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Acute Leukemias

Combined use of reverse transcriptase polymerase chain reaction and flow cytometry to study minimal residual disease in Philadelphia positive acute lymphoblastic leukemia

LUZ MUÑOZ, OLGA LÓPEZ, RODRIGO MARTINO, SALUT BRUNET, MAR BELLIDO, ENRIQUETA RUBIOL, JORGE SIERRA, JOSEP F. NOMDEDÉU
Hematology Division, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain

ABSTRACT

Background and Objectives. **The Philadelphia chromosome in acute lymphoblastic leukemia (Ph+ ALL) is associated with a poor prognosis given the high frequency of chemoresistance and leukemia relapse. Minimal residual disease (MRD) detection before cytogenetic and hematologic relapse could be useful in early therapy. The most suitable methods for detecting MRD in Ph+ ALL are flow cytometry (FC) and reverse transcriptase polymerase chain reaction (RT-PCR). However, since both techniques carry the risk of false-negative results the combined use of these two techniques could overcome this problem.**

Design and Methods. **We report our experience using this approach in 47 bone marrow samples obtained from 10 Ph+ ALL patients. Twenty-seven marrow aspirates were taken from patients in clinical remission (CR). The samples were considered positive for MRD by FC when two conditions were met: 1) detection of an abnormal B-cell differentiation pattern and 2) presence of more than $1x10^{-3}$ cells coexpressing CD22/CD34/CD45 or CD66/CD34/CD10. After FC analysis, RNA was purified using standard methods.**

Results. **FC was positive in 23/27 samples in CR (sensitivity 85%). RT-PCR was successfully performed in 23 samples in CR. RT-PCR was positive in 18/23 samples (sensitivity 78%). There were 5 samples with discordant results. FC was positive in 3 samples with a negative RT-PCR and FC was negative in 2 samples with a positive RT. All the 10 patients relapsed and only 1 is currently alive after an allogeneic stem cell transplantation. The median (range) time from MRD detection to relapse in patients treated with chemotherapy was 42 (39-71) days.**

Interpretation and Conclusions**. These data suggest that RT-PCR may be negative despite the presence of neoplastic cells identified by their immunophenotypic traits. We conclude that immunologic and molecular techniques can be used in tandem for monitoring MRD in Ph+ ALL.**

Keywords: acute lymphoblastic leukemia; bcr/abl; minimal residual disease; flow cytometry; RT-PCR

Correspondence: Josep F. Nomdedéu, M.D., Laboratori d´Hematologia, Hospital de la Santa Creu i Sant Pau, Avda Sant Antoni M. Claret 167, 08025 Barcelona, Spain.

The t(9;22)(q34;q11) translocation is the cytogenetic hallmark of chronic myeloid leukemia. This rearrangement is also detected in 25% of adults and in 5% of children with acute lymphoblastic leukemia (ALL). The presence of bcr/abl arising from this translocation confers a very bad prognosis. Patients with Ph+ ALL obtain complete remission less frequently than patients with other subtypes of ALL using standard chemotherapy. In addition, relapse invariably occurs when these patients are exclusively treated with chemotherapy. Allogeneic stem cell transplantation (alloSCT) is in practice the only curative option for these patients.[1] The identification of minimal residual disease (MRD) in the absence of cytogenetic and hematologic relapse may be of interest for early therapy. Of the methods available for detecting MRD in Ph+ ALL, flow cytometric (FC) identification of leukemia-associated immunophenotypes and bcr/abl detection by reverse transcriptase polymerase chain reaction (RT-PCR) have proven to be the most sensitive and reproducible.[2-5] However, both assays carry the risk of false negative results. We investigated the simultaneous use of RT-PCR and FC to study MRD in Ph+ ALL.

Design and Methods

Patients

The study group included 10 consecutive patients, 9 adults and 1 child diagnosed with Ph+ ALL in the Hospital de la Santa Creu i Sant Pau from 1997 to 1999. The patients' clinical and biological characteristics are shown in Table 1.

Cell lines

Four cell lines[6] were used for sensitivity assays (cell lines were purchased from DSMZ, Braunschweig Germany):[7] CML-T1, EM-2, TOM-1 and L363 expressing p210 protein (b_2a_2), p210 (b_3a_2), p190 (e_1a_2) and negative, respectively. The cell lines were maintained in RPMI 1640 medium (Gibco BRL, Life Technologies, USA) with 10% fetal calf serum (Gibco BRL) and L-glutamine (Gibco BRL) until RNA extraction.

Sample processing

Bone marrow (BM) samples were collected in heparin or EDTA anticoagulant. BM cellullarity and the criteria of complete remission were assessed by two independent observers following standard cri-

Table 1. Clinical and biological characteristics of Ph+ ALL patients.

