A multiplex reverse transcription-polymerase chain reaction strategy for the diagnostic molecular screening of chimeric genes: a clinical evaluation on 170 patients with acute lymphoblastic leukemia

Loredana Elia, Marco Mancini, Luisa Moleti, Giovanna Meloni, Sonia Buffolino, Mauro Krampera, Giulio De Rossi, Robin Foà, Giuseppe Cimino

Background and Objectives. In the last few years molecular methods have allowed the identification of leukemia-associated genetic lesions, which may represent the most accurate predictors of clinical outcome. These considerations strengthen the need for rapid identification of the abnormalities. Our aim was to demonstrate whether a modified multiplex reverse transcription polymerase chain reaction (RT-PCR) system might be successfully used to screen a large number of patients with acute lymphoblastic leukemia (ALL).

Design and Methods. In this study we adapted the multiplex RT-PCR assay, previously described by Pallisgaard *et al*., to detect all the most frequent genetic lesions with their characteristic splicing variants occurring in acute lymphoblastic leukemia, such as the *MLL*/AF4, *MLL*/ENL, *BCR/ABL* p190 (e1a2) and p210 (b2a2,b3a2) isoforms, *E2A/PBX1*, *TEL/AML1*, *SIL/TAL1* and the novel *NUP98/RAP1GDS1* transcript, recently described in a T-ALL leukemic subtype.

Results. We used the multiplex RT-PCR assay to screen 170 ALL patients (70 children and 100 adults). PCR positivity was detected in 67 (39%) of the 170 ALL patients studied. The comparison between cytogenetic and molecular analyses showed complete correspondence between the two assays in all patients with an evaluable karyotype. Finally, the observed incidence of genetic lesions in our ALL patients was similar to the frequency usually reported both in children and in adults with ALL.

Interpretation and Conclusions. These results show that, compared to single RT-PCR reactions, our multiplex RT-PCR system allows rapid, specific, simultaneous as well as a less expensive, laborious and time-consuming detection of the most frequent fusion transcripts in ALL patients. Therefore, it might be recommended for rapid diagnostic molecular screening of large numbers of patients, such as those enrolled in multicenter, co-operative studies. Furthermore, we have shown that multiplex RT-PCR is an *open system* that can easily be adapted to detect new leukemic genes.

Key words: acute lymphoblastic leukemia, molecular diagnostic screening, monitoring of minimal residual disease, multicenter studies.

Haematologica 2003; 88:275-279
http://www.haematologica.org/2003_03/88275.htm

Fom the Dipartimento di Biotecnologie Cellulari ed Ematologia, University "La Sapienza" of Rome (LE, MM, LM, GM, SB, RF, GC,), Cattedra di Ematologia, Policlinico "G.B. Rossi", Verona (MK), Italy and Ospedale Pediatrico "Bambino Gesù" Hematology Department, Città del Vaticano (GDR).

Correspondence: Giuseppe Cimino, MD, Dipartimento di Biotecnologie Cellulari ed Ematologia, Sezione di Ematologia, University "La Sapienza", via Benevento 6, 00161 Rome, Italy. E-mail: cimino@bce.med.uniroma1.it

In the past 10-15 years, several molecular methods have progressively allowed identification of leukemia-associated gene abnormalities.[1] These genetic lesions, which are currently detected in almost 50% of patients with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML), represent the most accurate predictors of clinical outcome.[2] These considerations, together with the recent availability of drugs that specifically target fusion proteins or their activity, highlight the need for a rapid and accurate identification of genetic lesions in patients with acute leukemia (AL).[3,4] In this respect, Pallisgaard *et al*.[5] recently proposed a multiplex reverse-transcription polymerase chain reaction (RT-PCR) system that allows simultaneous detection of 29 fusion genes and more than 80 breakpoints and splice variants in patients with acute leukemia. This result that would be extremely laborious and time consuming to obtain with single RT-PCR reactions.

