Leukemia (2005) 19, 628–635

www.nature.com/leu

Significant reduction of the hybrid *BCR/ABL* transcripts after induction and consolidation therapy is a powerful predictor of treatment response in adult Philadelphia-positive acute lymphoblastic leukemia

F Pane[1], G Cimino[2], B Izzo[1], A Camera[3], A Vitale[2], C Quintarelli[1], M Picardi[3], G Specchia[4], M Mancini[2], A Cuneo[5], C Mecucci[6], G Martinelli[7], G Saglio[8], B Rotoli[3], F Mandelli[2], F Salvatore[1] and R Foà[2], for the GIMEMA group

[1]*CEINGE – Biotecnologie Avanzate and Dipartimento di Biochimica e Biotecnologie Mediche, University 'Federico II di Napoli', Italy;* [2]*Dipartimento di Biotecnologie Cellulari ed Ematologia, University 'La Sapienza', Rome, Italy;* [3]*Divisione di Ematologia, University 'Federico II di Napoli', Italy;* [4]*Divisione di Ematologia, University of Bari, Italy;* [5]*Dipartimento di Scienze Biomediche e Terapie Avanzate, University of Ferrara, Italy;* [6]*Dipartimento di Medicina Clinica e Sperimentale, Universiy of Perugia, Italy;* [7]*Istituto di Ematologia 'L&A Seragnoli', University of Bologna, Italy; and* [8]*Dipartimento di Scienze Biomediche ed Oncologia Umana, University of Turin, Italy*

Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) has a dismal prognosis. We prospectively evaluated minimal residual disease (MRD) by measuring *BCR/ABL* levels with a quantitative real-time PCR procedure after induction and after consolidation in 45 adults with Ph+ ALL who obtained complete hematological remission after a high-dose daunorubicin induction schedule. At diagnosis, the mean *BCR-ABL/GUS* ratio was 1.55±1.78. A total of 42 patients evaluable for outcome analysis were operationally divided into two MRD groups: good molecular responders (GMRs; *n*=28) with >2 log reduction of residual disease after induction and >3 log reduction after consolidation therapy, and poor molecular responders (PMRs; *n*=14) who, despite complete hematological remission, had a higher MRD at both time points. In GMR, the actuarial probability of relapse-free, disease-free and overall survival at two years was 38, 27 and 48%, respectively, as compared to 0, 0 and 0% in PMR (*P*=0.0035, 0.0076 and 0.0026, respectively). Salvage therapy induced a second sustained complete hematological remission in three GMR patients, but in no PMR patient. Our data indicate that, as already shown in children, adult Ph+ ALL patients have a heterogeneous sensitivity to treatment, and that early quantification of residual disease is a prognostic parameter in this disease.

Leukemia (2005) **19,** 628–635. doi:10.1038/sj.leu.2403683
Published online 3 March 2005
Keywords: Ph-ALL; minimal residual disease; prognosis; real-time PCR

Introduction

Patients with Philadelphia chromosome (Ph) constitute the largest genetically defined group among adult acute lymphoblastic leukemias (ALL), with an overall frequency of 25–30%.[1] Most importantly, the Ph chromosome is known to confer a very poor prognosis, and entails a high risk of treatment failure.[2,3] Although high-dose chemotherapy induces complete hematological remission (CHR) in approximately 70% of Ph+ ALL patients, most of them experience early relapse.[1–4] At molecular level, the Ph translocation results in the juxtaposition of the 5′ part of the *BCR* gene (chromosome 22) to the second exon of the *ABL* gene (chromosome 9), leading to the formation, on the derivative chromosome 22, of a *BCR/ABL* fusion gene, which encodes an oncogenic protein with constitutive tyrosine kinase activity. Depending on the breakpoint location, the fusion gene encodes two oncogenic proteins that differ in size: the smaller 190-kDa protein ($P190^{BCR/ABL}$) is found in 65–80% of Ph+ ALL patients, whereas the larger 210-kDa protein ($P210^{BCR/ABL}$), typical of chronic myeloid leukemia, is found in 20–35% of Ph+ ALL patients.[5] The transforming power of the two hybrid proteins has been intensively investigated. Experimental evidences indicate that $P190^{BCR/ABL}$ has a higher tyrosine kinase activity and is more efficient than $P210^{BCR/ABL}$ in stimulating the growth of pre-B cells.[6] In addition, transfection of primary mouse bone marrow (BM) cells with $P190^{BCR/ABL}$-expressing constructs, but not with those expressing $P210^{BCR/ABL}$, results in a potent induction of lymphoid expansion independent of cytokine supplementation.[7] However, when conventional treatment regimens are used, no substantial differences in clinical response have been observed between Ph+ ALL patients carrying $P190^{BCR/ABL}$ compared with those carrying $P210^{BCR/ABL}$.[1–3]

In childhood ALL, molecular monitoring of minimal residual disease (MRD) during treatment has become a major tool to assess the outcome and to evaluate the risk of impending relapse.[8,9] These studies strongly suggested that the level of MRD can be added to other widely agreed prognostic features, that is, age, leukocytosis and cytogenetics, to stratify prognostic groups in ALL patients.

In this prospective study, we show that the levels of MRD in the BM of patients with Ph+ ALL, assessed with quantitative real-time PCR (RQ-PCR) after induction and after consolidation treatment, is a powerful indicator of prognosis.

