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Detection by Polymerase Chain Reaction of BCR/ABL Transcripts in Myeloproliferative Diseases at Time of Diagnosis and for Monitoring Chronic Myelogenous Leukaemia Patients After Bone Marrow Transplantation

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The Philadelphia chromosome t(9;22)(q34;q11) is a cytogenetic marker for chronic myelogenous leukaemia (CML), and is also present in some acute leukaemias. The translocation in CML gives rise to two *BCR/ABL* chimeric transcripts (b3a2 and b2a2) encoding a 210-kD tyrosine kinase protein. These leukaemia-specific transcripts can be detected easily by the reverse transcriptase polymerase chain reaction (PCR). PCR has improved the possibility of detecting minimal residual leukaemia cells in Ph-positive patients, especially after bone marrow transplantation (BMT). With PCR, we looked for *BCR/ABL* transcripts in 30 patients with CML and 4 with essential thrombocythaemia at time of diagnosis, finding a significant difference in the platelet counts of CML patients carrying b3a2 or b2a2 transcripts. The *BCR/ABL* transcript was monitored by PCR in 6 CML patients after BMT. The usefulness of PCR in clinical practice at time of diagnosis, and the biological and clinical significance of positive/negative PCR results, in patients with transplants, are discussed.

Key words: Philadelphia chromosome, BCR-ABL, fusion proteins, polymerase chain reaction, chronic leukaemia, myeloid leukaemia, essential thrombocythaemia, platelet count, bone marrow transplantation, graft versus host disease

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INTRODUCTION

THE PHILADELPHIA chromosome (Ph), a shortened long-arm chromosome 22 [1], is present in cells of more than 95% of patients with chronic myelogenous leukaemia (CML), approximately 25% of adult acute lymphocytic leukaemia (ALL) and a few (< 5%) with acute non-lymphocytic leukaemia and paediatric ALL [2]. The Ph abnormality is due to a reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11). At the molecular level, the translocation involves the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 [2]. The c-abl proto-oncogene product belongs to the Abelson subfamily of non-receptor protein tyrosine kinases, defined by the products of human, murine, *Drosophila ABL* gene [3] and human *ARG* gene [4]. The *BCR* gene product is a serine/threonine kinase protein [5]. The rupture of the *ABL* gene is usually located in the intron 5′ of exon II [6]. In Ph-positive CML and in approximately half of the Ph-positive ALL, the

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rupture of the *BCR* gene occurs within a region called the major breakpoint cluster region (bcr), encompassing four exons often referred to as exons b1–b4. During the rearrangement, exon b4 alone or exon b4 together with b3 can be lost, giving rise to two types of *BCR/ABL* fused transcripts [2, 6] (respectively defined as b3a2 and b2a2 transcripts). These fused *BCR/ABL* transcripts encode a 210-kD fusion protein (p210) that has an abnormally high tyrosine kinase activity [7], and has been shown to transform haematopoietic cells *in vitro* [8], and to induce a CML-like syndrome in transgenic mice [9]. Detection of the Ph chromosome, a cytogenetic marker for CML, has been used to confirm diagnosis and to monitor the disease. Because of the limited sensitivity of cytogenetic techniques (1–5% malignant cells in the sampled cell population), a more sensitive technique (one malignant cell in 10^6 normal cells or more) ([10], Figure 1), reverse transcriptase polymerase chain reaction (RT-PCR), has been used to detect the molecular rearrangement [11]. PCR has improved the possibility of detecting minimal residual leukaemia cells in Ph-positive patients, especially after bone marrow transplantation (BMT) [12]. However, variable data have been obtained about the clinical significance of *BCR/ABL*-positive cells detected by PCR in CML patients with transplants [13–17]. In this paper, we discuss the use of the PCR technique in clinical practice to detect the *BCR/ABL* transcript in patients with myeloproliferative disease, illustrating some of the important points, such as the use of PCR at time of diagnosis, the relation between the *BCR/ABL* transcripts and platelet count, and the serial PCR monitoring of CML patients after BMT.

