Detection of BCR/ABL Transcripts By Reverse Transcriptase Polimerase Chain Reaction in Pediatric Acute Lymphoblastic Leukemia: Incidence and Clinical Features

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ABSTRACT

BCR/ABL expression, which is the molecular equivalent of the Philadelphia chromosome, is an independent poor risk factor in acute lymphoblastic leukemia (ALL). We used a two-step (nested) reverse transcriptase polimerase chain reaction (RT-PCR) assay to examine BCR/ABL expression in the diagnostic bone marrow specimen of children with ALL, prospectively. Among 75 de novo ALL patients, 4 (%5.3) were found to be BCR/ABL-positive, whereas 4 of 17 relapsed patients (23.5%) were positive.This preliminary study in Turkish children showed an incidence similar to reports from Europe and the U.S.A. More intensive chemotherapies and allogeneic bone marrow transplantations (BMT) during the first remission were planned if a donor was available. Out of 8 BCR/ABL-positive patients, complete remission (CR) was achieved in 7 patients and partial remission (PR) was achieved in 1 patient. Three patients underwent allogeneic BMT during the first CR and 1 under went autologous BMT during the first PR. The Kaplan-Meier estimate of event-free survival (EFS) of BCR/ABL negative de novo ALL patients was 78.36% at 3 years, whereas the EFS of positive patients was 31.25% at 26 ± 6.4 months. Molecular screening for the Philadelphia chromosome should become a part of the routine diagnostic panel in ALL patients in order to predict which patients have a poor prognosis and need tailored therapy.

Key Words: BCR/ABL, RT-PCR, Children, Acute lymphoblastic leukemia.

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Since the beginning of chemotherapy (CT), 50 years ago by Farber and his colleagues, childhood ALL has been among the most responsive of all cancers studied[1]. Currently, more than 70% of children with acute lymphoblastic leukemia (ALL) are alive and disease free at 5 years[2]. But still, there is a group that is resistant to therapy. In recent years, with studies in molecular biology, it is now possible to identify this group that has a poor prognosis, although optimal therapies have not been readily available. The detection of BCR/ABL and ALL/AF_4 must be part of the routine diagnostic panel for all children with newly diagnosed ALL, and is essential for children with an early bone marrow relapse. Reverse transcriptase polimerase chain reaction (RT-PCR) allows the screening of a large number of patients and is highly sensitive and reliabile.

Among the many chromosomal abnormalities identified in leukemic blasts, BCR/ABL fusion product of translocation on chromosome 9 and 22, known as the Philadelphia (Ph) chromosome, is the most striking adverse prognostic factor in ALL[3].

In initially diagnosed cases of ALL, the incidence of a BCR/ABL transcript is found to be 3% to 5% in children, and 25% to 50% in adults[4-6]. Although the Ph chromosome in chronic myeloid leukemia (CML) and ALL seems are cytogenetically identical, the molecular analysis has established two different breakpoints on the chromosome[22]. M-bcr, known as the major breakpoint cluster region, encodes the chimeric p 210 protein, which is specific to CML. In Ph-positive childhood cases of ALL, the majority of cases have a minor breakpoint cluster region (m-bcr) on chromosome 22 which encodes protein p190[6,7].

In the Dana-Farber Cancer Institute, between 1981-1989, four-year event-free survival rates (EFS) and overall survival rates (OS) were found to be 0% and 20%, respectively, in patients with Ph positive childhood ALL, although they had been treated with the highest risk arm of chemotherapy (CT) protocols. In the same study, EFS and OS were 81% and 88%, respectively, in 419 children lacking this translocation[4].

Current concepts for the treatment of patients with Ph positive childhood ALL recommend administering intensive multi-agent CT for remission induction and referring patients to allogeneic BMT from matched siblings or unrelated donors during the first CR[8,9].

Therefore, in 1995, to detect patients with adverse prognostic factors and tailor the therapy, we initiated a molecular diagnostic study with nested RT-PCR as a part of routine diagnostic panel for children with newly diagnosed cases of ALL and with marrow relapse.

MATERIAL and METHODS

Patients: Diagnostic bone marrow samples were obtained from 75 de novo and 17 relapsed pediatric ALL patients (excluding B-cell ALL), between October 1995 and October 1998, for a molecular analysis of the BCR/ABL fusion transcripts. Subsequent samples of the BCR/ABL positive patients were also obtained for molecular monitoring. A diagnosis of ALL had been established according to standard morphological, cytochemical, and immunophenotypical criteria.