Materials and methods

Patients

Between January 1999 and October 2001, 235 adult patients aged 15–60 years with newly diagnosed ALL with the exclusion of B-ALL were consecutively enrolled, after providing informed consent, in the GIMEMA 0496 or LAL2000 protocols. Of these, 57 patients (24.3%) had the Ph and/or BCR/ABL gene and were considered for inclusion in this study. The diagnosis of ALL was based on standard hematological, morphological and immunophenotypic criteria. Both GIMEMA protocols contemplated central handling of cell samples at diagnosis and at

Correspondence: Professor F Pane, Onco-Hematology Unit – CEINGE, Università di Napoli Federico II, Via S Pansini, Naples 80131, Italy; Fax: +39 081 746 2404; E-mail: fabpane@unina.it
Supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), and COFIN, Ministero della Salute (Roma), Ministero dell'Istruzione e Ricerca Scientifica (MIUR), Regione Campania, Intas (Brussel), EurLeukemiaNet (Brussel), Biogem (Avellino).
Received 29 July 2004; accepted 10 January 2005; Published online 3 March 2005

predetermined time intervals during the clinical follow-up.[10] According to this program, molecular analyses on all patients at presentation and during follow-up were performed at the CEINGE Biotecnologie Avanzate (University of Naples Federico II) and the Department of Biomedical Sciences (University of Turin), while cytogenetic analyses were performed at the Department of Cellular Biotechnologies and Hematology (University 'La Sapienza', Rome), the Center of Medical Biotechnology, Dipartimento di Scienze Biomediche e Terapie Avanzate (University of Ferrara) and the Dipartimento di Medicina Clinica e Sperimentale (Universiy of Perugia). The diagnosis of Ph+ and/or *BCR/ABL*+ ALL was based on standard cytogenetic and/or molecular analysis at diagnosis. The patients enrolled in the study were selected from the whole cohort of patients with Ph+ ALL within the two protocols, on the basis of the following criteria: (a) achievement of CHR at the end of induction therapy and (b) availability of both postinduction and postconsolidation BM samples for molecular analysis. Patients were considered to be in CHR if, after induction treatment, they had normal peripheral blood counts (peripheral blood polymorphonucleates (PMN) $>1.5\times10^9$/l; Hb >10 g/dl; platelets $>100\times10^9$/l with no circulating blast cells) and $<5\%$ blast cells in the BM with a normal cellularity. Patients who did not achieve CHR after induction were considered resistant and excluded from this study.

Treatment protocols

All patients were treated according to the GIMEMA 0496 or LAL2000 protocols, both including Ph+ ALL patients in the high-risk group arm. Treatment schedule for the high-risk group was the same in both protocols. Induction treatment was administered for 6 weeks, and consisted of four drugs: prednisone (p.o. 60 mg/m^2 – days 1–30), vincristine (i.v. 1.4 mg/m^2, maximum dose = 2.0 mg – days 1, 8, 15, 22, 36), high-dose daunorubicin (i.v. 30 mg/m^2 – days 1→3, 22→24, 36→38, total dose 270 mg/m^2) and L-asparaginase (i.v. 6000 IU/m^2 – days 10→30). According to the protocols, all patients with a diagnosis of Ph+ and/or *BCR/ABL*+ received consolidation therapy (HAM schedule) according to the EORTC study, which includes a course of high-dose cytarabine (3-h i.v. 3000 mg/m^2/12 h – days 1, 2, 3, 4 – total eight infusions) and mitoxantrone (i.v. 10 mg/m^2 – days 3, 4, 5) followed by allogeneic or autologous stem cell transplantation (SCT), based on the availability of an HLA identical donor. Postconsolidation treatment in patients who lacked an HLA-matched donor included maintenance therapy with oral 6-mercaptopurine (90 mg/m^2/day) and i.m. methotrexate (15 mg/week) for 36 months. This therapy was stopped during periodic reinduction courses that consisted of two alternative schemes, reinduction scheme A (vincristine 1.4 mg/m^2, day 1/daunorubicin 30 mg/m^2, days 1–2/prednisone 60 mg/m^2, days 1–7) and B (vincristine 1.4 mg/m^2, day 1/cyclophosphamide 600 mg/m^2, days 1–2/prednisone 60 mg/m^2, days 1–7) given monthly during the first 6 months, then every 3 months up to 1 year, and finally every 6 months until the end of treatment. Daunorubicin was stopped when a total dose of 550 mg/m^2 was reached.

Molecular analysis

Mononuclear cells were centrally isolated in the laboratories in Rome from BM aspirates by centrifugation on a Ficoll–Hypaque gradient. Cells were washed twice in saline solution and resuspended in aliquots of 1×10^6 in 600 μl of 4 M guanidinium isothiocyanate solution (GITC). The aliquots were stored at -20°C until shipment to the other reference laboratories (Naples and Turin). An archive of patient cell pellets was also established in central laboratory to store aliquots of patient cells for further analysis. Total RNA was extracted in one of the three reference laboratories from cells cryopreserved in GITC using ion exchange chromatography on minicolumns (GeneElute, Total RNA Purification kit, Sigma, St Louis, MO, USA) according to the manufacturer's directions. The quality of RNA was assessed on ethidium bromide-stained 1% agarose gel containing 2.2 mol/l formaldehyde. RT-PCR specific for the *BCR-ABL* transcripts encoding either the P190$^{BCR/ABL}$ or the P210$^{BCR/ABL}$ proteins was performed on BM samples at baseline as part of a systematic molecular screening for fusion transcripts (*BCR/ABL*, *E2A/PBX1* and *MLL-AF4*). This diagnostic analysis was carried out with the standardized BIOMED-1 protocol.[11]

Quantitative real- time PCR analysis of minimal residual disease

MRD was quantified with a novel method based on an RQ-PCR procedure standardized within a Europe Union (EU) concerted action program.[12] The analysis was carried out in all patients at disease presentation and after completion of both induction and consolidation therapy upon recovery of blood counts.

The method independently measures in each sample by PCR the copy number of mRNAs encoding the *BCR/ABL* protein and, simultaneously, of a control gene, to verify sample-to-sample RNA quality variations. Of the three control genes used in the PCR standardization program, that is, abelson (*ABL*), beta-2microglobulin (*B2m*) and beta-glucuronidase (*GUS*), *GUS* was chosen for this study because its level of expression and its stability in hemopoietic cells were similar to those of *BCR/ABL*, and because PCR primers used for *ABL* would amplify both normal *ABL* and fusion *BCR/ABL* transcripts (total *ABL*).[12]

For each amplification run, *BCR/ABL* and *GUS* standard curves were independently generated by assaying, in parallel with the samples, 1:10 serial dilutions (from 10^6 to 10^2 copies, each in triplicate) of plasmid DNA calibrators containing the target sequences diluted in a solution of *Escherichia coli* RNA (20 ng/μl). The copy numbers of *BCR/ABL* and *GUS* transcripts were derived by the interpolation of cycle threshold (Ct) values (number of PCR cycles necessary to achieve a target-specific fluorescence detection threshold) to the appropriate standard curve, and the result was expressed for each sample as a ratio of *BCR/ABL* mRNA copies to *GUS* mRNA.[12] All amplification reactions were carried out in triplicate and the mean Ct values were used to interpolate standard curves and to calculate the transcript copy number. Reaction conditions, primers and probe sequences for RQ-PCR of *BCR/ABL* and *GUS* were designed, tested and standardized within the EU concerted action program.[12] Dilutions of plasmid containing the PCR target sequences of *BCR-ABL* and *GUS*, used to generate the standard curves of the assays, were from IPSOGEN Inc. (Marseilles, France).

