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DETECTION OF BCR/ABL TRANSCRIPTS BY RT-PCR AND THEIR COLORIMETRIC EVALUATION IN CHRONIC MYELOID LEUKEMIA PATIENTS RECEIVING ALLOGENEIC BONE MARROW TRANSPLANTATION

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ABSTRACT

Background. **Chronic myeloid leukemia (CML) is a disease characterized by the presence of a unique molecular marker, i.e. the fusion gene bcr-abl and its mRNA and protein products. This marker permits minimal residual disease follow-up after bone marrow transplantation (BMT) through cytogenetic or molecular analysis. Although the reverse transcriptase polymerase chain reaction (RT-PCR) method is largely employed, the clinical value and impact of a positive RT-PCR as a herald of hematological relapse has not yet been definitively ascertained.**

Methods. **In order to verify the frequency of bcr-abl positivity in CML patients who underwent alloBMT, we performed serial two-step RT-PCR on 63 peripheral blood and bone marrow specimens obtained at different times after non T-cell-depleted BMT from 16 CML patients treated in our Institution. After amplification, RT-PCR products were always checked by liquid hybridization with a specific probe. Median molecular follow-up after BMT was 38 months (range 2-144 months).**

Results. **None of the patients studied presented clinical hematological relapse after BMT. Six out of sixteen patients were found to be positive for bcr-abl. PCR positivity appeared in 4/6 patients more than one year post-BMT and in 2/6 patients within one year post BMT. In both instances PCR was an isolated finding in 5/6 patients and reverted to negativity in subsequent analysis; only one case was PCR positive twice. It is noteworthy that RT-PCR positivity appeared in five patients presenting acute or chronic graft versus host disease (GVHD) and in one patient who had received MUD-BMT.**

Conclusions. **In our cohort of patients, transient bcr-abl positivity had no clinical relevance and was also found in MUD-BMT without heralding hematological relapse. Our observations further stress the importance of applying only quantitative PCR methods during the post BMT follow-up of CML patients.**

Key words: chronic myeloid leukemia, bcr/abl, RT-PCR, allo-BMT

Chronic myeloid leukemia is a disease characterized by the proliferation of a clone of myeloid precursors endowed with growth advantages with respect to normal hematopoietic cells. The neoplastic clone retains the ability to achieve terminal maturation for a variable time. The natural course of the disease leads to a form of acute leukemia refractory to all chemotherapeutic regimens.[1] Notwithstanding the undoubted success of IFN-α therapy,[2] allogeneic bone marrow transplantation (allo-BMT) should still be considered the definitive treatment for chronic myeloid leukemia (CML).[3,4] Nevertheless, even after allo-BMT, relapse rates reach 9-24%.[5] The unique molecular marker present in CML, namely the presence of the bcr/abl fusion transcript,[6] renders minimal residual disease detection a relatively simple task, especially with the

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RT-PCR technique.[7] It still must be noted that the reappearance or persistence of bcr/abl transcripts after allo-BMT is an observation whose clinical significance as an early sign of relapse is still under careful evaluation. The experience of therapy with donor lymphocyte transfusions in the presence of cytogenetic or molecular evidence of relapse,[8] before frank hematological relapse, again raises this biological and clinical problem. We present here the results of a molecular follow-up of 16 CML patients who underwent allo-BMT. In all cases nested RT-PCR was performed at several time intervals after BMT. To assess the nature of the band visualized on agarose gels in PCR-positive cases, the PCR product was hybridized with two specific oligonucleotide probes and the hybridization was evaluated by the development of a color reaction quantifiable by spectrophotometric analysis.[9]

Materials and Methods

Patients

The study included 16 Ph1-positive CML patients who underwent allo-BMT (allogeneic = 12; MUD = 4) in our Institution. The clinical characteristics of the patients are summarized in Table 1. None of them received T-cell depleted marrow. The conditioning regimen included busulfan 16 mg/m^2 and cyclophosphamide 120 mg/kg for 7 pts; busulfan, cyclophosphamide and vepeside 25 mg/kg for 2 others and cyclophosphamide and total body irradiation (TBI) for the remaining 7. Patients received BMT between November 1983 and March 1995, and the molecular study was terminated on September 9th, 1995.

