Original article

Multiplex PCR approach for detection of fusion genes in acute lymphoblastic and myeloid leukemia

PCR multiplex pentru detectia genelor de fuziune in leucemia acuta mieloida si limfoblastica

Dumitru Jardan[1*], Rodica Talmaci[2], Cerasela Jardan[1], Daniel Coriu[2], Anca Colita[2], Andrei Colita[2], Constantin Arion[1]

1. Carol Davila University of Medicine and Pharmacy, Department of Pediatrics Clinical Institute Fundeni, Bucharest, Romania.
2. Carol Davila University of Medicine and Pharmacy, Department of Hematology Clinical Institute Fundeni, Bucharest, Romania.

Abstract

Acute leukemias are a group of heterogeneous diseases with respect to clinical features and acquired genetic aberrations. Since the discovery of BCR-ABL fusion gene a lot of other fusion genes were and are continuing to be discovered in leukemia patients. Many of these fusion genes are believed to be pathognomonic to disease and can be used as prognostic factors for the evolution of disease. In this study we performed development and optimization of a multiplex PCR assay for detection of 8 most common fusion genes in AML and ALL, namely: PML-RARα, AML1-ETO, CBFβ-MYH11, BCR-ABL, MLL-AF4, E2A-PBX1, SIL-TAL and TEL-AML1.Results: Our multiplex PCR assay allowed multiplexing of primers for detection of 8 most common fusion genes in acute leukemia. This allowed risk stratification for 36.5% of the patients. Furthermore identification of these fusion genes allowed MRD monitoring using nested PCR or real-time PCR for quantitative evaluation of MRD. Overall a very good performance was obtained with no non-specific amplifications and 100% correlation with commercial HemaVision kits. In comparison with HemaVision kits this assay allows identification of the most frequent 8 fusion genes at a considerably lower price per patient.

Keywords: *Fusion genes, multiplex RT-PCR, acute leukemia*

Rezumat

Leucemiile acute sunt un grup de boli heterogene in ceea ce priveste evolutia clinica si anomaliile genetice prezentate. De la descoperirea genei de fuziune BCR-ABL1 multe alte gene de fuziune au fost si sunt in continuare descrise la pacientii cu leucemie.O parte din aceste gene de fuziune sunt considerate patognomonice bolii si pot fi folosite ca factori de prognostic. In acests tudiu am dezvoltat si optimizat o metoda PCR multiplex pentru detectia a 8 gene de fuziune cu frecventa cea mai mare in LAM si LAL: PML-RARα, AML1-ETO, CBFβ-MYH11, BCR-ABL, MLL-AF4, E2A-PBX1, SIL-TAL si TEL-AML1. Rezultate: Metoda noastra, PCR multiplex, a permis combinarea

[*]**Corresponding author:**Dumitru Jardan, Departament of Hematology, Fundeni Clinical Institute, Sos. Fundeni 258, Sector 2, Bucharest, Romania.
Tel.: 0735300999, Email: d.jardan@gmail.com

primerilor pentru detectarea a 8 gene de fuziune in leucemiile acute. Aceasta permite stratificarea in grupe de risc a 36.5% din pacientii cu leucemie acuta. De asemenea, identificarea acestor gene de fuziune permite monitorizarea bolii minime reziduale folosind metoda nested si Real-time quantitative PCR. Aceasta este o metoda robusta ce nu a prezentat amplificari nespecifice, rezultatete fals positive si fals negative. Rezultatele obtinute au fost comparate cu rezultatele de la kit-urile comerciale Hemavision si a fost obtinuta o corelatie de 100%. In comparatie cu kit-urile Hemavision metoda noastra a permis identificarea genelor de fuziune la un pret scazut.

Cuvinte cheie: *gena de fuziune, RT-PCR multiplex, leucemie acuta*

Introduction

Acute leukemias are a group of heterogeneous diseases with respect to clinical features and acquired genetic aberrations (1). Current treatment agents and regiments have raised quality of life and overall survival of patients. Still the major challenge in any group of patients is to establish the most appropriate regiment (either blood marrow transplantation or standard chemotherapy) for the best clinical evolution of patients. In this regard a series of prognostic factors are used (2). These factors provide information about disease biology, identify potential treatment target and thus affect treatment decisions. The most common and accepted prognostic factors are: age, presenting white cell count, sex, primary or secondary leukemia, persistence of minimal residual disease and cytogenetics (2). There are many other prognostic factors described in the literature but many of them are not prospectively validated in large cohorts of patients and it is of critical importance to statistically validate all data before it can be used for patient risk stratification (2).