Sample results and all analytical series were checked as follows: (i) RNA samples that repeatedly gave *GUS* Ct values higher than 29.2 (corresponding to 1000 molecules) were operationally considered degraded and eliminated from further evaluation. In these cases, a further aliquot of BM cells from the same patient was withdrawn from the central archive for reanalysis. Thus, in all samples tested, the dynamic range of MRD detectability of the assay technique was at least 3 logs.

(ii) In the case of a *BCR/ABL* Ct above the intercept value of the relative standard curve of the run (Ct corresponding to one copy), the amount of the transcript was considered below the detection limit of the technique. (iii) When the slope of a standard curve was not within the mean ± 2 s.d. obtained during the standardization program of the method (3.46 ± 0.1 and 3.51 ± 0.17 for *BCR/ABL* and *GUS* mRNAs, respectively), all the samples of the series were reassayed.

Statistical analysis

The primary prognostic endpoints of this study were: overall survival (OS) and disease-free survival (DFS), cumulative incidence of relapse and duration of the first CHR (ie event-free survival (EFS)). Survival was defined as the time from diagnosis to death or date of the last follow-up. When calculating DFS, both relapse and death in CHR due to stem cell transplant-related mortality (nonrelapse mortality (NRM)) were counted as 'events'. The cumulative relapse rate was calculated using the same type of analysis that was used for DFS, except for patients who died in CHR, who were censored at the time of death. EFS was calculated from the time of achieving CHR to relapse or date of last follow-up. The probabilities of OS, relapse, RFS and DFS were estimated using the Kaplan–Meier method. The log-rank test was used to evaluate the prognostic impact of MRD assay. Median follow-up time was estimated by reversing the codes for the censoring indicator in a Kaplan–Meier analysis.

Results

Patients

Out of the 57 patients affected by Ph+ ALL and enrolled in the 0496 or LAL 2000 GIMEMA treatment protocols, 45 had achieved CHR after induction therapy, and were included in the prospective monitoring study of MRD. All patients expressed B-cell antigens (CD19 and cytoplasmic CD22). A total of 36 of them expressed membrane CD10, and were thus classified as common-ALL; the remaining nine were diagnosed as pre-B ALL based on cytoplasmic IgM. Other clinical and biological features of the patients are listed in Table 1. The median age of the patients at diagnosis was 42 years, and none was older than 58 years. As frequently observed in Ph+ ALL, median leukocyte counts were high, and 10 patients (23%) had a WBC of 50×10^9/l or more. Median follow-up was 440 days; in eight patients, it exceeded 2 years; 15 (34%) patients were alive at the end of follow-up. Three patients were lost to clinical observation with a clinical follow-up below 6 months and were excluded from further evaluation. Median survival probability of the whole cohort of patients was 445 days; relapse was the most common cause of treatment failure, occurring in 27 of the 42 evaluable patients (64%). All but one relapse occurred in BM; in two cases, BM relapse was associated with a meningeal localization of the disease. At univariate analysis, young age and low leukocyte count were associated with a slight but not statistically significant trend toward longer survival. Overall, 20 patients underwent SCT, in 13 cases from an HLA-identical sibling, in four cases from an HLA-matched unrelated donor and in three cases from a mismatched (haploidentical) donor. A total of 27 patients died: 19 due to disease progression and eight from SCT-related complications (mostly pneumonia or multiorgan failure).

Table 1 Characteristics of patients

No. of patients	45
M/F	24/21
Age (years), median (range)	42 (16–58)
$\geqslant 45$	13
WBC ($\times 10^9$/l), median (range)	30 (0.8–205)
$\geqslant 50$	10
RT-PCR	
$P190^{BCR/ABL}$	36
$P210^{BCR/ABL}$	6
$P190^{BCR/ABL}$+$P210^{BCR/ABL}$	3
Follow-up (days), median (range)	430 (150–1300)
$\geqslant 730$	17%
Alive after last follow-up	34%
No. of allo-SCT	20
Identical sibling donor	13/20
Haplo	3/20
MUD	4/20
TRM	8/20

MUD: matched unrelated donor SCT; haplo: SCT from haploidentical donor; TRM: transplant-related mortality.

Analysis of MRD after induction and consolidation treatment

Overall, RQ-PCR method used for the MRD analysis showed a high degree of reliability: only 11 out 135 (8.1%) BM samples were reassayed during the study – in seven cases, reanalysis was due to RNA degradation and the RQ-PCR assays were successfully repeated on a different aliquot of archival cell pellet from the same patient, whereas in the remaining four cases, repetition was due to a slope of the *GUS* standard curve outside the range established during the standardization program of the method (see Materials and methods).

The transcript encoding $P190^{BCR/ABL}$ was found in 34 of 42 evaluable patients (81%); the transcript encoding $P210^{BCR/ABL}$ was present in five. The remaining three patients expressed both types of transcripts at the diagnostic qualitative PCR, most likely due to alternative splicing of a primary transcript with a $P210^{BCR/ABL}$ type of junction.[13] Indeed, these patients had higher amounts of $P210^{BCR/ABL}$- than $P190^{BCR/ABL}$-encoding mRNA at RQ-PCR. Therefore, these three patients were operationally considered 'P210 positive', and the sum of the copy numbers of the two types of *BCR/ABL* mRNA was used to calculate MRD.