Pts.	Sex	Age	FAB	WBC count (x 10^9/L)	BCR/ABL transcript	Induction therapy	Salvage therapy	SCT	Outcome
1	F	79	L1	7	b_2a_2	No	No	No	Dead
2	F	50	L1	11	e_1a_2	No	No	No	Dead
3	F	15	L1	20	e_1a_2	SHOP-94	Yes	Allogeneic	TRM
4	M	21	L1	18	e_1a_2	PETHEMA	ReLAL	Allogeneic	TRM
5	F	45	L2	69	e_1a_2	PETHEMA	ReLAL	No	REL
6	F	43	L2	15	e_1a_2	PETHEMA	ReLAL	Allogeneic	REL
7	M	28	L1	84	e_1a_2	PETHEMA	ReLAL	Allogeneic	REL
8	M	47	L2	7.5	b_2a_2	PETHEMA	ReLAL	No	REL
9	F	66	L2	10.3	e_1a_2	PETHEMA	No	No	REL
10	M	61	L1	41	e_1a_2 (b_2a_2)*	PETHEMA	ReLAL	Allogeneic	Alive in CR

**Predominant e_1a_2 expression with a small amount of b_2a_2. Induction therapy: Pethema ALL. Salvage therapy: ReLAL. REL: relapse; TRM: transplant-related mortality.*

Table 2. Immunophenotype of Ph+ ALL patients.

Pts.	CD10	CD20	CD19	CD33	CD13	CD117	CD22	CD66	CD34	CD45	HLADR	MPO #	CD79a #	CD3 #	Tdt	μ #
1	+100%	+30%	+100%	+76%	+37%	-	+100%	ND	+100%	+25%	+100%	-	+100%	-	+100%	ND
2	+100%	-	+100%	+71%	+56%	-	-	ND	+100%	+100%	+100%	-	+100%	-	+100%	ND
3	+100%	+100%	+100%	-	-	-	+50%	ND	+100%	-	+100%	-	+100%	-	+100%	ND
4	+100%	+53%	+100%	+47%	+62%	-	+70%	ND	+100%	-	+100%	-	+100%	-	+100%	ND
5	+100%	+45%	+100%	+58%	+30%	-	+79%	+73%	+100%	+62%	+100%	-	+100%	-	+100%	ND
6	+100%	+62%	+100%	+42%	+25%	-	-	+50%	+100%	+44%	+100%	-	+100%	-	+100%	-
7	+100%	-	+100%	+43%	+35%	-	+49%	+86%	+100%	+60%	+70%	-	+100%	-	+100%	-
8	+100%	+42%	+100%	+40%	+50%	-	+92%	+90%	+100%	+62%	+70%	-	+100%	-	+100%	+100%
9	+100%	+22%	+100%	+35%	+85%	+31%	+82%	+100%	+100%	+25%	+90%	-	+100%	-	+100%	-
10	+100%	+74%	+100%	+32%	-	-	+80%	+100%	+100%	+26%	+52%	-	+100%	-	+100%	+100%

#Cytoplasmic; ND: not done.

teria.[8] After marrow aspiration, only 1 sample was obtained, and flow cytometric analysis was always carried out before RNA extraction.

Immunologic methods

Sample preparation. The number of total cells was quantified by microscopy and adjusted to $2x10^6$ in each tube. The immunophenotypic analysis was performed on lysed whole BM samples with direct conjugated monoclonal antibodies (MoAbs). To increase the possibility of detecting the maximum number of phenotypic aberrations present at diagnosis, antigen expresssion was analyzed using triple combinations of the following MoAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinine chlorophyll protein (PerCp) or phycoerythrin-cyanine 5 (PE/Cy 5) flourochrome tandem: CD 66 (Kat4c-FITC), CD22 (4KB128 FITC), CD34 (8G12-FITC, PE), HLA-Dr (L243 PetCp), CD10 (W8E7 FITC), CD 20 (L27 PE); CD2 (S5.2 FITC), CD33 (67.6 PE), CD7 (4H9 FITC), CD45 (2D1 PerCp), CD13 (L138 PE), CD3 (SK7 PerCp), CD4 (Leu 3ª FITC), CD5 (Leu 1 FITC), CD8 (Leu 2ª PE) purchased from Becton Dickinson (BDIS), San José, Ca, USA; CD19 (SJ25-C1 PE/Cy 5) and MPO (H-43-5 FITC) from Caltag Laboratories, Burlingame, USA; CD36 (FAG-52 FITC) from Immunotech, Marseille, France and TdT (VNF-830 and VTF-510 FITC) from Harlam Sera Lab, Sussex, England. Direct immunofluorescence was performed incubating $2x10^6$ cells with the specific MoAb for 15 min in the dark at room temperature. In all cases, isotype-matched immunoglobulins with no reactivity against BM cells and the CD4-FITC/CD8-PE/CD3-PerCP combinations were used as negative and positive controls, respectively. Cells were lysed (FACS lysis solution, BDIS) for 3 to 5 minutes and centrifuged at 250 g for 5 minutes. The cells were washed twice with phosphate buffered saline (PBS) before being resuspended in PBS and examined.