PCR Procedure

Total RNA was extracted from peripheral blood or bone marrow at various intervals post-BMT, according to the method of Chomczynski and Sacchi.[10] cDNA generation and the nested RT-PCR procedure were performed according to Guerrasio *et al.*[7] At the end of the two PCR steps, 10 uL of the mixture were run on 2% agarose gel for ultraviolet analysis, giving a fragment of 198 bp (b2a2) or 273 bp (b3a2).

PCR procedure check-outs

Table 1. Clinical characteristics of the 16 CML patients studied.

Case n.	*Age/sex*	*Stage*	*Molecular rearrang.*	*Lapse diagn./BMT*	*BMT*	*GVHD acute/chronic*	*Conditions*	*Current clinical status*
1	52/M	CP1	b3a2	8 mos	MSD	none/exten.	Bu/Cy	CR (+2 yrs)
2	31/F	AP	b2a2	1 yr	MSD	none/exten.	Bu/VP/Cy	CR (+5 yrs)
3	41/M	CP2	b2a2	6 mos	MUD	III/none	Cy/TBI	CR (+60 days)
4	15/M	AP	Ph pos.	1 yr	MSD	none	TBI/CCNU/Cy	CR (+11 yrs)
5	41/M	CP1	b2a2	7 mos	MUD	I/none	Cy/TBI	Deceased (+80 days)
6	27/M	CP1	b3a2	1 yr	MSD	none	Bu/Cy	CR (+3 yrs)
7	45/M	CP1	b2a2	1 yr	MSD	none	Bu/Cy	CR (+6 yrs)
8	42/M	CP2	b2a2	1 yr	MSD	none	Bu/VP/Cy	CR (+5 yrs)
9	51/F	CP1	b2a2	1 yr	MSD	none/exten.	Bu/Cy	CR (+3 yrs)
10	51/M	CP1	b2a2	1 yr	MSD	none	Bu/Cy	CR (+4 yrs)
11	32/F	CP1	b2a2	1 yr	MSD	none	Bu/Cy	CR (+4 yrs)
12	25/M	CP3	b3a2	1 yr	MSD	IV lethal	Bu/VP/Cy	Deceased (+62 days)
13	24/M	CP1	b2a2	7 mos	MSD	none	Bu/Cy	CR (+2 yrs)
14	46/F	CP1	b3a2	7 mos	MSD	none	Bu/Cy	CR (+2 yrs)
15	35/F	CP1	b3a2	1 yr	MUD	I/limited	Cy/TBI	CR (+2 yrs)
16	35/F	CP1	b2a2	7 yrs	MUD	I/limited	Cy/TBI	CR (+2 yrs)

Age refers to last clinical evaluation. Abbreviations: CP1&CP2: first and second chronic phase CML; AP: accelerated phase CML; MSD: marrow sibling donor; MUD: marrow unrelated donor; CR: complete clinical remission; Exten.: extensive chronic GVHD.

In order to avoid contamination manipulation of samples, RNA extraction and reverse transcription were carried out in different rooms. The pre-PCR room was routinely illuminated overnight with U.V. rays and check-outs of reactions were performed as recommended by Kwok and Higushi.[11] In addition, a positive (K562, RNA extracted with a different set of pipettes and in wood) and a negative (reagents without cDNA template) control were always included in the reactions. Amplification reaction data were discarded when controls were not giving the expected results.

Hybridization

Ultraviolet-positive PCR products were further checked with a colorimetric assay[9] (cTRAK, Raggio Italgene, Pomezia, Italy). In brief, after amplification of a translocated sequence, the reaction mixture was diluted, denatured and allowed to hybridize with a set of derivatized oligonucleotides complementary either to the bcr/abl junction region or to the abl region. These derivatized oligonucleotides are internal (nested) to the primers used for the amplification steps, and are referred to as *capture* probes and *reporter* probes (see Figure 1). The capture probes are conjugated to fluorescein isothiocyanate (FITC) and are used to separate bound specific target from unbound sequences by the use of an anti-FITC antibody coated on paramagnetic beads. The reporter probes are conjugated to the enzyme alkaline phosphatase (ALP) and allow visualization of the presence of the target sequence by using the appropriate substrate for ALP. Only when the amplified sequence (b2a2 or b3a2) was present in the reaction mixture could both the capture and the reporter oligonucleotides bind to the same target sequence and hence be bridged together. Following the reaction with the paramagnetic beads coated to the anti-FITC antibody and cycles of magnetic sedimentation and washing, complete isolation of target bound to the solid phase was achieved. A chromogenic substrate for the enzyme was then added to the magnetic particle suspension and a visible signal generated. The absorbance of the solution was measured by means of a spectrophotometer at 550 nm. A positive test result was indicated by an absorbance value significantly higher than that of negative controls (see Table 2). The sensitivity