MATERIALS AND METHODS

Study population

30 unselected patients with CML in chronic phase (CP) and 4 patients with essential thromobocythaemia (ET) (Table 1) were analysed at diagnosis. Cytogenetic analyses for detection of the Ph chromosome by established techniques were performed on bone marrow cells, and the same bone marrow aspirate samples were analysed by RT-PCR. 6 of the CML patients underwent allogenic non-T-cell-depleted BMT (Figure 2). 3 patients had marrow from related donors and 3 (patients 3, 5, 6) from unrelated HLA-matched donors (Figure 2). Patients 3 and 5 had BMT during the accelerated phase of the disease (patient 3 had Mar20, patient 5 had double Ph). Patient 2 had BMT during the second CP; patients 1, 4 and 6 had BMT during the first CP. The conditioning regimen and acute graft versus host disease (GVHD) prophylaxis were standard (cyclophosphamide, total body irradiation and short-term methotrexate plus cyclosporin). These patients were studied with cytogenetics and PCR before transplantation and at various serial post-transplant intervals (Figure 2).

Table 1. Study population at time of diagnosis

	CML	ET
Patients	30	4
Male/female	15/15	2/2
Age (years)		
Median	52	31
Range	22–75	25–81
Cytogenetics (Ph status)	28 + /2 −	0 + /4 −
RT-PCR (*BCR/ABL* status)	28 + /2 −	0 + /4 −

CML, chronic myelogenous leukaemia, chronic phase; ET, essential thrombocythaemia; + positive; − negative.

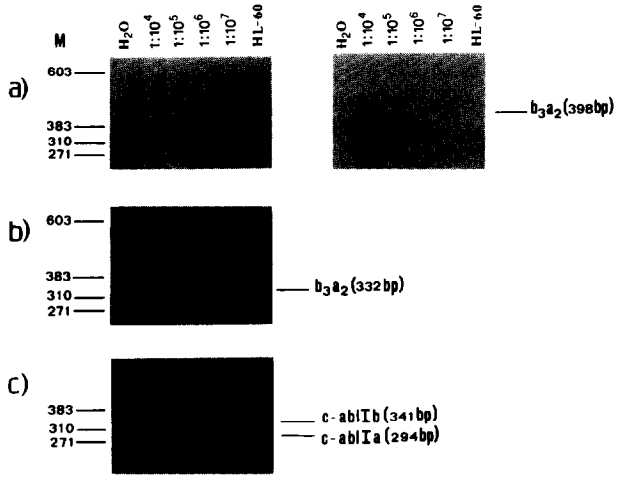

Figure 1. Sensitivity of PCR analysis. (a) Agarose gel-fractionated PCR product of *BCR/ABL* (b3a2 type; primers A–D) transcripts present in RNA extracted from K562 cells diluted at the ratios of a single K562 cell in 10^4, 10^5, 10^6, 10^7 *BCR/ABL* negative HL-60 cells; ethidium bromide staining (left), Southern blot hybridisation with 32P end-labelled primer B (3 h exposure) (right). (b) Nested PCR (primers A–C) of the samples loaded in panel (a). (c) The quality of RNA was established by amplifying the c-abl Ia/Ib transcripts (primers Ia, Ib and D in the first round PCR). M: ΦX174/HaeIII and pBR322/BstNI DNA molecular weight markers. H_2O: all the PCR components except RNA. HL-60: PCR amplification of mRNA extracted from HL-60 cells.