In this study, we used a multiplex RT-PCR system, derived from Pallisgaard's method, for the molecular diagnostic screening and monitoring of minimal residual disease (MRD) in 170 patients with ALL. We found that the multiplex RT-PCR allowed systematic and extensive molecular screening of AL patients, was more sensitive than conventional karyotype and less laborious and time consuming than single RT-PCR analyses. Moreover, we have shown that this assay can be adapted to detect new leukemic genes, such as the *NUP98/RAP1GDS1*, recently described in a subset of T-ALL by Hussey *et al*.[6] and by Mecucci *et al*.[7]

Design and Methods

Patients

Sequential bone marrow (BM) and/or peripheral blood (PB) samples were obtained at diagnosis and during the clinical follow-up from 170 patients (100 adults and 70 children) with ALL, studied in Rome between April 1999 and December 2001. The diagnosis of ALL was established according to standard morpho-cytochemical criteria and immunophenotypic analyses. Patients' samples were studied at the laboratories of molecular biology and cytogenetics of the Department of Cellular Biotechnology and Hematology of the University La Sapienza of Rome.

Mononuclear cells were obtained from BM and PB samples after centrifugation on a Ficoll-Hypaque gradient, washed and cryopreserved in 4M guanidiun

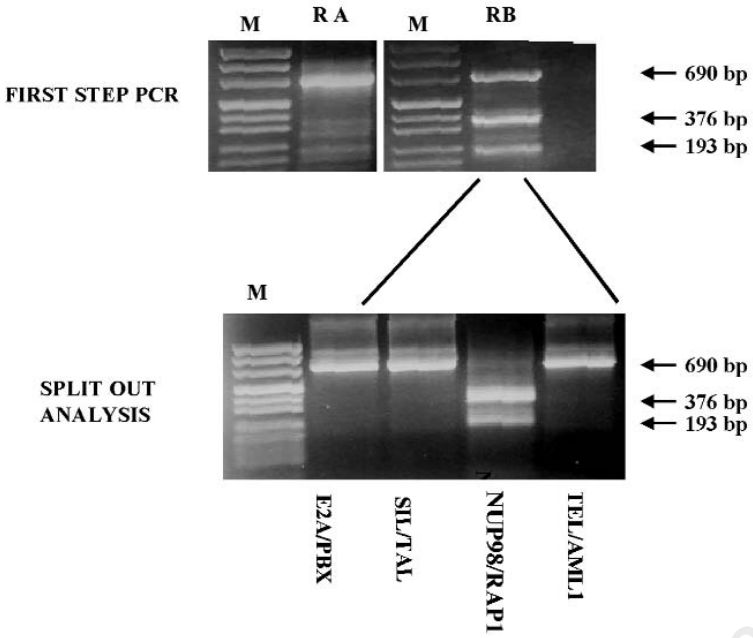

Figure 1. A representative example illustrating the first step multiplex RT-PCR reaction and the split-out analysis of a case expressing the *NUP98/RAP1GDS* fusion transcript. M=DNA molecular weight marker VIII (MBI Fermentas).

isothiocyanate at –20°C for total RNA extraction or as dry pellet for DNA extraction.

DNA analysis of the ALL1 locus

High molecular weight DNA was obtained from cell pellets following proteinase-K digestion and phenol-chloroform extraction, digested to completion with Bam-HI and Bgl-II, size fractioned by electrophoresis through an 0.8% agarose gel and transferred to nitrocellulose membranes. Filters were hybridized with the addition of the denatured B859 probe which is a cDNA insert that explores the entire MLL breakpoint cluster region, as reported by Gu *et al.*[8]

Multiplex RT-PCR

Total RNA was extracted from cells cryopreserved in guanidium isothiocyanate. The quality of RNA was assayed on an ethidium-bromide-stained 1% agarose gel containing 2.2 mol/L formaldehyde.