Separation of mononuclear cells: Bone marrow samples were centrifuged in a Ficoll-Hypaque gradient, washed and cryopreserved in 4M guanidium isothiocyonate in a liquid nitrogen tank until used for RNA extraction.

RNA extraction: The Acid Guanidium Thiocyanate-Phenol-Chloroform Extraction Method of Chomezynsky and Sacchi was used[10]. The sample was minced on ice and homogenized with 0.5 mL of 4 M guanidium thiocyanate, 25 mM of sodium citrate, and a PH of 7; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol at room temperature was used in a 1.5 mL Eppendorf tube. This suspension was shaken vigorously for 15 seconds and cooled on ice for 15 minutes. After centrifugation at 10.000 g for 20 min at 4°C, RNA was present in the aqueous phase, whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 mL of isopropanol, and then placed at -20°C for at least 1 hr to precipitate RNA. Sedimentation at 10.000 g for 20 minutes was again performed and the pellet was resuspended in 75% ethanol and again centrifuged at 10.000 g,

at 4°C for 1 minute, left for drying, and dissolved in 50 L sterile distilled water. The final 5 L RNA preparation is dissolved in 10 L water and quality control is done on 2% agarose-gel electrophorese stained with ethidium bromide.

RT-PCR analysis: 1 g of RNA was diluted in 10 L of water and was mixed with 1 L of random Hexamer (500 g/mL, Promega-Madison WI, USA) and heated to 70°C for 10 minutes, then rapidly cooled on ice. The cDNA synthesis was carried out at 37°C for 60 minutes after an adjustment of the mixture to contain 50 ng/ L RT buffer (10 mM Tris-Hcl pH: 8.3, 1.5 mM $MgCl_2$), 200 mM deoxynucleotide triphosphate, 20U of RNAse inhibitor (1 U/microliter, Promega- Madison WI, USA), 10 U/ L reverse transcriptase and 0.1 M DTT. A Perkin Elmer Cetus 480 DNA Thermal Cycler was used. The cDNA was tested with PCR amplification using a primer for Abelson (c-abl) proto-oncogens. The amplification of bcr-abl p-210 was performed in a 25 L reaction tube containing 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4), 1.5 mmol/L MgCl2, 0.001% (wt/vol) gelatin, 1.5 mmol/L each deoxynucleotide triphosphate (dNTP), 2 L cDNA and 1 U Taq-polymerase (Promega, Madison WI, USA), 7.5 mol/L each primer and overlaid with 1 volume of mineral oil. PCR was performed with a DNA thermal cycler. The first denaturation was at 94°C at 2 minutes, 1 cycle. Cycling times and temperatures for denaturation, annealing and synthesis for an external PCR were as follows: At 94°C at 1 minute, at 62°C at 1 minute, at 72°C 1 at minute for 35 cycles. The same conditions were used for the internal PCR. External primers for P210 were A+D. inner primers: B+C

Sequences of primers

primer A: 5'-GAA GAA GTG TTT CAG AAG CTT CTC CC-3'

primer D:5'- TGT GAT TAT AGC CTA AGA CCC GGA G-3'

primer B:5'- GTG AAA CTC CAG ACT GTC CAC ACG CA –3'

primer C:5'- TCC ACT GGC CAC AAA ATC ATA CAG T-3'

outer primers (p-190): F+D, inner primers: L+C

primer F:5'- AGA TCT GGC CCA ACG ATG GCG AGG GC-3'

primer L:5'- TCC ATG GAG ACG CAG AAG CCC TTC CAG CGG C-3'

15 microliters of the final product is visualised by ethidium bromide staining on 2% agarose gel under a UV lamp. DNA size markers are also visualised in the same gel. All necessary precautions to prevent cross contamination of the amplified material were taken. For each PCR amplification, negative and positive controls were used. Positive controls were samples in which the genetic confirmation is also done with cytogenetics. These samples were obtained from Milano University Tattiomoni Foundation Research Laboratory- Italy.

Treatment modalities: BCR/ABL positive patients whose first genetic study was done during relapse had a standard CT protocol (BFM-86 ALL) as a first line therapy, whereas newly diagnosed patients had more intensified protocols (BFM-90 HR or CCG-Augmented BFM). For patients with a matched related donor allogeneic BMT was planned during the first remission or an unrelated donor search was initiated. If a BMT donor was not available, either autologous BMT was performed or CT was continued. The CCG-1941 relapse protocol or BFM 90 HR protocol was used to induce second remissions. For second relapses, ifosphamid +VP-16 + carboplatinium + prednisolon was also used.