Polymerase chain reaction

Mononuclear cells were prepared from bone marrow by density gradient separation using lymphoprep (Nycomed). Total cellular RNAs were prepared from mononuclear cells [18]. RNA samples were then subjected to RT-PCR amplification. Briefly, 1 μg of total cellular RNAs was reverse transcribed, according to the manufacturer's directions, with M-MLV reverse transcriptase (Gibco BRL) and with an antisense primer D spanning between the second and third exons of human c-abl cDNA. For amplification, a sense primer A, which spans between exons b1 and b2 of human bcr cDNA, and 2.5 U of Thermus Aquaticus DNA polymerase (Perkin Elmer-Cetus) were added to cDNA in 100 μl final volume containing 2mM $MgCl_2$, again according to the manufacturer's directions. PCR was carried out in a DNA thermal cycler (Perkin-Elmer Cetus model 480) for 35 cycles (1 min 95°C, 1 min 60°C). One tenth of the PCR-amplified products was fractionated on a 3% agarose gel. Three microlitres of the amplification product, not visualised on agarose after the first PCR, were subjected to a second 35-cycle PCR (nested PCR), again using primer A and an internal c-abl primer C. With this approach, the sensitivity of PCR was one single Ph positive cell (K562) diluted in 10^6–10^7 Ph-negative cells (HL-60) in the nested PCR (Figure 1). At the start of this study, the RNA quality and the validity of negative PCR results were evaluated by amplification of human β-actin cDNA in all specimens, and later all the negative PCR results were validated by amplification of the more suitable normal human c-abl cDNA. Results with both methods were concordant. The c-abl Ia and Ib cDNA [6] were amplified using the antisense primer D and the sense primers specific for c-abl Ia and Ib exons, in the first round PCR. The results were verified by repeated analysis of different RNA or cDNA preparations from the same or from additional cell specimens. The *BCR/ABL* amplified products were transferred

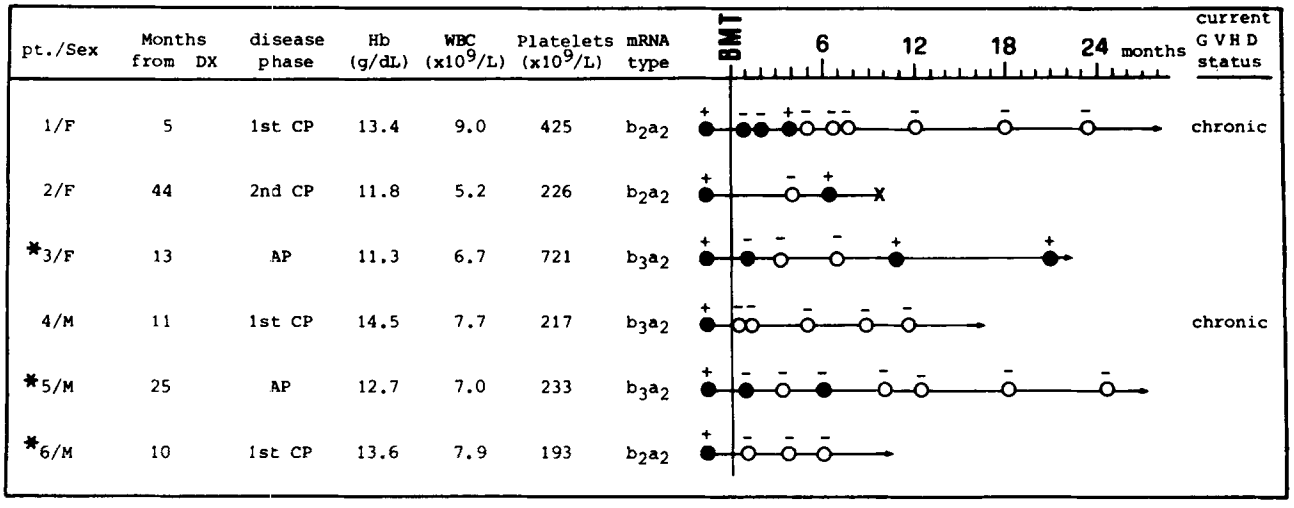

Figure 2. Clinical and cytogenetic/molecular status of CML patients immediately before allogenic BMT and the cytogenetic/molecular follow up after BMT. Pt, patient; Dx, diagnosis; CP, chronic phase; AP, accelerated phase. Patient 3 had Mar20, patient 5 had a double Ph chromosome. ● PCR positive; ○ PCR negative; + Ph positive; − Ph negative; ⟶ alive; ⟶× dead. * These patients had allogenic bone marrow from unrelated HLA matched donors.