Detection of intracellular antigens (cytoplasmic and nuclear) by FC. The cells were incubated for fixation and permeabilization with Fix & Perm solution (CALTAG, Burlingame, CA, USA). They were then washed twice with PBS and incubated for 15 min with 20 µL of antibody. After incubation, the cells were washed again with PBS before resuspension and examination.

Immunologic criteria for lineage involvement. Lineage affiliation was performed according to the EGIL recommendations. The diagnosis of B-cell lineage acute leukemia was established when cytoplasmic CD79a (cyCD79a) was positive regardless of the pattern of surface markers. Acute myelogenous leukemia (AML) was excluded from this series by the lack of cytoplasmic myeloperoxidase expression. Cytoplasmic CD3 was tested in every case.

Data acquisition and analysis. A FACScan flow cytome-

ter (BDIS) was employed. Calibration of the instrument was performed before data acquisition using well-established protocols with CD4/CD8/CD3 positive controls and bead standards (Calibrite, BDIS).[9] For data acquisition the LYSIS-II (BD) software program (BDIS) was used. At least 10,000 events/tube were measured. The PAINT-A-GATE PRO software program (BDIS) was used for further data analysis. Thresholds for positivity were based on isotype negative controls. The positivity threshold was 20% for all markers except for surface CD117 and cytoplasmic or intranuclear antigens for which a 10% threshold was used.

MRD analysis

The strategy for MRD detection included two criteria based on previously published results:[11,12] i) an abnormal B-cell pattern as revealed by the percentages in the cell population identified using the CD10/CD20/CD19 tube and ii) detection exceeding $1x10^{-3}$ cells coexpressing an aberrant phenotype. For MRD studies three triple-antigen combinations were employed: CD10/CD20/CD19; CD22/CD34/CD45 and CD66/CD34/CD10 or CD66/CD34/CD45 (Table 3). Evaluation of cells displaying leukemia-associated phenotypes was performed using a two-step acquisition procedure according to a previously defined method.[21]

Cytogenetic analysis

Cytogenetic analysis was performed on bone marrow cells after short-term culture without stimulation.

RT-PCR analysis

Total RNA was prepared either by the guanidinium thiocyanate-phenol chloroform method[13] (Ultraspec, Biotecx laboratories, Inc., Houston, Texas, USA) (27 samples) or by using a RNeasy Kit[14] (Qiagen Gmbh, Hilden, Germany) (17 samples), following the manufacturers' recommendations. The RNA was dissolved in RNase-free water and 7 µL were withdrawn for quantification on a GeneQuant RNA/DNA (Pharmacia Biotech, Sollentuna, Sweden) and stored until use at –80ºC.

The conditions for reverse transcription and nested-PCR to quantify bcr/abl cDNA were those described by Saglio *et al*.[15] with minor modifications. Briefly, 5 µg of RNA were reverse-transcribed into cDNA using a specific antisense primer in a final reaction volume of 50 µL with 10 µL of buffer 5x (Gibco BRL), 5 µL of DTT 0.1M (Gibco BRL) 200 U of M-MV reverse transcriptase (Gibco BRL), 3 µL of each dNTP (1.5 mM each in final concentration) (Promega, Madison, USA) and 40 U of RNAsin (Promega). The reaction mixture was incubated at 37ºC for 1h 25'.

To rule out contamination by DNA,[16] we repeated the experiments for all the samples showing PCR positivity in the second step of the nested PCR. Repeat cDNA synthesis was carried out for some samples without adding reverse transcriptase. None of these samples produced any bands after PCR amplification.

We used specially dedicated pipettes and filtered tips and included non-nucleic acid containing blanks during all reactions to decrease the possibility of PCR product carry over and cross-contamination. Quality control tests were carried out with 10µL of cDNA in a one-step PCR amplification of normal ABL and E2A sequences.