At diagnosis, the median ratio *BCR/ABL-GUS* of the whole cohort of patients was 1.15 (range 0.23–10.28); there was no significant difference between the levels in patients with the P210- and patients with the P190-encoding *BCR/ABL* gene (Table 2). MRD levels were quantitatively assessed at two time points: after completion of induction treatment and after completion of consolidation treatment. On both occasions, BM was sampled when the WBC was $>500 \times 10^9$/l and the platelet count was $>20\,000 \times 10^9$/l. MRD was assessed at a median of 71 and 124 days postinduction and postconsolidation, respectively. When patients were stratified according to *BCR/ABL* gene junction ($P190^{BCR/ABL}$ *vs* $P210^{BCR/ABL}$), there were no differences in MRD level at either time points (Table 2). This indicates that the two groups of patients had a similar initial chemosensitivity. However, the MRD levels of patients were

Table 2 *BCR/ABL-GUS* ratios at diagnosis and during follow-up

	Diagnosis	*Post-induction*	*Post-consolidation*
$P190^{BCR/ABL}$ ($n=36$)			
Mean ± s.d.	1.59 ± 1.82	0.032 ± 0.059	0.0094 ± 0.0243
Median (min–max)	1.23 (0.23–10.27)	0.005 (0.00002–0.28570)	0.00011 (0.00001–0.0769)
$P210^{BCR/ABL}$ ($n=9$)			
Mean ± s.d.	1.36 ± 1.20	0.017 ± 0.040	0.00016 ± 0.00022
Median (min–max)	1.06 (0.30–3.92)	0.0014 (0.00001–0.118)	0.00002 (0.00001–0.0005)

highly variable at both time points, suggesting heterogeneity of blast clearance among patients (Table 2). Therefore, we stratified the whole cohort of patients into two MRD groups on the basis of the reduction of *BCR/ABL* transcript levels at these two time points. Given the high variability of *BCR/ABL* expression at diagnosis, to express MRD levels, patient transcript ratios at completion of both induction and consolidation were converted in a logarithmic (base 10) scale of reduction from individual base line ratio *BCR/ABL-GUS* assayed at diagnosis before the start of treatment. Patients with 2 or more logs of MRD reduction postinduction, and 3 or more logs of MRD reduction post-consolidation, were considered 'good molecular responders' (GMRs). The remaining patients, that is, those who had MRD reductions less or equal to 2 logs postinduction, and 3 logs postconsolidation, were considered 'poor molecular responders' (PMRs). There was postinduction/postconsolidation concordance of the MRD classification in all patients (Figure 1). On the basis of MRD levels, 28 were GMRs and 14 PMRs. The eight patients expressing the $P210^{BCR/ABL}$ transcript were evenly distributed between the two MRD groups: six were classified as GMR and two as PMR.

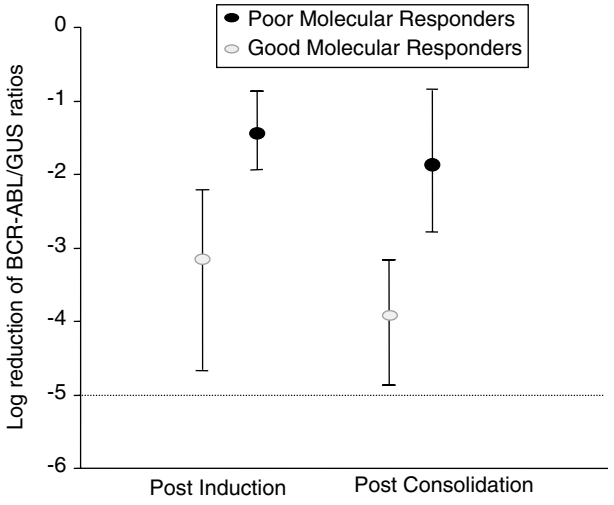

Figure 1 MRD in Ph+ ALL patients. Patients were stratified *a posteriori* into two MRD groups based on *BCR/ABL-GUS* ratios after treatment. MRD levels are expressed as log reduction from the individual baseline levels of *BCR/ABL-GUS* ratio at diagnosis. GMRs = MRD levels <0.01 at the postinduction time point, and <0.001 at the postconsolidation time point; poor molecular responders = MRD levels $\geqslant 0.01$ at postinduction, and $\geqslant 0.001$ at post-consolidation. Median and 95% CI are given for the two groups of patients at each time point. The dotted line indicates the lower limit of sensitivity of the RQ-PCR technique.

MRD-based prognostic stratification

To assess the prognostic value of the MRD classification, the clinical follow-up was recorded for all patients. GMRs had a significantly longer survival (Kaplan and Meyer probability) compared with PMRs (Figure 2a). Overall, 12/14 PMRs (86%) and 15/28 GMRs (54%) had died by the end of the study. The probability of survival at 2 years was 48% (CI 29–65%) for GMRs and 0% (CI 0–0%) for PMRs (Table 3). The longer survival probability of GMRs is due to a lower probability of relapse (Figure 2b) and to the longer probability of CR duration (Figure 2c). Indeed, 12 PMRs relapsed, none maintaining remission at 2 years (Table 3). By contrast, 15/28 GMRs relapsed (54%), and GMRs had a 38% (CI 24–62%) probability of remaining in first remission at 2 years (Table 3). Moreover, 6/15 GMRs (and none of the PMRs) who relapsed were induced into a second CHR by salvage treatment, which included SCT in three cases; a sustained CHR was maintained by all three (one treated with an unrelated donor SCT) up to the end of the study.

The differences in prognosis between the two MRD groups are not attributable to a different percentage of patients undergoing SCT (Table 4). Indeed, about half the patients in the two groups underwent SCT within a median of 10 and 9 months for GMRs and PMRs, respectively. Mortality due to transplant complication was rather high in both groups, accounting for five and three deaths in GMRs and PMRs, respectively. Even when transplant complication mortality was computed as an event in the Kaplan–Meier estimation of DFS, GMRs maintained a better survival than PMRs ($P=0.0076$; Table 3).

Median age and baseline WBC, the most common prognostic indicators in ALL, did not differ between the two MRD groups of patients (40 *vs* 45 years and 27.8 *vs* 27.8×10^9/l, in GMRs and PMRs, respectively), which indicates that MRD level is an independent prognostic index in Ph+ ALL.