Statistical analysis

Notable events considered for event free survival (EFS) determination included failure to achieve remission, death during therapy or remission, and relapse. The probability of survival was calculated according to the Kaplan Meier method. The relationship between BCR/ABL expression and the classical prognostic factors was calculated with the Chi-square and Fischer exact test.

RESULTS

Among 75 de novo ALL patients 4 (5.3%) we-

re found to be BCR/ABL positive whereas 4 of 17 relapsed patients (23.5%) were BCR/ABL positive. p< 0.05. Figure 1 shows a photograph of the ethidium bromide-stained PCR products on 2% agarose.

The clinical characteristics and outcome of BCR/ABL positive patients (n:8) are provided in Table 1. None of the patients had extramedullary involvement at diagnosis. Patient 1 had a leukemic ophtalmopathy, and patient 5 had CNS involvement preceeding the first marrow relapse.

The characteristics and outcome of BCR/ABL negative patients in the de novo group and in the relapsed groups are shown on table 2. Age, WBC, phenotype at diagnosis and event-free-survival (EFS) rates were not significant (p> 0.05) between BCR/ABL positive and negative patients due to the small number of BCR/ABL positive patients and the relatively short follow-up of the last three BCR/ABL positive patients.

Out of 8 BCR/ABL positive patients, 7 achieved CR at the end of induction, showing a CR rate of 87.5%. In the de novo group, 10 of the BCR/ABL negative patients were referred to different centers after diagnosis and there were three induction failures due to infections. The remission rate of de novo BCR/ABL negative patients was (58/61) 95.08%.

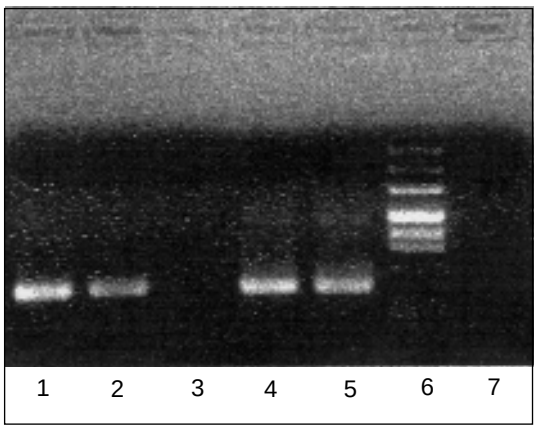


Figure 1. RT-PCR products of BCR/ABL transcripts on ethidium bromide stained agarose gel. Track 1, 2, and 4 belong to diagnostic samples of patients 6, 7, and 8 recpectively. Track 3 belongs to a patient with a negative PCR. Track 5 is the positive control. Track 6 codes marker 8 (pucmix MBI). Track 7 belongs to the negative control.

In the BCR/ABL positive group, patients 1, 4, 5, and 7 did not have an available BMT donor and the first three died following a second relapse. Patients 3, 6, and 8 had allogeneic BMT (3 and 8 from matched-sibling donors, 6 from a matched-unrelated donor in a BMT Center abroad). Patient 2 also did not have an available donor and underwent unpurged autologous BMT during the first partial remission, but had a resistant relapse in the first month of BMT, and died. Patient 3 died of acute graft versus host disease. Finally, patients 6 and 8 are alive in the 12th month after BMT and patient 7 is alive in the 19th month of the first CR.

The results of the PCR monitoring of BCR/ABL positive patients are shown in Figure 2. PCR was positive in all relapse samples. In patient 5, PCR detected the residual disease during the CNS relapse when the bone marrow morphology still showed remission. In repeated samples of this patient obtained during CT, the BCR/ABL transcript persisted. In patient 6, the BCR/ABL transcript was eradicated with allogeneic BMT, whereas in patient 7 and 8 it was eradicated after CT.

DISCUSSION

In this study the incidence of BCR/ABL positive cases in de novo acute leukemia is, 5%, which is consistent with other reports[3,11,12]. Johansson et al[13] reported the geographic heterogenity of neoplasia, which may be due to genetic or environmental factors. The incidence in our study is similar to that of Eastern Europe (5.4%), while in Northern Europe, a higher incidence (19%) is reported. The incidence of BCR/ABL gen rearrangment in relapse is considerably higher (23.5%) than at the time of the initial diagnosis. Beyerman et al[14] also found an incidence of 12% in the molecular screening of 170 children in the first bone marrow relapse of ALL.