The remaining cDNA from each sample was then divided into 2x100 µL PCR tests for the first-step amplification of a given fusion gene transcript followed by a second-step (nested) amplification of 6 µL of first-step products. Separate amplifications of p210 and p190 BCR-ABL transcripts were performed under the same conditions: each PCR mixture contained 10 µL of buffer (KCl 50 mM, Tris-HCl pH 10 mM, $MgCl_2$ 25 mM, dNTPs 0.2 mM each, BSA 0.2 mg/mL final concentration), 0.3 µL of Taq polymerase (Perkin-Elmer 5 U/µL, Branchburg, New Jersey, USA) and two specific primers for p210 and p190 amplification. Nested-PCR amplification of the bcr/abl gene was performed sequentially with two internal specific primers for p210 and p190 detection. Amplification reactions were performed on a temperature controller (Perkin Elmer GeneAmp PCR System 9700 Thermocycler). Amplified PCR products from PCR-1 and PCR-2 were separated by electrophoresis on a 2% agarose gel.

In order to assess the sensitivity of this technique, CML-T1 (b_2a_2), EM-2 (b_3a_2) and TOM-1 (e_1a_2) cells were diluted with another cell line (non-Ph+ cells). These preparations represented dilutions of 1/10, $1/10^2$, $1/10^3$, $1/10^4$, $1/10^5$, $1/10^6$ and 0 Ph cells in 10^6 non-Ph cells. Each aliquot was processed as described above.

MRD analysis with RT-PCR

RNA isolation from MRD samples and the conditions and specific primer sequences used in reverse transcription/PCR amplification were as described above.

Results

Immunophenotypic characteristics

All the cases were CD10, CD19, CD34, HLA-DR, CD79a and TdT positive. In our series there was a high incidence of myeloid antigen expression : CD66 in 6 out of 6 cases tested, CD33 in 9 out of 10 cases, CD13 in 8 out of 10 cases, and CD117 in 1 out of 10 cases. Another interesting feature was the expression of the CD45 antigen, which was negative in 2 patients and showed a dim expression in 3 cases[10] (Table 2).

Table 3. MoAbs combinations used to detect MDR and their distribution at diagnosis in Ph+ ALL patients.

Aberrant phenotypes	*No. of cases*
CD22FITC/CD34PE/CD45PerCP	8/10
CD66FITC/CD34PE/CD45PerCP	6/6
CD10FITC/CD20PE/CD19PerCP	10/10

Table 4. Discordant findings between FC and RT-PCR results of MRD detection in our series.

Patient	Timing	Flow cytometry	# of aberrant cells* detected by FC	RT-PCR
3	After AlloSCT	-		+
6	After AlloSCT	+	$1x10^{-3}$	-
8	After salvage chemotherapy	-		+
8	After salvage chemotherapy	+	$2x10^{-3}$	-
10	After induction chemotherapy	+	$2x10^{-3}$	-

**of the total nucleated cells.*

MRD analysis by FC

We analyzed 27 BM samples in CR with FC. The criteria used to define MRD have been described previously. MRD was detected in 23 out of 27 samples. Both criteria, an abnormal B-cell differentation pattern and a population with CD22/CD34/CD45 or CD66/CD34/CD10 $>10^{-3}$ cells were positive in 20 samples. In one sample MRD was only detected by an abnormal B-cell differentation pattern and in another sample it was only associated with $>1x10^{-3}$ cells coexpressing CD66/CD34/CD10. The median (range) of residual leukemic cells detected was $4x10^{-3}$ ($0.6\text{-}40x10^{-3}$) of total nucleated cells. The median (range) time between MRD detection and relapse in patients treated with chemotherapy was 42 (39-71) days. After MRD detection two patients treated with allogeneic stem cell transplantation relapsed 2.5 and 4 months later. The sensitivity of FC in aberrant phenotype detection using cell dilutions was $1x10^{-4}$. The sensitivity for detecting B-cell differentation pathway abnormalities has not been established.

Molecular characteristics

Ten Ph+ ALL patients were tested by qualitative bcr/abl RT-PCR. Eight patients expressed p190 transcript (e_1a_2) and two showed p210 (b_2a_2).