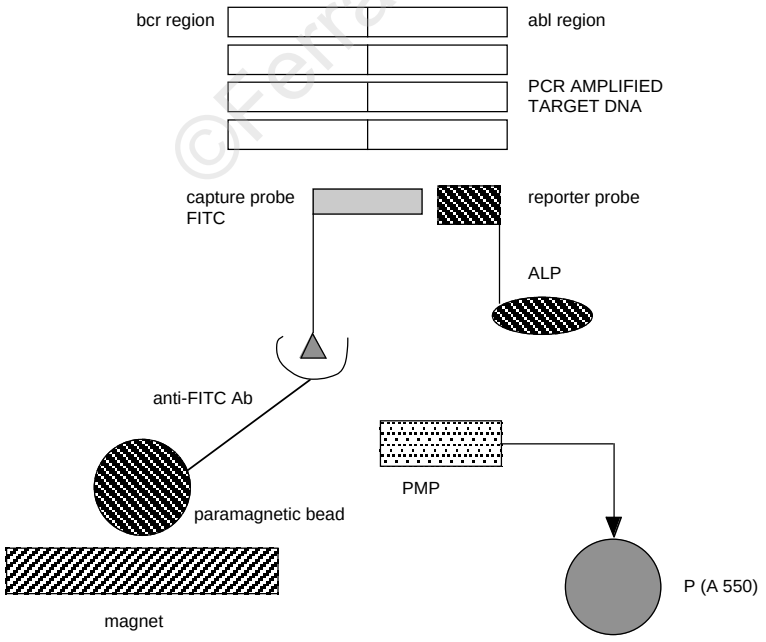

Figure 1. Schematic representation of the principle of the c-trak t(9,22) translocation analysis system. After amplification, the target DNA was diluted, denatured and allowed to hybridize with oligonucleotides complementary either to the bcr-abl junction region or to the abl region. These derivatized oligonucleotides are internal (nested) with respect to the primers used for the amplification steps and are referred to as *capture* and *reporter* probes. Capture probes are conjugated to fluorescein isothiocyanate (FITC) and are used to separate bound specific targets from unbound (irrelevant) sequences by means of an anti-FITC antibody coated on paramagnetic beads. The reporter probes are conjugated to the enzyme alkaline phosphatase (ALP) and allow visualization of the target sequence by using the appropriate chromogenic substrate for ALP. The absorbance (A 550) of the solution can be measured with a spectrophotometer at 550 nm and will be proportional to the amount of amplified target present in the reaction mixture.
Other abbreviations used: PMP = phenolphthalein monophosphate; P= phenolphthalein.

of the method was assessed by diluting cells from the K 562 (b3a2 positive) and BV 173 (b2a2 positive) cell lines with lymphocytes from healthy blood donors and submitting them to the RT-PCR procedure described by the manufacturer (non nested PCR primers).[9] The results showed a sensitivity beyond 1:50,000.

Results

RT-PCR and clinical follow-up

Bcr-abl fusion transcripts were present in 6 out of the 16 cases after BMT (Figure 2). Of these, 5 had a b3a2 breakpoint and only one a b2a2. In 5 out of these 6 cases RT-PCR positivity was a single event during the molecular follow-up. Median duration of the molecular follow-up was 38 (2-144) months. There were 7 positive PCR reactions out of a total of 63; each case had a minimum of three and a maximum of six samples consecutively studied. We performed PCR on 37 bone marrow samples, and of these 6 were found to be positive for bcr-abl, whereas only one out of the 26 peripheral blood samples was positive. PCR positivity appeared more than a year after BMT in 4/6 cases, among which was the one with the b2a2 breakpoint. In these patients PCR results reverted to negativity in the following analysis. On the other hand, in two cases (#12 and #14) PCR positivity appeared within one year after BMT, but here too we observed reversion to negativity. None of the patients included in our study showed any sign of hematological relapse. Patient #14, despite two consecutive positive PCR of BM samples, never relapsed and the five subsequent analyses carried out from BM and PB samples were always negative (see Figure 2). We never observed alternating positive and negative

Table 2. Absorbance values of RT-PCR products.