Since the discovery of BCR-ABL fusion gene a lot of other fusion genes were and are continuing to be discovered in leukemia patients. Many of these fusion genes are believed to be pathognomonic to disease and can be used as prognostic factors for the evolution of disease. The genes involved in genetic aberrations usually are key genes involved in development, regulation and function of lymphoid and myeloid cells. These genes frequently encode transcription factors, signal transduction proteins and receptors (3).

In acute myeloid leukemia (AML) prognostic factors described, besides the standard ones (such as age, presenting white blood count, etc.), include fusion genes (ex. AML1-ETO) (4), gene mutations (ex. FLT3-ITD, NPM1, CEBPα) (5,6) and overexpression of different genes (ex. EVI1, BAALC). The most common fusion genes described for AML are PML-RARα, AML1-ETO and CBFβ-MYH11 (7). These free fusion genes are detected in ~25-30% of cases and usually confer a favorable prognostic (7). Still mutations in c-KIT, which are detected in 20-30% of core binding factor leukemias, confer a higher risk of relapse for patients with AML1-ETO and CBFβ-MYH11 fusion genes (8). On the other hand FLT3 mutations are detected more frequently in PML-RARα cases and are associated with higher white cell counts at presentation (6).

In acute lymphoblastic leukemia (ALL) there is a different spectrum of fusion genes described. The prevalence of fusion genes and prognostic associated in ALL is different in children and in adults. The most common fusion genes are: BCR-ABL, MLL-AF4, E2A-PBX1, SIL-TAL and TEL-AML1 (7).

BCR-ABL is the most common fusion gene detected in adults (~25-30%) and it is associated with aggressive disease (7). Therapy with kinase inhibitors (ex. Imatinib) can lead to the development of resistance and thus testing for ABL kinase mutations is crucial in this type of patients. TEL-AML1 on the other hand is the most common pediatric translocation (~25-30%) and rare in adults and is associated with a favorable outcome. Favorable outcome conferred by this fusion gene is believed to be related to the greater chemotherapy susceptibility of leukemic cell harboring this translocation. E2A-PBX1 is detected in precursor-B-ALL at the frequency of

3-5% in pediatric and 3% adult patients. Detection of this fusion gene is associated with poor prognostic. MLL-AF4 fusion gene is the most frequent abnormality of 11q23 locus and is usually detected in precursor-B-ALL. It is detected in ~5% of pediatric and adult cases but it has a much higher prevalence in infants and in therapy related ALL – 40-60%. Detection of this fusion gene is associated with a poor prognosis. SIL-TAL is detected in 10-15% of T-ALL and is more prevalent in pediatric patients. There is no prognostic associated with detection of this fusion gene and is mainly used for minimal residual disease (MRD) monitoring (2,7).

In this study we performed development and optimization of a multiplex PCR assay for detection of 8 most common fusion genes in AML and ALL, namely: PML-RARα, AML1-ETO, CBFβ-MYH11, BCR-ABL, MLL-AF4, E2A-PBX1, SIL-TAL and TEL-AML1.

Patients and methods

Samples were collected from patients referred to Fundeni Clinical Institute, Department of Pediatrics and Department of Hematology and Coltea Hospital, Department of Hematology in the period between January 2008 and September 2011. There are a total of 192 samples analyzed, of which 92 were AML samples and 100 were ALL samples from both pediatrics and adult cases. Of 92 AML - 79 AML samples were from adults and 13 AML samples were from pediatric patients. Of 100 ALL samples - 67 ALL samples were from pediatric patients and 33 ALL samples were from adults. Experimental protocol was approved by local ethics committee and informed consent from all the patients was obtained.