Discussion

In adults, as well as in children with ALL, the Ph+ subgroup is associated with the worst prognosis due both to a reduced rate of initial response to treatment and to a high risk of early relapse, and long-term survival rates range from 35 to 40% in children to less than 20% in adults.[2,3,14] This prospective study shows that quantitative MRD assessment was a prognostic indictor in 45 adult patients affected by Ph+ ALL who underwent homogeneous treatment programs, the GIMEMA protocols 0496 and LAL2000.[10] Refractoriness to induction treatment is an obvious parameter of dismal prognosis in Ph+ ALL, but early relapses can also occur in patients who have a CHR to initial treatment. Therefore, we enrolled in our study only Ph+ ALL patients who had a CHR after induction treatment and who would benefit

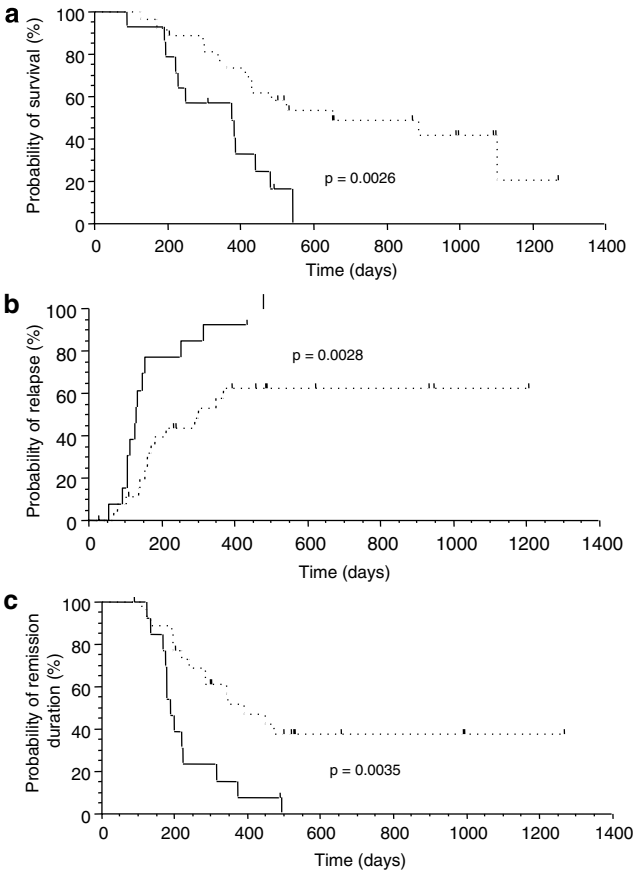

Figure 2 Outcome of Ph+ ALL patients according to MRD stratification. (a) OS probability. (b) Cumulative incidence of relapse. (c) Remission duration probability (EFS). Censored events are indicated in the two groups on the curves. PMRs, poor molecular responders; GMRs, good molecular responders.

Table 3 Outcome at 2 years

	Good molecular responders (n = 27)	*Poor molecular responders (n = 14)*
OS (%), median (95% CI)	47 (28–65)	0 (0–0)
Relapse rate (%), median (95% CI)	61 (40–79)	100 (100–100)
EFS (%), median (95% CI)	44 (26–66)	0 (0–0)
DFS (%), median (95% CI)	29 (11–47)	0 (0–0)

OS, overall survival; EFS, event-free survival; DFS, disease-free survival.

Table 4 Outcome of SCT in the two groups of patients

	Good molecular responders (n = 14)	*Poor molecular responders (n = 6)*
SCT no.		
Identical sib	10	3
MUD	2	2
Haploidentical	2	1
Time to SCT (months)		
Median	10	9
Range	5–17	5–18
Pre-SCT status		
CR1 or CR2	14	4
Relapse	0	2
Transplant-related mortality	5 (2 MUD, 1 haplo)	3 (1 MUD, 1 haplo)

MUD: matched unrelated donor SCT; Haplo: SCT from haploidentical donor; CR: complete remission.

from the identification of a new predictor of treatment outcome. Here, we applied a rapid, accurate and sensitive real-time RT-PCR assay of the *BCR/ABL* transcripts that was developed by us within a European network of experienced centers,[12] to study the MRD kinetics during the early phases of treatment in those patients who showed CHR after induction treatment. Although MRD values assayed by the RQ-PCR may be expressed as absolute ratios of fusion gene to a control gene mRNA levels, here we express the MRD data as individual log reduction of *BCR/ABL-GUS* ratios to compensate the high variability of *BCR-ABL* levels in ALL leukemic blasts at diagnosis,[12] and the lab-to-lab variability. The use of this type of data presentation might lead to loss of some clinical information related to variability of *BCR-ABL* expression at diagnosis and to possible discrepancies between classification of individual patient responses to treatment using thresholds based on absolute value of *BCR-ABL/GUS* ratios and that based on relative log reductions. A multicentric prospective study aimed at a formal comparison between these two types of MRD calculations is still lacking;[15] however, the use in our patients of two absolute values of *BCR/ABL-GUS* ratio (0.01 and 0.001 after induction and after consolidation treatment, respectively) as cutoff for MRD-based stratification did not change significantly the prognostic value of this parameter (data not shown).

In children affected by ALL, MRD monitoring and the quality of early response to treatment are independent prognostic factors of outcome: patients who rapidly clear MRD clearance have a significantly better outcome than patients who have a delayed or insufficient response.[16–19] We provide evidence also in the adult setting that Ph+ ALL is a heterogeneous disease with regard to response to treatment. Among the Ph+ ALL patients who achieved CHR, we identified two subgroups on the basis of MRD levels, which differed in clearance rates of Ph+ cells and sensitivity to chemotherapy. Specifically, patients who had an MRD reduction of less than 2 logs after induction treatment had no chance of being alive and in remission at 2 years, and their median remission duration was 6 months. Overall, these PMR patients had a low sensitivity to chemotherapy; indeed, most of them had only slight additional MRD decreases after the administration of cytosine arabinoside plus mitoxantrone used as consolidation regimen, and the MRD level never decreased to below 0.001 (3 log reduction compared to the diagnosis values) at the second time point. Thus, such patients are candidates for experimental treatments as soon as possible after remission induction, because in no case did salvage treatment including SCT result in long survival. Differently, GMRs, that is, patients with an MRD reduction of more than 2 logs after induction treatment, included all patients highly sensitive to chemotherapy and to cytarabine-based treatment of the HAM protocol. All these patients had an MRD reduction of 1 log after subsequent consolidation

treatment with the HAM schedule. The rapid and effective MRD clearance observed in this subgroup correlates significantly with outcome: the Ph+ ALL patients with the best outcome were GMRs. In this subgroup, OS and EFS probability at 2 years was 48 and 38%, respectively.