Diagnosis	*I pos. PCR*	*II pos. PCR*	*Neg. PCR*	*Case #*
2.76	8.34 (b3a2)	9.33 (b3a2)	0.090	14
ND	5.323 (b3a2)	—	0.049	12
ND	4.939 (b3a2) 5.751 (b2a2)	—	0.026	1
ND	8.368 (b2a2)	—	0.085	9
2.76	1.525 (b3a2)	—	0.022	6
2.74	7.940 (b3a2)	—	0.041	15
			0.017 (b2a2)	Neg. control
			0.091 (b3a2)	
	2.731 (b2a2)			Pos. control
	2.140 (b3a2)			

After RT-PCR, an aliquot of the reaction was hybridized with two complementary oligonucleotides (capture and reporter). The development of a color reaction was measured by a spectrophotometer at 550 nm. Positive samples showed significantly higher absorbance values than negative controls.

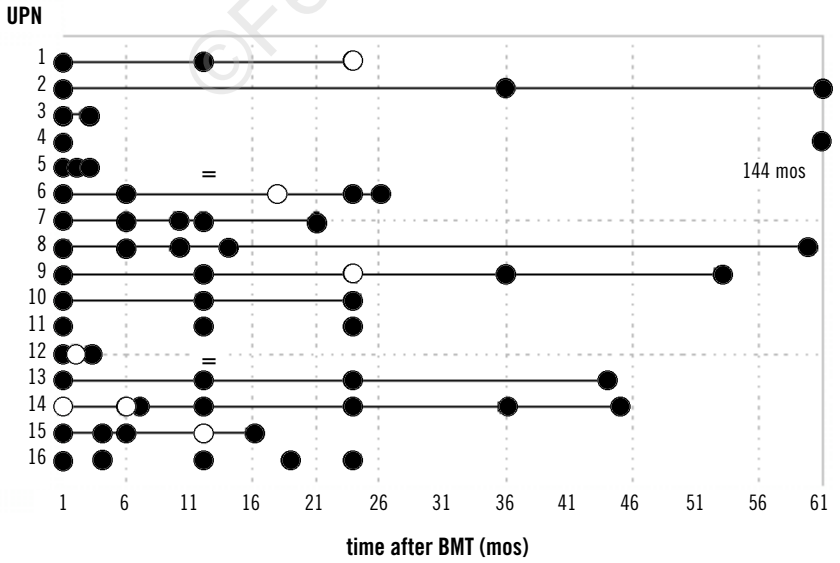

Figure 2. Molecular follow-up with RT-PCR of 16 CML patients after allo-BMT. (m) PCR positive for the bcr-abl rearrangement; (l) bcr-abl negative PCR; = deceased.

results or a second return to positivity in any of the patients. In patient #15, RT-PCR reverted to positivity more than 12 months after MUD-BMT.

All but one of the patients with the bcr-abl fusion transcript developed a graft versus host reaction, either acute (cases #5, 12, 15, 16) or chronic. Patient #9, who developed extensive chronic GVHD, contemporarily reverted to PCR negativity from 25 to 40 months after BMT. The only long-term remission and survival patient (#4) was constantly negative. Positive PCR post-BMT occurred in three female and three male patients.

Breakpoint analysis

In 15 out of the 16 patients, pre-transplant samples were available and RT-PCR was performed. In ten cases the junction was between exon 2 of the breakpoint cluster region and abl exon 2 (b2a2), in five between bcr region exon 3 and abl exon 2 (b3a2). Samples analyzed post-BMT and showing PCR positivity carried the same breakpoints as pre-BMT.

All b3a2 presenting-cases had at least one positive PCR after BMT, whereas only 1 out of 10 b2a2 was PCR-positive in hematological CR. In patients #1 and #5 both fusion products, i.e. b2a2 and b3a2 (although the latter with a fainter band at U.V. detection), were present in pre-BMT samples. No positive PCR was detected after BMT (MUD) for patient #5, whereas case #1 had a positive PCR 24 months after BMT, still with the presence of both transcripts.