RNA extraction

For RNA extraction white blood cells were transferred to TRIZOL reagent. Briefly – white cells were separated by lysis of red blood cells using RBC (Red Blood Cell) lysis buffer prepared in house (for 1L of 10x solution dissolve 8.26 g ammonium chloride (NH_4Cl), 1 g potassium bicarbonate ($KHCO_3$) and 0.037 g EDTA in ddH_2O). Cells were pelleted at 5000 rpm for 10 min, and washed once with PBS 1x. After washing pellet is resuspended in 1 mL of PBS and cells counted on COULTER® LH 750 Hematology Analyzer (Beckman Coulter) and aliquoted 20 000 000 cell/per tube. Cells were pelleted at 5000 rpm for 10 min. and supernatant removed. 1 mL of TRIZOL reagent per tube was added and cells were lysed by passing through a fine syringe needle. RNA was extracted following manufacturer's protocol.

Reverse transcription

RNA was reverse transcribed as modified from (2) using MMLV reverse transcriptase (Sigma) - 4 μg of RNA in 30 μl of H_2O was incubated at 65°C for 10 min and other reagents were added to final volume of 40 μl: RT buffer, DTT for a final concentration of 10 mM, random hexamers for a final concentration of 25 μM, RNAasin 20 units (Promega), RT enzyme MMLV 200 units (Sigma) and dNTP for a final concentration of 1 mM (Qiagen). Reaction mixture was incubated for 2h at 37°C and MMLV was denatured at 95°C for 5 min.

Multiplex PCR

For multiplex PCR, primers were used as previously described (7). Two tubes were used with the following primers in each:

Tube 1: primers for following fusion genes were mixed: PML-RARα, AML1-ETO, CBFβ-MYH11 and ABL-1 for internal control (*Table 1*). Primers concentration was: 1.6 mM for fusion genes and 0.8 mM ABL internal control.

Tube 2: primers for following fusion genes were mixed: BCR-ABL, MLL-AF4, E2A-PBX1, SIL-TAL, TEL-AML1 and ABL-1 for internal control (see *Table 1*). Primers concentration was: 1.6 mM for fusion genes and 0.8mM for ABL internal control.

PCR conditions: to final volume of 20 μl add 2 μl of cDNA (or water for negative control), 400 nM final concentration of primers for fusion genes and 200 nM for ABL internal control (5 μl of

Table 1. Primers used in multiplex mixes

Tube 1		
CBFB-A	5' GCAGGCAAGGTATATTTGAAGG 3'	CBFB-MYH11
MYH11-B2	5' TCCTCTTCTCCTCATTCTGCTC 3'	
MYH11-B1	5' TGAAGCAACTCCTGGGTGTC 3'	
AML1-A	5' CTACCGCAGCCATGAAGAACC 3'	AML1-ETO
ETO-B	5' AGAGGAAGGCCCATTGCTGAA 3'	
PML-A1	5' CAGTGTACGCCTTCTCCATCA 3'	PML-RAR
PML-A2	5' CTGCTGGAGGCTGTGGAC 3'	
RARA-B	5' GCTTGTAGATGCGGGGTAGA 3'	
Tube 2		
SIL-A	5' TCCCGCTCCTACCCTGCAA 3'	SIL-TAL
TAL1-B	5' CGCGCCCAGTTCGATGAC 3'	
E2A-A	5'CACCAGCCTCATGCACAAC 3'	E2A-PBX1
PBX-B	5'TCGCAGGAGATTCATCACG 3'	
MLL-A	5' CCGCCTCAGCCACCTAC 3'	MLL-AF4
AF4-B	5' TGTCACTGAGCTGAAGGTCG 3'	
BCR-e1-A	5' GACTGCAGCTCCAATGAGAAC 3'	BCR-ABL
ABL-a3-B	5' GTTTGGGCTTCACACCATTCC 3'	
BCR-b1-A	5' GAAGTGTTTCAGAAGCTTCTCC 3'	
TEL-A	5' TGCACCCTCTGATCCTGAAC 3'	TEL-AML1
AML1-B	5' AACGCCTCGCTCATCTTGC 3'	
ABL-r	5' TCCACTTCGTCTGAGATACTGGATT 3'	Control ABL fragment