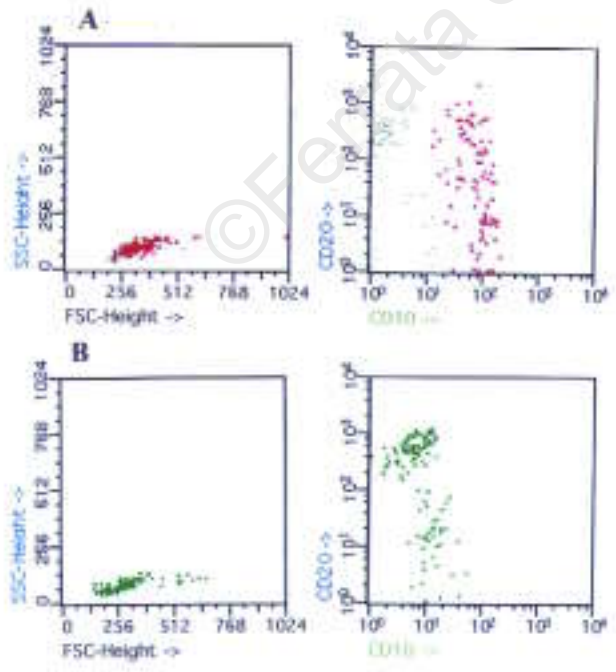
MRD analysis by RT-PCR

All samples tested at diagnosis were positive for some type of bcr/abl transcript. Eighteen patients in CR were bcr/abl positive.

We found a sensitivity of 10^6 for bcr/abl p210 and 10^5 for bcr/abl p190.

MRD detection combining both methods

The presence of MRD was simultaneously assessed by FC and RT-PCR in 23 samples from patients in CR. FC was performed on 27 CR samples. FC was positive in 23 of these 27 samples. RT-PCR was successfully performed in 23 samples from patients in CR. RT-PCR was positive in 18/23 samples. The results of the two methods showed a good correlation. However, 5 samples showed discordant findings: FC was positive in 3 samples with a negative RT-PCR and FC was negative in 2 samples with a positive RT-PCR (Table 4, Figure 1). The clinical outcome suggests that these discordant results were false-negatives. These disparate results always preceded hematologic relapse. FC and RT-PCR were negative in only two samples taken consecutively from the same patient who was allografted and suffered from acute graft-versus-host disease (GvHD). All the patients in this series relapsed and only one is currently alive after allogeneic stem cell transplantation.



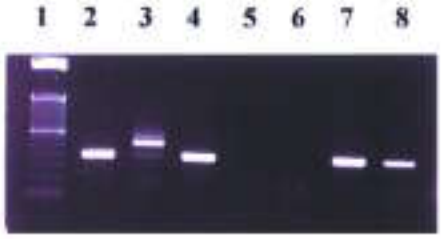


Figure 1. Left: flow cytometric detection of abnormal B-cell maturation (red: lymphoblasts, green: normal B-lymphocytes). B-cell gate was established on the basis of CD19 expression and low right angle light scatter (SSC) and additional analysis of B-cell differentiation pathways. We acquired 10^3 of total events and CD19+ cells represent 2% of the total nucleated cells. Right: RT-PCR analysis in Ph+ ALL. Lane 1: Molecular weight marker. Positive controls b2a2, b3a2, b2a2 and e1a2, lanes 2,3,4 and 7, respectively. Lane 5: Negative control. Lane 6: RT-PCR negativity in one case with MRD detected by FC (dot plots A). Lane 8: RT-PCR positivity in one case with normal immunophenotype (dot plots B).

Discussion

MRD studies are currently performed to estimate the total burden of leukemic cells during clinical remission. A number of techniques have been recently developed to improve the morphologic methods in assessing response to therapy. These include immunophenotype analysis and molecular methods, which are the most sensitive and reproducible techniques to date. However, both methods require technical expertise and are not problem free.[5,17-19]

We used two FC criteria to study MRD: detection of an abnormal B-cell maturation pattern and the presence of a cell population >1x10^{-3} with an aberrant phenotype. We chose these criteria because both are reproducible and can be applied to all ALL samples.[11,12,20] We found that CD66 is a good marker in ALL and that it could be used to detect residual leukemic cells in a large number of cases of B-cell lineage ALL.[21]

The main reasons for false-negative results are: 1) uneven distribution of leukemic cells during clinical remission leading to sampling variability, and 2) clonal evolution, during and after treatment which may lead to the disappearance of one or more markers detected at diagnosis. Sample variability could be resolved by taking samples from multiple sites, whereas immunophenotypic shifts could be avoided by increasing the number of marker combinations.[22]

However, major phenotypic changes did not seem to be common in Ph+ ALL.

The possibilities and limitations of molecular methods such as RT-PCR assay for detecting minimal residual bcr/abl depend not only on their specificity, sensitivity and reproducibility but also on their simplicity and speed.

