Detection of BCR-ABL T315I mutation by peptide nucleic acid directed PCR clamping and by peptide nucleic acid FISH

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it is far from being 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.

Peptide Nucleic Acid (PNA) is a potent DNA mimic in terms of sequence specific hybridization. PNA/DNA is thermally more stable than DNA/DNA or DNA/RNA duplexes, [8] but PNA sequences cannot be extended by DNA polymerase [9]. As consequence, PNA/DNA duplex suppresses DNA 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 [10] 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 (Fig. 1). When perfect matching occurs PNA-template hybridization is favoured more than primer template duplex and DNA amplification is suppressed. Conversely, a single mismatch destabilizes the PNA-template duplex, favouring 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 favoured.

In addition, in order to identify the presence of BCR-ABL1 T315I mutation at the single-cell level, we set up a fluorescently-labelled PNA probe, coupled to FISH technology.

This method allows to distinguish single mutated cells from wild-type cells in the context of Ph positive hematopoiesis. Here we report the two strategies (PNA

![Fig. 1 Experimental design: perfect PNA/DNA hybridization occurs when template sequence is w.t., thus leading to suppression of PCR amplification. By contrast, when in presence of single base-pair mismatch (i.e. T315I, indicated by x), PNA/DNA duplex is strongly destabilized allowing template amplification. Empty and filled arrows represent DNA primers used for PCR amplification and PNA competitor, respectively.](image)

![Fig. 2 Abl kinase domain T315I point mutation detection analysis by PNA directed PCR clamping. A representative result of the analysis carried out on cDNAs isolated from patients affected by imatinib resistance CML is represented in panel (a). PCR amplification was carried-out in absence (−) or in presence (+) of competitor PNA, at a concentration 3× greater than primer FWD. The amplification performed without (−) PNA represents an internal positive control displaying the efficiency of template amplification. As result only when PNA-template duplex stability is weakened because of the mutation an efficient template amplification occurred. L: DNA ladder. Sensitivity was assessed mixing, at different ratio, mutated (T315I) and w.t. template panel (b). Dilutions were as follow: 100, 20, 10, 5, 1, 0.5 and 0 % mutated (T315I) versus w.t. template](image)
clamping and PNA FISH) for a highly sensitive and very specific detection of BCR-ABL1 T315I, applied in the clinical setting of BCR-ABL T315I monitoring.

Methods
PNA-PCR clamping for BCR-ABL1 T315I mutation
The study was approved by the local ethic committee of San Luigi Hospital, Orbassano, Turin. After written informed consent BM aspirates were obtained from 17 imatinib resistant CML patient and 1 Ph + Acute Lymphoblastic Leukemia (ALL), all displaying T315I mutation detected by Sanger Sequencing. In addition, as negative control, 25 CML patients without mutations and 15 healthy subjects were examined. Detection of BCR-ABL1 was performed by capillary Sanger Sequencing method and analyzed by sequencing with BigDye terminator v3.1 (Applied Biosystem, Foster City, California CA) and capillary electrophoresis on ABI PRISM 3130XL (Applied Biosystem, Foster City, California CA). The sensitivity of this method was previously estimated by serial dilutions experiments to be approximately 10%.

Primer Sequences were as follow:
FWD: 5’-tatcatcactgtcatgacc-3’;

![Fig. 3 Detection of BCR-ABL T315I mutation by PNA. Detection of BCR-ABL T315I mutation by PNA (green signal) in CD34+ cells enriched from CML patients carrying T315I mutation. Panel a and b show T315I positive cells from a patient with 60% of mutated cells positive cells, panel c and d show T315I positive cells from a patient with 45% of mutated cells. Negative control (e, f) is represented by a CML patient without T315I mutation. No specific PNA green signal can be detected in the absence of the mutation.](image-url)
in situ

Acute lymphoblastic leukemia; CML: Chronic myeloid leukemia; mutations thus by (2015) 3:15 allele usually confer et al. Biomarker Research transcript during dasatinib treatment and versus BCR-ABL T315 BCR-ABL1 BCR-ABL1 cDNA. The single nucleo- compound mutants confer different levels of BCR-ABL1 T315I − cells were transcript. hybridization; KD: Kinase domain; MACS: Magnetic BCR-ABL1 T315I + match WT template sequence. Surprisingly, the method displays a quite high sensitivity, performed keeping constant the total template amount. dilutions with WT and mutated T315I templates were amplification. To test the sensitivity of the method, serial dilutions with WT and mutated T315I templates were performed keeping constant the total template amount. Surprisingly, the method displays a quite high sensitivity, allowing to detect amount of mutated template as low as 0.5 % (Fig. 2, panel b), which are not identified by classical sequencing allowing the identification of T315I mutation even when present at low amount. This method is highly sensitive, specific and cheap and could be applied even in laboratory not equipped for more sophisti- cated analysis. Five out of 17 CML patients carrying T315I, 1 Ph + ALL and 10 WT CML patients have been tested by PNA FISH.

This technique allowed us to identify T315I mutation at a single cell level. In particular we applied this tech- nique to CD34+ cells to investigate the mutation in the progenitor cell compartment.

More in details, we found a residual amount of 3 % of CD34+ positive cells in a patient who became negative for the T315I mutation by Sanger sequencing after ponatinib treatment and acquired T317 mutation at the time of evaluation by PNA. In additional 4 patients positive for T315I mutation by Sanger sequencing, PNA FISH detected the mutation in respectively 20, 35, 45 % (Fig. 3 panel c, d) and 60 % (Fig. 3 panel a, b) of CD34+ cells. Finally in the case of Ph + ALL with increasing values of BCR-ABL1 transcript during dasatinib treatment and negative for T315I by Sanger sequencing we found about 2 % of cells with T315I mutation by PNA FISH. These cells were undetectable after hematopoietic stem cell transplant, in accordance with a progressive decrease of BCR-ABL1 transcript.

Conclusions

We suggest that this approach could be extended to other relevant and frequent BCR-ABL1 mutations thus allowing to drive clinical decisions after TKI failure. Important- ly, it is now known that the emergence of compo-und mutations in a BCR-ABL1 allele usually confer ponatinib resistance. More in general, it was shown that BCR-ABL1 compound mutants confer different levels of TKI resistance [11], thus requiring a rational and patient adapted selection of drugs to optimize the clinical out- come. PNA-FISH allows to identify compound mutation in single cells and to predict response to therapy. Fur- thermore, PNA-FISH technology allows to provide an answer to many questions including the possibility of the persistence of mutated stem/progenitor cells in pa- tients in MMR and the significance of the presence of small mutated clones at diagnosis. Finally, it allows to follow up clonal evolution during TKI therapy.

Abbreviations

ALL: Acute lymphoblastic leukemia; CML: Chronic myeloid leukemia; FISH: Fluorescence in situ hybridization; KD: Kinase domain; MACS: Magnetic activated cell sorting; MMR: Major molecular remission; PCR: Polymerase chain reaction; Ph: Philadelphia; PNA: Peptide nucleic acid; TKI: Tyrosine kinase inhibitors; WT: Wild type.

Competing interests

The authors have no competing financial interests.
Authors’ contributions
VR and RP performed the experiments. ES designed PNA and wrote the manuscript. CC, SC and JP processed samples and analysed data. FF and GS contributed to manuscript writing and provided final approval. DC designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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