A cytogenetic analysis retains its importance in the detection of the Philadelphia chromosome, but RT-PCR allows the study of a large number of patients and is highly sensitive. In many studies, a cytogenetic analysis failed to detect RT-PCR positive cases or the cytogenetic analysis was un-

Table 1. Characteristics and outcome of bcr/abl positive patients

Patient	Age/sex year	WBC FAB	Immuno phenotype	bcr/abl junction type	First line therapy CT/BMT	First rem.	Duration of first rem.	Salvage therapy	Second rem.	Outcome
1 py	3.5, F	14 000 L_1	B precursor, CD_{10}^+	p-190	BFM-86 ALL	CHR	44 m.	BFM 90 HR, ICE	+	Died (47 mo) on therapy in third rem. CVC complication (pulmonary embolism)
2	5, F	9100 L_2	B precursor, CD_{10}^+, MY +	p-210	BFM-86 ALL ABMT	VGPR	10 m.	ICE + prednisolon	-	Died (14 mo) resistant leukemia
3	14, M	800 L_1	B precursor, CD_{10}^-	p-190	BFM-90 ALL High risk, Allo BMT*	CHR		-		Died (10 mo) BMT related complications. (acute GVHD)
4	2, M	4000 L_2	B precursor, CD_{10}^-, MY+	p-190	BFM-86 ALL	CHR	26 m.	CCG-1941 relaps protocol	+	Died (38 mo) in second relapse, resistant disease
5	14, M	7000 L_1	T-cell	p-190	BFM-86 ALL	CHR	13 m.	CCG-1941 relaps protocol	+	Died (36 m) in second relapse, resistant disease
6	14, M	15 600 L_2	B precursor, CD_{10}^- MY+	p-190	Augmented BFM Allo BMT-MUD	CHR		-		Alive (22 m) in CCR
7	10, M	3000 L_1	B precursor, CD_{10}^-	p-190	Augmented BFM	CHR		-		Alive (19 m) in CCR
8	6, M	95 000 L_1	T cell MY+	p-190	Augmented BFM Allo BMT*	CHR		-		Alive (21 m) in CCR

CHR: Complete hematologic remission, VGPR: Very good partial remission, rem: Remission, mo: Month, MY: Myeloid antigen, ABMT: Autologous bone marrow transplantation, Allo BMT: Allogeneic bone marrow transplantation, CVC: Central venous catheter, ICE: Ifosphamide, carboplatinium, eto-

Table 2. Characteristics and outcome of BCR/ABL negative patients

	De novo group n= 71	Relapsed group n= 13
Sex (n)		
Male	47 66.1%	8
Female	24 33.8%	5
Age (year) mean	6.05 ± 3.68	8.77 ± 3.61
Minimum-maximum	3 month-14 year	4-14
WBC count (/ L) mean	33 572 ± 52 056	33 480 ± 44 058
Minimum-maximum	1200-200 000	1200-130 000
Immunophenotype (n)		
B-precursor	56 80%	8 61.5%
T-cell	14 20%	5 38.4%
Remission rate	95.08%	100%*
Relapse rate	8.45%	100%
Event-free survival rates		
(3-years)	78.36%	23.08%
(4-years)		15.38%
Overall survival rates		
(3-years)	80.05%	46.15%
(4-years)		27.69%