In our study, all patients were monitored for both the p210 and p190 transcripts. Two patients had the p210 transcript (both b_2a_2) and seven had the p190 transcript (e_1a_2). We observed that one case presented two bcr/abl transcripts simultaneously with a predominant e_1a_2 expression and a small amount of b_2a_2 expression. A false negative bcr/abl RT-PCR analysis could result from an inadequate template, poor RNA isolation or failed reverse transcription or PCR amplification.[23,24]

Although RT-PCR has been shown to be a highly sensitive assay for bcr/abl detection, we encountered a number of false-negative samples. We attributed this discordance to our limited sample volume and technical errors, in part because in our study RNA was obtained after FC analysis. RT-PCR could have been improved by obtaining a separate BM sample. However, the high sensitivity described for RT-PCR is not always achieved routinely and variations in each sample could arise.[25,26]

Both methods were good at predicting relapse. In our study, positive MRD almost invariably persisted in

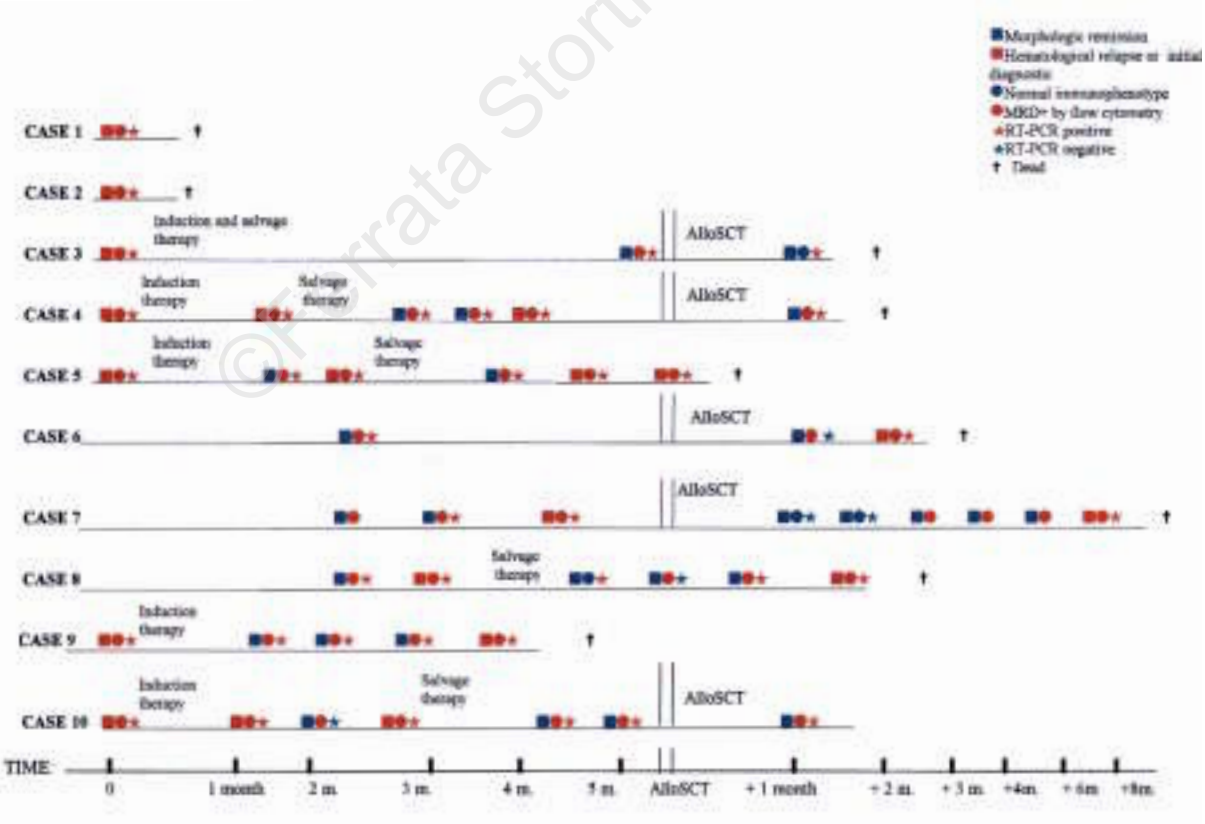


Figure 2. Clinical course and MRD results combining flow cytometry and RT-PCR in 10 Ph+ ALL patients.

all chemotherapy-treated patients. These results in conjuction with prior studies[27,28] suggest that chemotherapy cannot eradicate the neoplastic clone in Ph+ ALL. AlloSCT is the only curative treatment although there is a high frequency of relapses.[29,30] In our series of patients treated with alloSCT, we detected MRD in all the samples before this procedure. After alloSCT, all but two samples were MRD positive. Of these patients two relapsed at 3 and 4 months after the transplantation and two patients died with MRD and CR. Only one patient yielded two consecutive negative samples, at 7 and 8 weeks after transplantation. This patient suffered from acute GvHD. Residual leukemic cells were detected by FC a month later and the patient relapsed and died 9 months after alloSCT. The other 4 patients treated with alloSCT did not suffer from GvHD. The negative MRD findings in this patient with acute GvHD could have been due to a graft-versus-leukemia effect[31] which could have reduced the neoplastic clone below the detection sensitivity threshold.