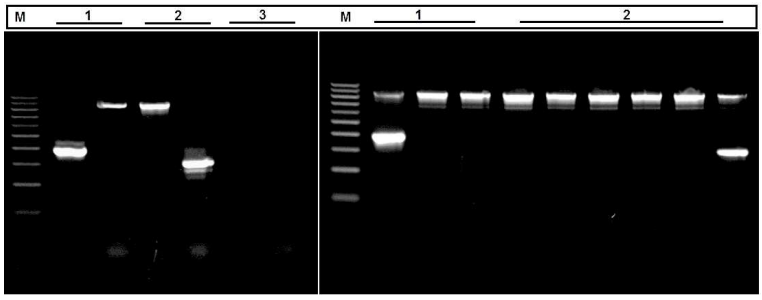
primer mix from either tube 1 or 2), dNTP 200 μM final concentration, PCR buffer, MgCl2 2.5 mM, 1 unit of HotStartTaq (Qiagen). PCR temperatures and cycle times - initial melting and enzyme activation 95°C for 15 min, PCR cycles melting 94°C for 30 s, annealing 60°C for 60 s and extension 72°C for 60 s for 35 cycles, no final extension. Amplicons were run on 2% agarose gel in 1x TAE buffer 1h at 100V, stained with etidium bromide and visualized using an UV transiluminator.

Control amplification amplifies a region 863 bp of ABL gene that serves as control for RNA extraction, reverse transcription and PCR amplification (9). Positive band corresponding to it should be amplified in all samples (except for the negative control). In case of samples which are positive for one of the fusion genes intensity of the control band can be greatly diminished. Control amplification was chosen in such a manner that it does not amplify nonspecific products in the presence of the other primers, amplify a ubiquitously expressed gene and have a lower efficiency of amplification then specific primers. Lower efficiency of amplification of control primers is given by the higher than optimal annealing temperature, lower concentration of primers and longer amplicon.

In case of identification of positive amplification a split-out reaction is performed using the same primers as in the mix but in individual reactions as described (7).

Performance testing and evaluation

Reliability of multiplex PCR assay described was tested using control RNA for: PML-RARα, AML1-ETO, CBFβ-MYH11, BCR-ABL, MLL-AF4, E2A-PBX1 andTEL-AML1 purchased from DnaTechnology. All control RNA amplifications were positive for the corresponding fusion genes.



1A 1B

Figure 1 A. Examples of 2 patient samples amplified using multiplex PCR. Patient 1 is positive for a fusion gene in tube 1; patient 2 is positive for a fusion gene in tube 2; lanes 3: the negative control. **1B.** Lanes 1: split-out for tube 1 for patient 1 identifying AML1-ETO fusion gene; lanes 2: split-out for tube 2 for patient 2 identifying TEL-AML1 fusion gene.

Using patient samples our assay consistently amplified control band in all reaction and there was no nonspecific amplification observed. All the specific bands were confirmed by split-out reactions (*Figure 1*).

Furthermore, a comparison of multiplex PCR assay and commercial kits was performed – 2 commercial kits were used – HemVision Full and HemaVision 7 kit (DnaTechnology). 25 clinical samples were tested in parallel with HemVision Full kit and our multiplex assay and 25 clinical samples were tested in parallel with HemaVision 7 kit and our multiplex assay using manufacturer's protocol.

Results

Of 25 samples of acute leukemia assayed in parallel with HemVision Full kit there were no discrepant results obtained – there were identified 4 cases with BCR-ABL, 3 cases with PML-RARα, 1 case with TEL-AML1 and 1 case with CBFβ-MYH11 fusion gene. The rest of the samples were negative.

Of 25 samples of acute leukemia assayed in parallel with HemVision 7 kit there were detected following fusion genes – 2 cases with AML1-ETO, 2 cases with PML-RARα, 1 case with MLL-AF4 and 1 case with BCR-ABL. There was one discrepancy found – 1 sample with SIL-TAL fusion gene was detected using our assay which was not detected by the HemaVision 7 assay (as it detects only 7 fusion genes- PML-RARα, AML1-ETO, CBFβ-MYH11, BCR-ABL, MLL-AF4, E2A-PBX1 and TEL-AML1) thus it is a normal finding. The rest of the samples were negative.