We used the multiplex RT-PCR system derived from that described by Pallisgaard *et al.*[5] which are adapted for the detection of the following fusion transcripts of ALL: *MLL1/AF4*, *MLL/ENL*, *BCR/ABL* p190 (e1a2) and p210 (b2a2,b3a2) isoforms, *E2A/PBX1*, *TEL/AML1* and *SIL/TAL1*, together with the *NUP98/RAP1GDS1* transcript, recently identified in a small subset of T-ALL.[6,7] Oligonucleotides and cycle parameters were those previously described by Pallisgard *et al.*[5] For the novel *NUP98/RAP1GDS1* fusion gene, not included in patients with Pallisgaard's system, we first defined the following primers near the *NUP98/RAP1GDS1* breakpoint gene sequence: RAPGDS1 = 5' TCC AGC ATC CAC 3', for reverse transcriptase; NUP1-198U20 = 5'-TGC TGT TGG TTC GAC CCT GT-3'and RAP1-209L20 = 5' TGG CTA CTT CTG CTA TGA TG 3'; and NUP1-253U20 = 5'-ACA ACC AGT GCA CCT TCA TT-3' and RAP1-169L20 = 5'-GCA GAC AAT CCA AGC ATC CT-3', for the first and second step of reaction, respectively. These primers were chosen to allow identical conditions to those of the RT-PCR multiplex assay. Prior to including these oligonucleotides in the multiplex RT-PCR system, we tested the primer pairs in separate PCR reactions using RNA samples from cases known to carry the *NUP98/RAP1GDS1* genetic abnormality, as reported elsewhere.[7] Once the correct performance of these primers had been checked, the new oligonucleotides were added in the multiplex RT-PCR reaction. cDNA synthesis and PCR amplification were performed as two parallel nested multiplex master reactions (preliminary analysis), following the conditions extensively described by Pallisgaard *et al.*[5] Each master reaction contains several pairs of primers specific for the following fusion transcripts: *BCR/ABL* p190 (e1a2) and p210 (b2a2,b3a2) isoforms, *MLL/AF4*, *MLL/ENL* (reaction A) and *E2A/PBX1*, *TEL/AML1*, *SIL/TAL*, *NUP98/RAP1GDS1* (reaction B). Moreover, each master reaction contains a pair of control primers that amplify the ubiquitously expressed *E2A* gene. At the end of the PCR amplification, 15 μL of each PCR reaction were electrophoresed in a 1.5% agarose gel for 60 min at 100 V and stained with ethidium bromide. To determine and verify the possible presence of a specific fusion gene after the preliminary analysis, a split-out analysis consisting

Table 1. Comparison between multiplex RT-PCR, cytogenetics and FISH analyses in the 170 ALL patients analyzed by multiplex RT- PCR assay.

Multiplex RT-PCR		*Karyotype*								*FISH*
		t(1;19) ± others (no. pts)	*t(12;21) ± others (no. pts)*	*t(9;22) ± others (no. pts)*	*t(4;11) ± others (no. pts)*	*t(11;19) ± others (no. pts)*	*Other structural and/or numerical abnormalities (no. pts)*	*Normal (no. pts)*	*Not evaluable (no. pts)*	
Negative pts.	**103**	–	–	–	–	–	33	38	32	–
Positive pts.	**67**									
E2A-PBX1	7	4	–	–	–	–	–	–	3	–
TEL/AML1	9	–	–	–	–	3	6	–	–	9
SIL/TAL1	3	–	–	–	–	–	3	–	–	–
BCR/ABL	31	–	–	25	–	–	–	–	6	–
MLL-/AF4	14	–	–	–	11	–	–	2	1	–
MLL-ENL	3	–	–	–	–	1	–	2	–	–

of single RT-PCR reactions using individual translocation-specific primer sets was performed. Negative controls without DNA template were included for all PCR reaction mixtures. A representative example of preliminary and split out analyses, related to a case with *NUP98/RAP1GDS1* fusion, not included in present series, is illustrated in Figure 1.

Cytogenetic and fluorescence in situ hybridization (FISH) analyses

Cytogenetic analyses were performed on BM cells after 24h of unstimulated culture. GTG bands with tryspin were obtained. Karyotypes were reviewed and defined according to the ISCN criteria. The cryptic translocation t(12;21)(p13;q22) was studied by FISH analysis, directly using fluorochromes-labeled TEL (SpectrumGreen) and AML1 (SpectrumOrange) probes from Vysis Inc. (France).