*First remission

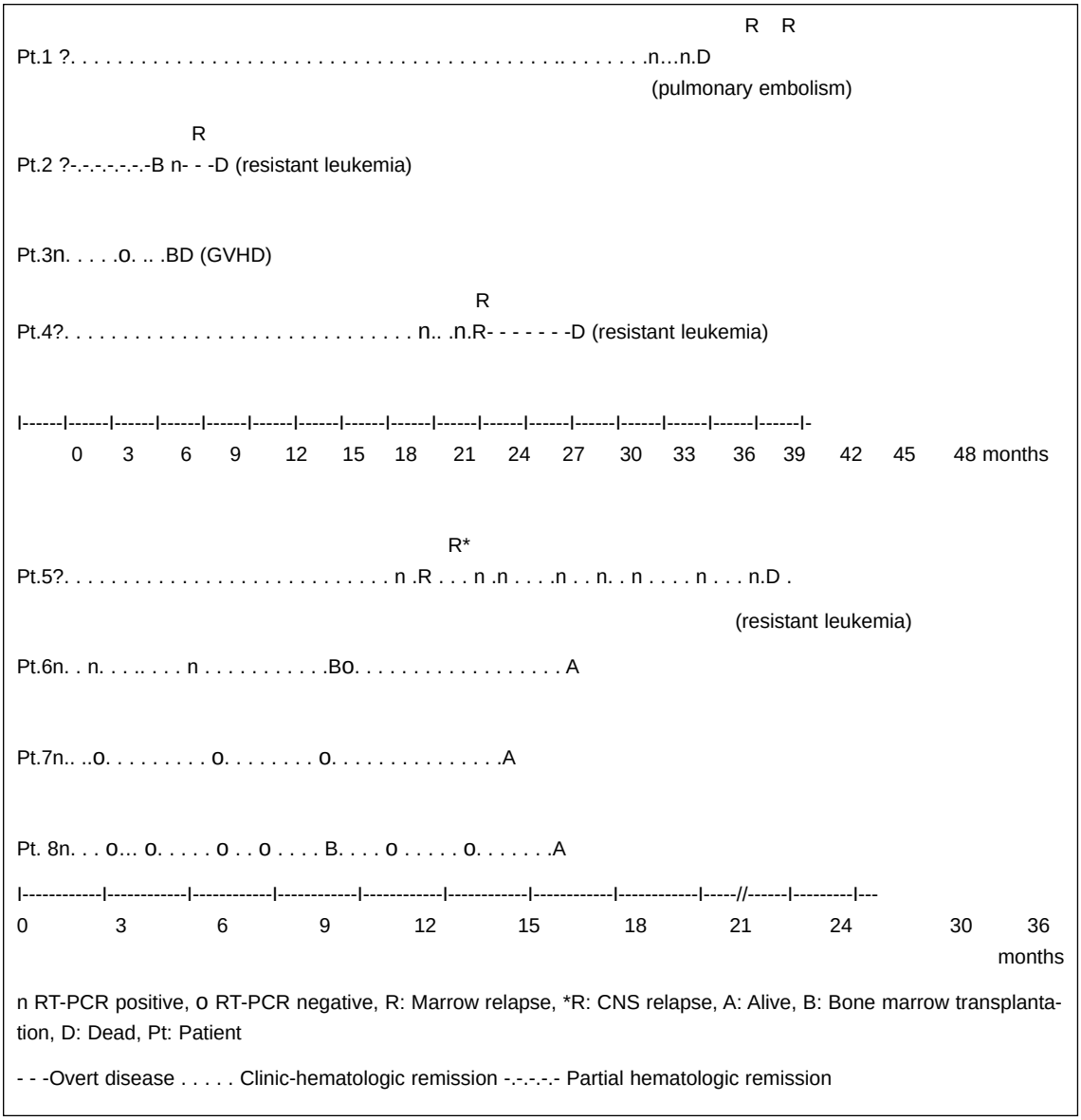
successful due to technical problems[15,16].

Many studies suggest a high leukocyte count among the associated features of this subgroup[3,16]. In contrast to a German pediatric multicenter study including 673 ALL cases, no significant difference in WBC counts could be found between BCR/ABL positive and negative cases[17]. In our study seven of the eight BCR/ABL positive patients had a WBC count below 20.000/ L.

BCR/ABL expression in T-cell leukemia is rare[17]; B precursor ALL is identified in most of the cases. Two out of 8 patients had the T-cell immunophenotype. In a POG study of 42 patients with the Philadelphia chromosome, 9% showed the T-cell immunophenotype[18], whereas in another multi-center study, there was a lower incidence (4.9%, 3/61)[6].

Four of our patients had myeloid markers. The coexpression of the myeloid antigen was reported both in common ALL and in T-lineage ALL[17]. In Carbonell et al.'s[19] series, 47% of Ph positive patients had biophenotypic leukemia and in Schrappe et al's series[16] 28.8% had biphenotypic leukemia. In a study of children with B precursor ALL expressing myeloid antigens (CD_{13}, CD_{33}), 63% expressed TEL/AML1 fusion transcript whereas t(9;22), t(4;11) and t(11;19) translocations were found in only 14% of the cases[20]. The prognostic significance of myeloid antigen positivity should be re-evaluated because it may be associated with both good and poor risks.