We detected only a small aberrant cell population in all the immunophenotypic samples but we were not able to detect the slope of increase before the hematologic relapse. This could be explained by the high division rate of the leukemic cells in Ph+.[30,31] New methods which allow quantification of the tumor population such as quantitative PCR,[32-34] and the shortening of the interval between samples could provide insights into disease evolution.

In conclusion, our findings suggest that MRD detection in Ph+ ALL is associated with a high probability of hematologic relapse within a short time.

Our results show a correlation between FC and RT-PCR but 5 samples were positive using only one of the two techniques. Bearing this in mind, we propose the use of both methods to study MRD in Ph+ ALL, thereby minimizing the risk of false-negative results. We also believe that therapeutic strategies such as immunotherapy or tyrosine kinase inhibitors[35-37] should be investigated when a small residual leukemic cell population is detected in patients in complete remission.

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Contributions and Acknowledgments

LM was responsible for the flow cytometric analysis, interpretation of the data and writing the manuscript. OL was responsible for the molecular studies, interpretation the data and writing the manuscript. RM, SB and MB were responsible for the flow cytometric technique. JS revised the final version. JFN was responsible for the conception of the manuscript and supervised the entire study and revised the final version of the paper.

Disclosures

Conflict of interest: none

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential implications for clinical practice

- Chemotherapy cannot eradicate the neoplastic clone in Ph+ ALL patients.
- The most suitable methods for detecting MRD in these patients are flow cytometry and RT-PCR. However, both assays carry the risk of false negative results. Their use in tandem may reduce false results.
- MRD detection in Ph+ ALL is associated with a high probability of hematologic relapse within a short time.

References

1. Hoelzer D. Treatment of acute lymphoblastic leukemia. Semin Hematol 1994; 31:1-15.
2. Honda H, Oda H, Suzuki T, et al. Development of acute lymphoblastic leukemia and myeloproliferative disorder in transgenic mice expressing p210bcr/abl: a novel transgenic model for human Ph1-positive leukemias. Blood 1998; 6:2067-75.
3. Faderl S, Talpaz M, Kantarjian HM, Estrov Z. Should polymerase chain reaction analysis to detect minimal residual disease in patients with chronic myelogenous leukemia be used in clinical decision making? Blood 1999; 9:2755-9.
4. Brisco MJ, Hughes E, Neoh SH, et al. Relationship between minimal residual disease and outcome in adult acute lymphoblastic leukemia. Blood 1996; 87:5251-6.
5. Coustan-Smith E, Behm FG, Sanchez J, et al. Immunological detection of minimal residual disease in children with acute lymphoblastic leukaemia. Lancet 1998; 351:550-4.
6. Drexler HG, Matsuo Y. Guidelines for the characterization and publication of human malignant hematopoietic cell lines. Leukemia 1999; 13:835-42.
7. Drexler HG, MacLeod RA, Uphoff CC. Leukemia cell lines: in vitro models for the study of Philadelphia chromosome-positive leukemia. Leuk Res 1999; 23: 207-15.
8. Cheson BD, Cassileth PA, Head DR, et al. Report of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in acute myeloid leukemia. J Clin Oncol 1990; 8:813-9.
9. Strauss K, Hannet I, Engels S, et al. Performance evaluation of the FACSCount system: a dedicated system for clinical cellular analysis. Cytometry 1996; 26:52-9.
10. Ratei R, Sperling C, Karawajew L, et al. Immunophenotype and clinical characteristics of CD45-negative and CD45-positive childhood acute lymphoblastic leukemia. Ann Hematol 1998; 77:107-14.
11. Lucio P, Parreira A, van den Beemd MW, et al. Flow cytometric analysis of normal B cell differentiation: a frame of reference for the detection of minimal residual disease in precursor-B-ALL. Leukemia 1999; 13: 419-27.
12. Ciudad J, Orfao A, Vidriales B, et al. Immunophenotypic analysis of CD19+ precursors in normal human adult bone marrow: implications for minimal residual disease detection. Haematologica 1998; 83:1069-75.
13. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-