Results

Sixty-seven of the 170 (39.4%) ALL cases studied were positive for a fusion protein after diagnostic multiplex RT-PCR. In particular, a fusion transcript was detected in 27 (38.5%) of the 70 childhood samples and in 40 (40%) of the 100 adult samples. In childhood ALL, the *TEL/AML1* fusion transcript was detected in 8/70 cases (11.4%), *E2A/PBX* in 5 cases (7.1%), *MLL/AF4* in 4 case (5.7 %), *SIL/TAL* in 3 cases (4.2 %), *MLL/ENL* in 2 cases (2.8 %), *p190 BCR/ABL* in 2 cases (2.8 %) and *p190/p210 BCR/ABL* in 3 cases (4.2%). By contrast, in the group of 100 adult ALL, the *BCR/ABL* fusion transcript was detected in 26 cases (26%) (p190 transcript = 22 cases, p190/p210 = 2 and p210 = 2 patients), *MLL/AF4* in 10 cases (10%), *MLL/ENL* in 1 case (1%), E2A/PBX in 2 cases (2%) and *TEL/AML1* in 1 case (1%). No case with *NUP98/RAP1GDS1* fusion transcript was identified in either group. Negative multiplex RT-PCR results were documented in 43/70 (61.4 %) and in 60/100 (60%) of children and adults, respectively.

Among the 67 multiplex RT-PCR positive patients, 57 had an evaluable cytogenetic analysis, whereas karyotype was not evaluable in three cases and it was not performed in the remaining 7 cases. Among the 57 multiplex RT-PCR positive cases for whom cytogenetic information was available, the cytogenetic analysis showed that 41 patients had the corresponding translocation, 13 had a normal karyotype (*TEL/AML1* = 6; *SIL/TAL1* = 3; *MLL/ENL* = 2; *MLL/AF4* = 2) whereas the 3 remaining patients with *TEL/AML1* fusion transcript had other structural and/or numerical abnormalities. The presence of the *TEL/AML1* fusion transcript was also confirmed by FISH analysis in all the 9 cases. With regard to the group of the 103 ALL patients with a negative multiplex RT-PCR test, an evaluable cytogenetic analysis was available for 71 of them. Among these cases, karyotype was normal in 38 patients and altered, because of the presence of either numerical and/or structural alterations, in the remaining 33 patients. We did not observe any cases negative by the multiplex RT-PCR analysis which were then shown cytogenetically to have one of the alterations evaluated by the molecular assay. A summary of the results achieved by multiplex RT-PCR, cytogenetics and FISH analyses is reported in Table 1.

With respect to the alterations of the *MLL* gene located at the 11q23 cytogenetic band, Southern blot analysis demonstrated genomic *MLL* gene rearrangements in 17 of the 170 cases (10%). All

these latter cases were positive in the multiplex RT-PCR assay, which detected the expression of *MLL/AF4* in 14 (82.4%) patients and MLL/*ENL* in the 3 remaining cases (17.6%).

Discussion

This study is a clinical demonstration of the usefulness of multiplex RT-PCR analysis for the rapid screening of a large number of ALL patients. As demonstrated by the present data, our system fulfils the criteria of specificity recently stated at the BIOMED concerted action for the RT-PCR molecular analysis of genetic lesions in acute leukemias patients.[1] In fact, in our series of patients the observed incidence of genetic lesions was similar to the frequency usually reported both in children and in adults with ALL,[9,10] whereas in all patients with a multiplex RT-PCR positivity and an evaluable karyotype we observed complete correspondence between the two analyses and, conversely, we did not observe any case with a positive cytogenetic analysis and a negative multiplex RT-PCR analysis. Moreover, the split-out analysis that followed a positive preliminary step RT-PCR multiplex assay and that allowed precise identification of the fusion product present in the sample, is by itself an indirect confirmation of the multiplex PCR specificity. In addition, with respect to the 17 *MLL* positive patients of this series, in all cases there was concordance between Southern-blot and multiplex RT-PCR analyses. In particular, RT-PCR showed the expression of the *MLL/AF4* fusion product in 14 (82.4%) patients and of the *MLL/ENL* in the 3 remaining cases (17.6%). It is worth noting that these data may indicate that the ALL subset shows a less marked level of promiscuity of the *MLL* gene than do acute myeloid leukemias. In fact, fusion of the *MLL* gene in ALL is limited mainly to the two *AF4* and *ENL* partner genes. This suggestion is further supported by similar observations (*unpublished*) in 494 adult ALL patients enrolled in the Italian multicenter GIMEMA 0496 study who were routinely screened for the MLL genomic configuration. As a consequence, we suggest that, for a rapid screening of ALL patients, searches for *MLL/AF4* and *MLL/ENL* (as in our assay), may be sufficient to detect almost all the *MLL* gene alterations, reserving the other more tedious and time-consuming methods such as Southern-blot and single RT-PCRs for the less common *MLL* fusion genes, etc. for selected cases only.