The majority of BCR/ABL positive childhood ALL cases express P-190 chimeric protein[14]. Studies have shown that the P-190 protein compared to the P-210 protein has greater in vitro tyrosine kinase activity[22] and produces a more virulent pattern of leukemia in both the mouse



n RT-PCR positive, o RT-PCR negative, R: Marrow relapse, *R: CNS relapse, A: Alive, B: Bone marrow transplantation, D: Dead, Pt: Patient

- - -Overt disease . . . . . Clinic-hematologic remission -.-.-.-.- Partial hematologic remission

Figure 2. Longitudinal monitoring of residual disease by RT-PCR analysis of BCR/ABL transcripts.

transplantation model and in the transgeneic mice systems[23,24]. In a report evaluating post-BMT relapses of Ph positive adults the, relapse rate of P-190 positive patients was 88%, whereas the rate for P-210 positive patients was only 12% and patients expressing both P-210 and P-190 had a relapse rate of 50%[21]. This may explain the poorer prognosis for Ph positive children as compared to Ph positive adults. Adults have P-190 and P-210 chimeric proteins of nearly equal rates[6].

The majority of Ph positive children achieve CR, but the duration of remission is short. In Scrape et al.'s 16 series, 25% (15/61) of patients had a resistant disease after 5 weeks of induction. Only one of our patients with a p-210 protein expression could not achieve remission.

The duration of the first remission is shorter than in the BCR/ABL negative group[14]. The duration of first remissions were 10, 13, 26, and 44

months in our relapsed patients, respectively. The rates of second remissions rates were also low in these patients. In Bayermann et al.'s[14] study, 11/18 patients (39%) achieved a second remission, which was significantly lower than in the BCR/ABL negative group. The one year EFS rate was 24% and none of the BCR/ABL positive patients reached an EFS of 5 years[14]. Three of our relapsed patients achieved second remissions which did not las. In a study of 170 relapsed children the incidence of a second relapse during salvage CT was also higher in BCR/ABL positive patients.

Transplantation from a matched-related donor (MRD) during the first CR is the best approach for improving the survival rate in these patients. Transplantation using matched-unrelated donors (MUD) is another alternative for patients without an available MRD. However, fatal complications often occur after this type of BMT procedure and the results are not always very encouraging[25]. In Scrappe et al.'s[16] study, EFS at 4 years was 0.38 and the (probability of survival) pSUR was 0.49. When post-induction therapy is compared (CT versus BMT), MRD BMT has been more successful because of the lethal toxicity of the MUD BMT procedure. EFS at 4 years after MRD BMT was 0.83, and for the CT group it was only 0.28 ($p = 0.01$). However, patient 6 had a succesful MUD BMT.

Transplantation with a purged autograft may also be an alternative for patients lacking a suitable donor. Patient 2 had an unpurged autologous BMT in PR because the purging procedure was not available in our center and remission could not be achieved. A negative PCR and a prolonged CR can be achieved with purged transplants, even in patients with a positive PCR at the time of the bone marrow harvest[26].

RT-PCR analysis shows a good correlation with relapse[26]. In patient 5, central nervous system (CNS) relapse preceded marrow relapse. The PCR in the bone marrow sample was positive during the CNS relapse, whereas the morphology still showed remission. Relapse is generally preceded by a switch to a positive PCR by 4 to 6 months[26]. In our patients, all relapse samples revealed a positive PCR. In patient 5, the PCR was persistantly positive during salvage CT and the patient suffered another relapse. In patient 6, the autologous transplantation, and in patients 7 and 8, CT seemed to eradicate the minimal disease, but a longer follow-up period is necessary to reach a final conclusion.

The sensitivity of PCR is lower in blood than in the bone marrow[26]. PCR is a very sensitive method of molecular genetics, but careful laboratory work is necessary to avoid contamination.

It has been confirmed with a matched-pair analysis of the BFM Study Group[27] that the Philadelphia chromosome is an independent poor risk factor, both in childhood and in adult ALL. Although the number of BCR/ABL positive patients is small, and the median observation time is not long enough for statistical significance, it indicates that patients expressing BCR/ABL transcripts have a poor prognosis. Screening for BCR/ABL must be a part of the routine diagnostic study, and BMT from MRD in the first remission must be pursued, because even with intensive chemotherapies, survival rates for these patients are low.

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