which constituted by denaturation 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 wilde-type, has been recognized on the amplification of increasing amounts of PNA (5-30 µM), in presence of cDNA of K562 (50 ng/µL).

TABLE 2. Primers and oligo-PNA sequences used for screening BCR/ABL samples (Genbank Accession number NM_005157).

Name	Sequences (5'-3')	Position	Bases
Ex6 fw T315 M	CCCCGTTCTATATCATCATTGAGTT	Ex6 929 m	25
Ex6 rev M351 M	RCTTCTCCAGGTACTCCGTG	Ex6 1074 m	20
PNA wt T315	Acetyl-AGT GAC TCA AST ACT G	Ex 6 936	16

R: purine (A or G) replacements; S: strong 3H bonds (C or G) replacements

Mutational screening thought PNA Clamping PCR

The reaction of PNA clamping PCR has been executed using the following protocol in a volume total of 25 µL for reaction. The mix of reaction have been performed with 1 µl of cDNA in a mixture of 25 µL of 10x Buffer (Roche, Monza, Italy) in which is contained 1.5 mm $MgCl_2$, 200 nM of both primers, 200 nM dNTPs, 5U " Accura" High Fidelity Taq DNA polymerase (TEMA research, Italy) and 25 µM of PNA wt (Table 2).

Process begins with step of denaturation of 2 minutes to 95°C, followed by amplification step that happens in 35 cycles, everyone of which constituted by denaturation for 30 sec to 95°C; annealing of PNA by grading temperature across 70°C and 64°C 5 sec for single graduation, annealing primer of 58°C for 30 sec; extension for 1 min and 20 sec to 72°C. At the end, must add a final step on 68°C for 5 mins.

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-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 on 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.

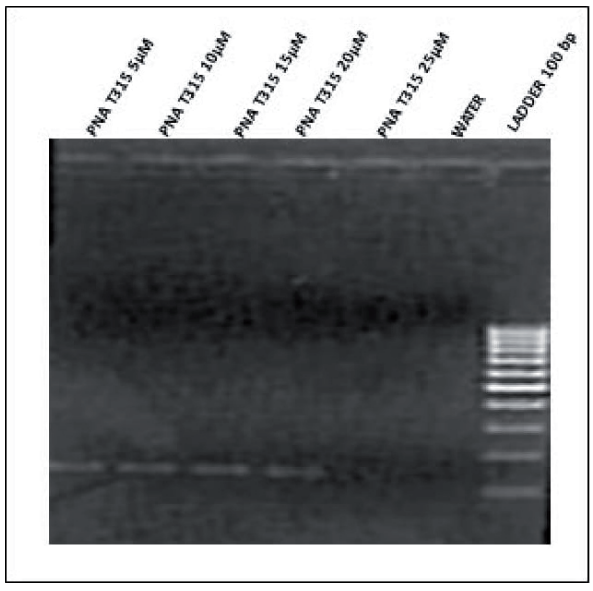

Fig. 1. Clamping-PCR with PNA T315 in concentration of 25 µM with K562 cDNA 50ng/ul.

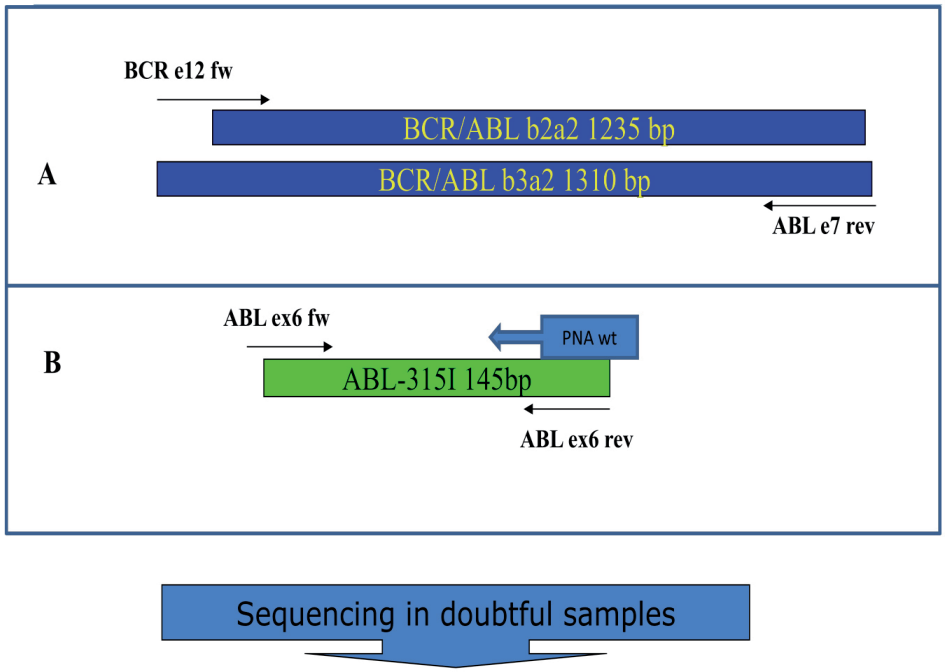

Fig. 2. Study design. *A,* Production of cDNA by RT reaction. *B,* The clamping PCR was obtainsed using 25 μM PNAwt T315. The primers used in this reaction is ABL ex6 Fw and ABL ex6 rev to amplified amplicon. ONly mutant minor allele produce an amplification product of 145bp. When results is doubtful, a confirmation next sequencing step is required.

DISCUSSION

The fully automated "Sanger sequencing"still remains as a gold method to identify acquired mutations. Furthermore, it suffers of a low sensitivity and it is costly and laborious procedure for the routine practice[29]. In our specific case, we set the aim to validate a more sensitive method, simple, economic and easy to use as the first screening, with the purpose of limiting the sequencing only for samples that were doubtful to the PNA clamping PCR method (Figure 2). The PNA clamping PCR methodology allows to view the amplification only when mutated gene is present in the detriment of the wild-type[30]. Oligo-PNA object of our study was designed to capture the wild-type fragment at the point where the 315I mutation responsible for Imatinib resistance is often described. Such screening model turns out to be simple to use, inexpensive and does not require the use of sophisticated equipment (only PCR machine). In the study performed was obtained a clamping PCR on the wild-type for the PNA at a specified concentration and this result to be reproducible over time. This result was also confirmed by amplifying ABL exon from 4 to7 (containing the mutations in the study) to the optimal concentration of PNA (25 μM to T315), which highlights the complete Clamping of the wild-type regions and the clear positivity of the changed sample.

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 plain the personal treatment for their patients in order to maximize benefits and minimize toxicity[31]. Therefore, it is fundamental that pharmaceutical and biotechnology companies join, in order to develop an extensive study on the standardization method to validated tests suitable for routine diagnostics in pharmacogenomics[32].

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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