Hybridization

Positive PCR after BMT were all analyzed for specificity by solution hybridization, magnetic separation and subsequent visualization of the *bound* enzyme-linked reporter by the addition of a chromogenic substrate (see *Methods*). Absorbance values are shown in Table 3.

It is clear that positive RT-PCR have a significantly higher absorbance value than controls. It is also apparent that there is a wide variability in these values. Indeed we did not encounter false positive results, that is the lack of concordance among ethidium bromide stained, UV visible bands and absorbance values after specific probe hybridization. Nor did we detect false negative samples when we randomly checked negative PCR.

Discussion

We studied the pattern of bcr-abl expression in 16 CML patients who underwent allo-BMT by two-step nested RT-PCR, followed by hybridization with probes internal to the transcript and visualization with a colorimetric reaction evaluable by spectrophotometric analysis. This study gives further support to the notion that it is essential to carry out RT-PCR analysis at several time points during post-BMT follow-up of CML patients, in order to carefully evaluate the persistence of bcr-abl transcripts.

Notwithstanding the finding of positive PCR, none of the CML patients included in our study showed any sign of hematological relapse. It should be stressed that all but one of the patients reverted to PCR negativity in the following analysis, indicating only a transient presence of detectable minimal residual disease. The most interesting data we gathered seem to be the constant finding of at least one positive PCR post-BMT in all cases bearing a b3a2 translocation (n = 6). This observation can only lead to a cautious conclusion because of the small number of patients studied. It has been widely discussed whether the difference in the breakpoint and thus in the fusion gene expressed could have some clinical significance.[12,13] Although later studies have not confirmed it completely,[14,15] the (dated) observation that the b3a2 translocation was correlated with a poorer prognosis, even after BMT,[16] supports our hypothesis.

Five of the 16 patients suffered GVHD. Quite surprisingly, this important immunological reaction did not seem to influence the possibility of detecting positive bcr-abl PCR samples. This finding, although only in a few patients, is in contrast to what has been observed elsewhere,[17] but it does not exclude the role of donor cells in arresting the expansion of the leukemic clone present in the chimeric bone marrow. In fact, patient #9, who had extensive chronic GVHD, reverted from positive PCR to negativity. Acute GVHD I was present in the

other cases (#5, #15, #16) that switched to PCR positivity one year after BMT. In these latter cases one can hypothesize that the early antileukemic effect of the donor cells could have been exhausted at the time of reversion. Interestingly, among these cases, case #15 was an unrelated donor BMT. MUD-BMT are burdened with a high mortality rate because of GVHD-related complications. Anyway, because of GVHD one would expect a complete absence of minimal residual disease in long survivors in such cases. Apparently this is not the case, and MUD-BMT does not appear to be completely effective in eradicating CML. On the other hand, the reappearance of positive RT-PCR one year after MUD-BMT may be explained by the aggressive immunosuppressive treatment applied. Further studies focused on this question are of course required. All the amplified samples in which the bcr-abl sequence could be detected were further analyzed by hybridization in solution with specific *capture* and *reporter* probes that developed a color which was measurable by spectrophotometric absorbance. The absorbance values of samples in which bcr-abl transcripts were detected on agarose gels were significantly higher than negative controls supplied by the manufacturer, and significantly ($p < 0.05$) higher than the absorbance values of RT-PCR-negative samples obtained from the same patient. In fact, *background* absorbance was not relevant (see Table 2). The importance of this procedure lies in the fact that it is possible to employ non radioactive probes endowed with a high specificity and sensitivity.

Possibly, our observations may indicate that the casual finding of one positive PCR post BMT does not necessarily herald hematological relapse, nor does it exclude later reversion to negativity. Connections between the reappearance of the molecular marker and clinical relapse can probably be drawn only after careful evaluation of the quantitative fluctuations in the transcript levels. In fact, there is a general consensus in judging the success of therapies for CML (whether BMT related or not) on the grounds of merequantitation of residual bcr/abl transcripts.[18-21] These evaluations are particularly important at a moment when one has to decide whether to risk GVHD in performing prophylactic therapy with allo-lymphocytes or to employ classic IFN α-therapy.[22]

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