It is generally thought that the only curative option in adults with Ph+ ALL is SCT, even though evidence for this comes from small retrospective studies.[20,21] In our study, about half the patients in each MRD subgroup underwent SCT. Therefore, SCT given as therapy intensification after consolidation did not bias the clinical outcome of the two groups of patients.

In a multicentric study of the GET-LALA group of 154 Ph+ ALL patients, high leukocyte count was recognized as an adverse prognostic parameter for CHR at 3 months, but not for OS.[3] On the other hand, in the same 154 patients, advanced age at baseline was a prognostic factor that inversely correlates with OS but not with CHR achievement.[3] Neither high leukocyte count nor advanced age was significantly associated, in our patients, to an adverse outcome; thus, the stratification based upon the MRD levels appears to be the principal prognostic parameter. Importantly, in our study, we excluded from the evaluation those patients who did not reach CHR after initial treatment and who, in the GET-LALA study, tended to have the oldest age and the highest leukocyte counts, and this may account for the discrepancy.

It is not clear whether the different responses to treatment identified by measuring MRD by the RQ-PCR technique in our Ph+ ALL patients reflect intrinsic biological characteristics of the Ph+ clone or whether they are due to other host features. However, Ph+ cells from ALL patients reacted differently *in vitro* to three widely used therapeutic agents,[22] and the gene expression pattern of the Ph+ ALL was recently found to be heterogeneous, unlike other ALL genetic subsets gene expression pattern.[23] Interestingly, a multicenter study of children with Ph+ ALL showed that patients can be stratified into a high or low risk of relapse on the basis of the clinical response to pretreatment with 7-day prednisone monotherapy.[24] Heterogeneity of Ph+ ALL children was confirmed by a large cooperative study in which prednisone pretreatment, age and the leukocyte count proved to be independent predictors of relapse risk.[14] More recently, in the same subset of childhood ALL patients, RT-PCR monitoring identified different risk groups of patients, thereby indicating a high heterogeneity in blast clearance in childhood Ph+ ALL.[25] These findings provided additional support to the results of our study.

In all previous studies of MRD monitoring of Ph+ ALL patients, qualitative PCR was used to measure *BCR/ABL* mRNA, and it was found that even intensive chemotherapy does not eradicate *BCR/ABL*-positive cells in a large proportion of patients.[3,26–28] In 36 Ph+ ALL adult patients treated by SCT, multivariate analysis showed that qualitative PCR assay of MRD was the best prognostic index for continuous complete remission.[29] Moreover, the type of *BCR/ABL* transcript was an important predictor of post-SCT relapse. In fact, patients with $P190^{BCR/ABL}$+ MRD had a relapse risk 11.2 times higher than patients with $P210^{BCR/ABL}$+ MRD.[29] However, the latter finding was not confirmed in a subsequent study[30] or by our observation that MRD levels did not differ between $P190^{BCR/ABL}$- and $P210^{BCR/ABL}$-positive patients. Competitive quantitative PCR has been used to monitor the dynamics of residual leukemic cells in 16 Ph+ ALL patients after conventional chemotherapy and SCT.[31] Although the data were not conclusive, it emerged that chemotherapy generally results in a limited, variable decrease (2 or 3 logs) in the number of transcripts in the BM of patients, *vs* decrease >4 logs after auto- and allo-SCT. The prognostic value of MRD has been documented in 154 Ph+ adult patients. Qualitative PCR assessment of MRD in 63 of those Ph+ adult ALL patient cases after two courses of treatment showed that MRD is an efficient prognostic tool.[3] The foregoing data support our observations that quantitative assessment of residual leukemic cells in Ph+ ALL patients is a better diagnostic tool for clinical decision-making than qualitative assessment.

Conclusion

In conclusion, RQ-PCR quantification of MRD in the initial phases of Ph+ ALL treatment allows for the prognostic stratification of patients. Indeed, early kinetic assessment of Ph+ cell reduction may help to identify patients who have no chance of being cured by current chemotherapy and who are thus candidates for prompt SCT from the best available donor and for other experimental therapeutic strategies. On the other hand, RQ-PCR quantification of MRD reveals patients who will respond to treatment with a rapid clearance of Ph+ cells and who will benefit from intensive initial chemotherapy. Imatinib, the novel therapeutic agent specifically designed to target tyrosine kinase activity of the *BCR/ABL* oncoprotein, is very effective against chronic myeloid leukemia.[32,33] In Ph+ ALL, imatinib given as single agent induces CHR in about 60% of relapsed or refractory patients. Unfortunately, clinical resistance develops rapidly and the median time-to-progression in these patients is only 2.5 months.[34]

Therefore, combining imatinib to polychemotherapy protocols seems a promising therapeutic approach to Ph+ ALL and is currently under evaluation in previously untreated patients.[35] It would be interesting to verify whether the inclusion of imatinib into polychemotherapy schedules modifies the overall response to the treatment of the two MRD groups of Ph+ ALL patients. Should this not be the case, MRD assessment would identify the Ph+ ALL patients who would benefit most from the imatinib inclusion into the treatment schemes thereby sparing them from the potentially life-threatening complications frequently associated with SCT from allogeneic donors.