Another relevant point showing that the multiplex system is faster and cheaper than single RT-PCR is the finding that two simple PCR reactions (i.e. the preliminary multiplex RT-PCR step) were sufficient to rule out the presence of the eight transcripts in 60% of ALL cases.

In conclusion, the multiplex RT-PCR strategy described here allows rapid, specific and simultaneous detection of several fusion transcripts in ALL patients, at a time when the early knowledge of the presence or absence of given genetic lesions is paramount for treatment decisions. Moreover, compared to single PCRs, multiplex PCR is significantly less expensive, laborious and time-consuming. Finally, as we have demonstrated for the detection of the *NUP98/RAP1GDS1* gene, this assay can be easily adapted to incorporate novel fusion genes. Multiplex RT-PCR might be recommended for the molecular screening of large numbers of patients, such as those enrolled in multicenter, cooperative studies.

References

1. van Dongen JJ, 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: investigational of minimal residual disease in acute leukaemia. Leukemia 1999;13:1901-28.
2. Look AT. Oncogenic transcription factors in the human acute leukemias. Science 1997;278:1059-64.
3 Mandelli F, Diverio D, Avvisati G, Luciano A, Barbui T, Bernasconi C, et al. Molecular remission in PML/RARα-positive acute promyelocyte leukemia by combined all-trans reitnoic acid and idarubicin (AIDA) therapy. Blood 1997;90:1014-9.
4. Mauro MJ, Druker BJ. STI571: targeting BCR-ABL as therapy for CML. Oncologist 2001;6:233-8.
5. Pallisgaard N, Hokland P, Rishoj DC, Pedersen B, Jorgensen P. Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. Blood 1998;92:574-88.
6. Hussey DJ, Nicola M, Moore S, Peters GB, Dobrovic A. The (4;11)(q21;p15) translocations fuses the NUP98 and RAP1GDS1 genes and is recurrent in T-cell acute lymphocytic leukaemia. Blood 1999;94:2072-9.
7. Mecucci C, La Starza R, Negrini M, Sabbioni S, Crescenzi B, Leoni P, et al t(4;11) (q21;p15) translocation involving NUP98 and RAP1GDS genes: characterization of a new subset of T acute lymphoblastic leukaemia. Br J Haematol 2000;109:788-93.
8. Gu Y, Nakamura T, Alder H, Prasad R, Canaani O, Cimino G, et al. The t(4 ;11) chromosome translocation of human acute leukemias fuses the ALL1 gene, related to Drosophila trithorax, to the AF4 gene. Cell 1992;71:701-8.
9. Pui CH. Acute lymphoblastic leukemia in children. Curr Opin Oncol 2000;12:2-12.
10. Hoelzer D. Acute Lymphoblastic leukemia in adults. In: Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, Mc Glave P, editors. Hematology: basic principles and practice. Churchill Livingstone; 2000. p. 1089-105.

Pre-publication Report & Outcomes of Peer Review

Contributions

GC was responsible for the conception of the study, its design, and ethical approval; he also wrote the paper. LE and SB, analyzed and interpreted the molecular data; MM analyzed and interpreted the cytogenetics data; LM, GM, MK and GDR were responsible for the patients' care and RF was responsible for the final approval of the version to be submitted. All the authors contributed to the manuscript and approved its final version.

Funding

This work was partially supported by the "Associazione Italiana per la Lotta contro le Leucemia - Sezione di Roma" (ROMAIL-ONLUS), Consiglio Nazionale delle Ricerche (CNR), Legge 27 dicembre 1997, n°449/97, and by Proggetto Finalizzato 2001 Min. Salute "Alterazioni geniche nelle leucemie acute".

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two externbal referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received August 14, 2002; accepted January 21, 2003.

In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

Molecular characterization of leukemic cells is relevant to clinical decision making in patients with acute lymphoblastic leukemia.

What this study adds

This study describes a multiplex RT-PCR system that allows rapid and specific detection of the fusion transcripts most frequently found in acute lymphoblastic leukemia.