chloroform extraction. Anal Biochem 1987; 162:156-9.
14. Pallisgaard N, Hokland P, Riishoj C, Pederden 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.
15. Saglio G, Pane F, Gottardi E, et al. Consistent amounts of acute leukemia-associated p190BCR/ABL transcripts are expressed by chronic myelogenous leukemia patients at diagnosis. Blood 1996; 87:1075-80.
16. Radich JP, Kopecky KJ, Boldt DH, et al. Detection of BCR/ABL fusion genes in adult acute lymphoblastic leukemia by the polymerase chain reaction. Leukemia 1994; 8:1688-95.
17. Tuszynski A, Dhut S, Young BD, et al. Detection and significance of bcr-abl mRNA transcript and fusion proteins in Philadelphia-positive adult acute lymphoblastic leukemia. Leukemia 1993; 7:1504-8.
18. Foroni L, Harrison CJ, Hoffbrad AV, Potter MN. Investigation of minimal residual disease in childhood and adult acute lymphoblastic leukaemia by molecular analysis. Br J Haematol 1999; 105:7-24.
19. Campana D, Coustan-Smith E, Janossy G. The immunologic detection of minimal residual disease in acute leukemia. Blood 1990; 76:163-71.
20. Ciudad J, San Miguel JF, López-Berges MC, et al. Prognostic value of immunophenotypic detection of minimal residual disease in acute lymphoblastic leukemia. J Clin Oncol 1998; 12:3774-81.
21. Carrasco M, Muñoz L, Bellido M, et al. CD66 expression in acute leukemia. Ann Hematol 2000; 79:299-303.
22. Campana D, Coustan-Smith E. Detection of minimal residual disease in acute leukemia by flow cytometry. Cytometry 1999; 38:139-52.
23. Melo JV, Yan XH, Diamond J, Lin F, Cross NC, Goldman JM. Reverse transcription/polymerase chain reaction (RT/PCR) amplification of small numbers of transcripts: the risk in misinterpreting negative results. Leukemia 1996; 10:1217-21.
24. Jung R, Lübcke C, Wagener C, Neumaier M. Reversal of RT-PCR inhibition observed in heparinized clinical specimens. Biotechniques 1997; 23:24-8.
25. Bose S, Deininger M, Gora-Tybor J, Goldman JM, Melo JV. The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. Blood 1998; 92: 3362-7.
26. Kozubek S, Lukásová E, Ryznar L, et al. Distribution of ABL and BCR genes in cell nuclei of normal and irradiated lymphocytes. Blood 1997; 89:4537-45.
27. Mitterbauer G, Födinger M, Scherrer R, et al. PCR-monitoring of minimal residual leukaemia after conventional chemotherapy and bone marrow transplantation in BCR-ABL-positive acute lymphoblastic leukaemia. Br J Haematol 1995; 89:927-41.
28. Brisco MJ, Sykes PJ, Dolman G, et al. Effect of the Philadelphia chromosome on minimal residual disease in acute lymphoblastic leukemia. Leukemia 1997; 11:1497-500.
29. Sierra J, Radich J, Hansen JA, et al. Marrow transplants from unrelated donors for treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia. Blood 1997; 4:1410-4.
30. van Dongen JJ, Breit TM, Adriaansen HJ, Beishuizen A, Hooijkaas H. Detection of minimal residual disease in acute leukemia by immunological marker analysis and polymerase chain reaction. Leukemia 1992; 6(Suppl 1):47-59.
31. Appelbaum FR. Graft versus leukemia (GVL) in therapy of acute lymphoblastic leukemia (ALL). Leukemia 1997; 11(Suppl. 4):S15-7.
32. Mensink E, van de Locht A, Schattenberg A, et al. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. Br J Haematol 1998; 102:768-74.
33. Lion T, Izraeli S, Henn T, Gaiger A, Mor W, Gadner H. Monitoring of residual disease in chronic myelogenous leukemia by quantitative polymerase chain reaction. Leukemia 1992; 6:495-9.
34. Cross NC, Feng L, Chase A, Bungey J, Hughes TP, Goldman JM. Competitive polymerase chain reaction to estimate the number of BCR/ABL transcripts in chronic myeloid leukemia patients after bone marrow transplantation. Blood 1993; 82:1929-36.
35. Sausville EA. A BCR/ABL kinase antagonist for chronic myelogenous leukemia: a promising path for progress emerges. J Natl Cancer Inst 1999; 91:102-3.
36. Carroll M, Ohno-Jones S, Tamura S, et al. CGP 57148, a tyrosine kinase inhibitor, inhibits the growth of cells expressing BCR-ABL, TEL-ABL, and TEL-PDGFR fusion proteins. Blood 1997; 90:4947-52.
37. Mahlmann S, McLaughlin J, Afar DE, Mohr R, Kay RJ, Witte ON. Dissection of signaling pathways and cloning of new signal transducers in tyrosine kinase-induced pathways by genetic selection. Leukemia 1998; 12:1858-65.