Overall our assay exhibited 100% concordance with HemaVision assays. To further validate this assay we tested another 142 of acute leukemia samples (both AML and ALL). There were identified following fusion genes: PML-RARα 9 cases, AML1-ETO 8 cases, CBFβ-MYH11 4 cases, BCR-ABL 10 cases, MLL-AF4 9 cases, E2A-PBX1 2 cases and TEL-AML1 12. A total of 70 cases were identified as harboring fusion genes (including those tested in parallel with HemaVision).

Combined there were identified 14 cases of PML-RARα, 5 cases of CBFβ-MYH11, 10 cases of AML1-ETO, 13 cases of TEL-AML1, 15 cases of BCR-ABL1, 10 cases of MLL-AF4, 2 cases of E2A-PBX1 and 1 case of SIL-TAL1. This allowed risk stratification for 36.5% of the patients. Furthermore identification of these fusion genes allows MRD monitoring using nested PCR (7) or real-time PCR (2,10) for quantitative evaluation of MRD. MRD monitoring provides important clues about the clinical evolution of patients and allows early relapse detection.

For AML patients combing findings from this assay and determination of mutational status of NPM1 and FLT3 (FLT3-ITD and FLT3-TK) allows risk stratification and thus better disease management for more than a half of the patients.

Conclusions

Our multiplex PCR assay allowed multiplexing of primers for detection of 8 most common fusion genes in acute leukemia.

Overall a very good performance was obtained with no non-specific amplifications and 100% correlation with commercial HemaVision kits. In comparison with HemaVision kits this assay allows identification of the most frequent 8 fusion genes at a considerably lower price per patient.

Aknowledgements

This work was supported by the grants PN 41-087 /2007, PN 42-099/ 2008 and CEEX 76/2006 from the Romanian Ministry of Research and Technology.

Conflict of interest declaration

Authors declare no conflict of interests.

References

1. Campana D. Minimal Residual Disease in Acute Lymphoblastic Leukemia. Hematology, 2010, vol 1, 7-12.
2. Gabert J, Beillard E, van der Velden VHJ, Bi W, Grimwade D, Pallisgaard N et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – A Europe Against Cancer Program. Leukemia, 2003, 17, 2318–2357.
3. Campana D. Minimal Residual Disease Studies in Acute Leukemia. Am J Clin Pathol, 2004, 122 (Suppl 1):S47-S5.
4. Viehmann S, Teigler-Schlegel A, Bruch J, Langebrake C, Reinhardt D and Harbott J. Monitoring of minimal residual disease (MRD) by real-time quantitative reverse transcription PCR (RQ-RT-PCR) in childhood acute myeloid leukemia with AML1/ETO rearrangement. Leukemia, 2003, 17, 1130–1136.
5. Krönke J, Schlenk RF, Jensen KO, Tschürtz F, Corbacioglu A, Gaidzik VI et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. J Clin Oncol., 2011 Jul, 29(19):2709-16.
6. Beretta C, Gaipa G, Rossi V, Bernasconi S, Spinelli O, Dell'Oro M G et al. Development of a quantitative-PCR method for specific FLT3/ITD monitoring in acute myeloid leukemia. Leukemia, 2004, 18, 1441–1444.
7. van Dongen JJM, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G et al. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease Report of the BIOMED-1 Concerted Action: Investigation of minimal residual disease in acute leukemia. Leukemia, 1999, 13, 1901–1928.
8. Wakita S , Yamaguchi H, Miyake K , Mitamura Y , Kosaka F , Dan K and Inokuchi K. Importance of c-kit mutation detection method sensitivity in prognostic analyses of t(8;21)(q22;q22) acute myeloid leukemia. Leukemia, 2011, 25, 1423–1432.
9. Iland H, Hertzberg M. and Marlton P. Myeloid Leukemia: Methods and Protocols (Methods in Molecular Medicine). Humana Press, Nov. 15, 2005, 69-93.
10. Beillard E, Pallisgaard N, van der Velden VHJ, Bi W, Dee R, van der Schoot E. et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) – a Europe against cancer program. Leukemia, 2003, 17, 2474–2486.