References

1 Kantarjian HM, Talpaz M, Dhingra K, Estey E, Keating MJ, Ku S *et al.* Significance of the P210 *vs* P190 molecular abnormalities in adults with Philadelphia chromosome-positive acute leukemia. *Blood* 1991; **78**: 2411–2418.
2 Gleissner B, Gokbuget N, Bartram CR, Janssen B, Rieder H, Janssen JW *et al.* Leading prognostic relevance of the BCR-ABL translocation in adult acute B-lineage lymphoblastic leukemia: a prospective study of the German Multicenter Trial Group and confirmed polymerase chain reaction analysis. *Blood* 2002; **99**: 1536–1543.
3 Dombret H, Gabert J, Boiron JM, Rigal-Huguet F, Blaise D, Thomas X *et al.* Outcome of treatment in adults with Philadelphia chromosome-positive acute lymphoblastic leukemia – results of the prospective multicenter LALA-94 trial. *Blood* 2002; **100**: 2357–2366.
4 Annino L, Ferrari A, Cedrone M, Giona F, Lo Coco F, Meloni G *et al.* Adult Philadelphia-chromosome-positive acute lymphoblastic leukemia: experience of treatments during a ten-year period. *Leukemia* 1994; **8**: 664–667.
5 Pane F, Intrieri M, Quintarelli C, Izzo B, Muccioli GC, Salvatore F. BCR/ABL genes and leukemic phenotype: from molecular mechanisms to clinical correlations. *Oncogene* 2002; **21**: 8652–8667.
6 Li S, Ilaria Jr RL, Million RP, Daley GQ, Van Etten RA. The P190, P210, and P230 forms of the BCR/ABL oncogene induce a similar chronic myeloid leukemia-like syndrome in mice but have

different lymphoid leukemogenic activity. *J Exp Med* 1999; **189**: 1399–1412.
7 Quackenbush RC, Reuther GW, Miller JP, Courtney KD, Pear WS, Pendergast AM. Analysis of the biologic properties of p230 Bcr-Abl reveals unique and overlapping properties with the oncogenic p185 and p210 Bcr-Abl tyrosine kinases. *Blood* 2000; **95**: 2913–2921.
8 Brisco MJ, Condon J, Hughes E, Neoh SH, Sykes PJ, Seshadri R *et al.* Outcome prediction in childhood acute lymphoblastic leukaemia by molecular quantification of residual disease at the end of induction. *Lancet* 1994; **343**: 196–200.
9 Gruhn B, Hongeng S, Yi H, Hancock ML, Rubnitz JE, Neale GA *et al.* Minimal residual disease after intensive induction therapy in childhood acute lymphoblastic leukemia predicts outcome. *Leukemia* 1998; **12**: 675–681.
10 Cimino G, Elia L, Mancini M, Annino L, Anaclerico B, Fazi P *et al.* Clinico-biologic features and treatment outcome of adult pro-B-ALL patients enrolled in the GIMEMA 0496 study: absence of the ALL1/AF4 and of the BCR/ABL fusion genes correlates with a significantly better clinical outcome. *Blood* 2003; **102**: 2014–2020.
11 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: investigation of minimal residual disease in acute leukemia. *Leukemia* 1999; **13**: 1901–1928.
12 Gabert J, Beillard E, van der Velden VH, 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.
13 Saglio G, Pane F, Gottardi E, Frigeri F, Buonaiuto MR, Guerrasio A *et al.* Consistent amounts of acute leukemia-associated P190BCR/ABL transcripts are expressed by chronic myelogenous leukemia patients at diagnosis. *Blood* 1996; **87**: 1075–1080.
14 Arico M, Valsecchi MG, Camitta B, Schrappe M, Chessells J, Baruchel A *et al.* Outcome of treatment in children with Philadelphia chromosome-positive acute lymphoblastic leukemia. *N Engl J Med* 2000; **342**: 998–1006.
15 Hochhaus A. A further milestone towards comprehensive standardization of quantitative RT-PCR protocols for leukemic fusion gene transcripts has been reached. *Leukemia* 2003; **17**: 2383–2384.
16 van Dongen JJ, Seriu T, Panzer-Grumayer ER, Biondi A, Pongers-Willemse MJ, Corral L *et al.* Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *Lancet* 1998; **352**: 1731–1738.
17 Cave H, van der Werff ten Bosch J, Suciu S, Guidal C, Waterkeyn C, Otten J *et al.* Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer – Childhood Leukemia Cooperative Group. *N Engl J Med* 1998; **339**: 591–598.
18 Biondi A, Valsecchi MG, Seriu T, D'Aniello E, Willemse MJ, Fasching K *et al.* Molecular detection of minimal residual disease is a strong predictive factor of relapse in childhood B-lineage acute lymphoblastic leukemia with medium risk features. A case control study of the International BFM study group. *Leukemia* 2000; **14**: 1939–1943.
19 Panzer-Grumayer ER, Schneider M, Panzer S, Fasching K, Gadner H. Rapid molecular response during early induction chemotherapy predicts a good outcome in childhood acute lymphoblastic leukemia. *Blood* 2000; **95**: 790–794.
20 Chao NJ, Blume KG, Forman SJ, Snyder DS. Long-term follow-up of allogeneic bone marrow recipients for Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* 1995; **85**: 3353–3354.
21 Thomas X, Thiebaut A, Olteanu N, Danaila C, Charrin C, Archimbaud E *et al.* Philadelphia chromosome positive adult acute lymphoblastic leukemia: characteristics, prognostic factors and treatment outcome. *Hematol Cell Ther* 1998; **40**: 119–128.
22 Hongo T, Yamada S, Yajima S, Watanabe C, Fujii Y, Kawasaki H *et al.* Biological characteristics and prognostic value of *in vitro* three-drug resistance to prednisolone, L-asparaginase, and vincristine in childhood acute lymphoblastic leukemia. *Int J Hematol* 1999; **70**: 268–277.
23 Fine BM, Stanulla M, Schrappe M, Ho M, Viehmann S, Harbott J *et al.* Gene expression patterns associated with recurrent chromosomal translocations in acute lymphoblastic leukemia. *Blood* 2004; **103**: 1043–1049.
24 Schrappe M, Arico M, Harbott J, Biondi A, Zimmermann M, Conter V *et al.* Philadelphia chromosome-positive (Ph+) childhood acute lymphoblastic leukemia: good initial steroid response allows early prediction of a favorable treatment outcome. *Blood* 1998; **92**: 2730–2741.
25 Cazzaniga G, Lanciotti M, Rossi V, Di Martino D, Arico M, Valsecchi MG *et al.* Prospective molecular monitoring of BCR/ABL transcript in children with Ph+ acute lymphoblastic leukaemia unravels differences in treatment response. *Br J Haematol* 2002; **119**: 445–453.
26 Radich JP, Kopecky KJ, Boldt DH, Head D, Slovak ML, Babu R *et al.* Detection of BCR-ABL fusion genes in adult acute lymphoblastic leukemia by the polymerase chain reaction. *Leukemia* 1994; **8**: 1688–1695.
27 Brisco MJ, Sykes PJ, Dolman G, Neoh SH, Hughes E, Peng LM. Effect of the Philadelphia chromosome on minimal residual disease in acute lymphoblastic leukemia. *Leukemia* 1997; **11**: 1497–1500.
28 Annino L, Ferrari A, Lamanda M, Mandelli F, Lo Coco F, Pane F. PCR-detectable transcripts in long-term remission of P190(BCR/ABL)-positive acute lymphoblastic leukemia. *Blood* 2000; **95**: 4018–4019.
29 Radich J, Gehly G, Lee A, Avery R, Bryant E, Edmands S *et al.* Detection of bcr-abl transcripts in Philadelphia chromosome-positive acute lymphoblastic leukemia after marrow transplantation. *Blood* 1997; **89**: 2602–2609.
30 Kroger N, Kruger W, Wacker-Backhaus G, Hegewisch-Becker S, Stockschlader M, Fuchs N *et al.* Intensified conditioning regimen in bone marrow transplantation for Philadelphia chromosome-positive acute lymphoblastic leukemia. *Bone Marrow Transplant* 1998; **22**: 1029–1033.
31 Mitterbauer G, Nemeth P, Wacha S, Cross NC, Schwarzinger I, Jaeger U *et al.* Quantification of minimal residual disease in patients with BCR-ABL-positive acute lymphoblastic leukaemia using quantitative competitive polymerase chain reaction. *Br J Haematol* 1999; **106**: 634–643.
32 O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F *et al.* Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 2003; **348**: 994–1004.
33 Rosti G, Martinelli G, Bassi S, Amabile M, Trabacchi E, Giannini B *et al.* Molecular response to imatinib in late chronic-phase chronic myeloid leukemia. *Blood* 2004; **103**: 2284–2290.
34 Ottmann OG, Druker BJ, Sawyers CL, Goldman JM, Reiffers J, Silver RT *et al.* A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. *Blood* 2002; **100**: 1965–1971.
35 Thomas DA, Faderl S, Cortes J, O'Brien S, Giles FJ, Kornblau SM *et al.* Treatment of Philadelphia chromosome-positive acute lymphocytic leukemia with hyper-CVAD and imatinib mesylate. *Blood* 2004; **103**: 4396–4407.

Appendix A1

Participating centers and physicians in the GIMEMA study were Alessandro Levis, Ospedale SS Antonio e Biagio, Alessandria; Pietro Leoni, Nuovo Ospedale Torrette, Ancona; Ettore Volpe, AOSG Moscati, Avellino; Umberto Tirelli, CRO Unita' Operativa Leucemia, Aviano; Vincenzo Liso, Ematologia-Universita' degli Studi, Bari; Michele Baccarani, Istituto di Ematologia L&A Seragnoli, Bologna; Giovanni Quarta, Azienda Ospedaliera 'A: Di Summa', Brindisi; Rosario Giustolisi, Ospedale Ferrarotto, Catania; Antonio Peta, Ospedale A Pugliese, Catanzaro; Andrea Gallamini, Ospedale S Croce, Cuneo; Gianluigi Castoldi, Arcispedale S Anna, Ferrara; Alberto Bosi, Ematologia-Policlinico di Careggi, Florence; Ruggerro Mozzana, Ospedale S

Antonio Abate, Gallarate, Gino Santini, Ospedale S Martino, Genoa; Franco Patrone, Ematologia-Universita' degli Studi, Genoa; Angelo De Blasio, Ospedale S Maria Goretti, Latina; Giulio Nalli, Ospedale Maggiore, Lodi; Marco Bregni, Istututo di Ricerca e Cura a Carattere Scientifico San Raffaele, Milan; Giuseppe Torelli, Universita' degli Studi, Modena; Marco Montanaro, Ospedale Civile, Montefiascone; Eustachio Miraglia, Ospedale S Giovanni Bosco, Napoli; Felicetto Ferrara, Ospedale A Cardarelli, Napoli; Bruno Rotoli, Universita' Federico II, Napoli;Vincenzo Mettiver, Divisione TERE-Ospedale A Carderelli, Napoli; Enrica Morra, Ospedale Niguarda 'Ca' Grande', Milan; Giancarlo Avanzi, Ospedale Maggiore della Carita', Novara; Attilio Gabbas, Ospedale S Francesco, Nuoro; Giuseppe Saglio, AOS Luigi Gonzaga, Orbassano-Turin; Salvatore Mirto, Ospedale Cervello, Palermo; Guglielmo Mariani, Universita' degli Studi, Palermo; Pietro Citarella, Universita' degli Studi, Palermo; Edoardo Ascari, IRCCS S Matteo, Pavia; Massimo Martelli, Policlinico Monteluce; Perugia, Giuseppe Visani, Ospedale S Salvatore, Pesaro; Giuseppe Fioritoni, Ospedale Civile, Pescara; Francesco Ricciuti, Ospedale S Carlo, Potenza; Francesco Nobile, AO Bianchi-Melacrino-Morelli, Reggio Calabria; Luigi Gugliotta, Arcispedale S Maria Nuova, Reggio Emilia; Franco Mandelli, Universita' 'La Sapienza', Rome; Giuseppe Leone, Universita' Cattolica del Sacro Cuore, Rome; Sergio Amadori, Ospedale S Eugenio, Rome; A Michele Carella, Ospedale Casa Sollievo Della Sofferenza, San Giovanni Rotondo; Maurizio Longinotti, Universita' degli Studi, Sassari; Francesco Lauria, AOA Sclavo, Siena; Dr Patrizio Mazza, Ospedale SS Annunziata, Taranto; Mario Boccadoro, Ospedale S Giovanni, Turin; Eugenio Gallo, Opsedale 'le Molinette', Turin and Giovanni Pizzolo, Policlinico GB Rossi, Verona; Italy.