to nitrocellulose and hybridised overnight with a 32P end-labelled internal bcr primer B, at 42°C in 10% formamide, 5× SSC, 5× Denhart, 0.1% SDS, 100 μg/ml ssDNA. The membrane was then washed three times with 6× SSC/0.1% SDS for 30 min at 42°C. Autoradiography was carried out for 3–24 h. Special precautions were adopted to minimise contamination while performing PCR, as recommended by Kwok and Higuchi [19]. Two negative controls were run: a normal RNA sample and a sample with all the required reagents except RNA. The oligonucleotide sequences were:

BCR [20]: (A) 5'GAAGAAGTGTTTCAGAAGCTTCTCCC3' (sense)
(B) 5'GTGAAACTCCAGACTGTCCACAGCA3' (sense)

c-abl [6]: (C) 5'TCCACTGGCCACAAAATCATACAGT3' (antisense)
(D) 5'TGTGATTATAGCCTAAGACCCGGAG3' (antisense)
(Ia) 5'ATGTTGGAGATCTGCCTG3' (sense)
(Ib) 5'AGCAGCCTGGAAAAGTAC3' (sense)

The A and D primers have an additional 5' eight-nucleotide tail containing the BamHI restriction site. In the case of *BCR/ABL* rearrangement, the b3a2 amplified fragment is 398 bp long (332 bp with nested PCR) and the b2a2 fragment is 323 bp long (257 bp with nested PCR). The Ia and Ib c-abl PCR fragments are, respectively, 294 and 341 bp long.

RESULTS

We report the analysis, at time of diagnosis, of bone marrow cell mRNA from 30 CML patients in CP and from 4 ET patients, using the reverse transcriptase PCR technique to demonstrate the *BCR/ABL* transcripts. The sensitivity of our PCR assay is shown in Figure 1.

At diagnosis, 28 CML patients showed the *BCR/ABL* transcript in the marrow cells. The 2 Ph-negative CML patients were also PCR-negative for the *BCR/ABL* message. The *BCR/ABL* transcript was not detected in any of the ET patients (Table 1). 10 of the 28 Ph-positive CML patients had the b2a2 transcript and 18 patients the b3a2 type.

The relationship between the two types of *BCR/ABL* transcript and the patients' haematological findings at diagnosis was studied. We found that the platelet count was significantly higher in patients with the b3a2 transcript than those expressing the b2a2 transcript (Table 2) in accordance with results of other published studies [21, 22]. There were no significant differences in the other haematological parameters in relation to *BCR/ABL* type transcript.

Table 2. Clinical and haematological parameters at diagnosis of Ph-positive CML patients in CP expressing the b2a2 or b3a2 type of the BCR/ABL *rearranged mRNA*

	b2a2	b3a2
No. of cases	10	18
Male/female	5/5	9/9
Age (years)		
Mean ± S.E.M.	47 ± 4.0	52 ± 3.5
Median	48	52
Range	23–66	22–75
Hb (g/dl)		
Mean ± S.E.M.	12.1 ± 0.3	12.2 ± 0.4
Median	12.4	12.0
Range	8.8–14.7	8.3–16
WBC (× 10⁹/l)		
Mean ± S.E.M.	128.8 ± 20.3	145.4 ± 38.8
Median	125	103
Range	31.6–240	10.7–700
Platelets (× 10⁹/l)		
Mean ± S.E.M.	263.6 ± 17.8	653.9 ± 132.7*
Median	247.5	486.5
Range	199–363	158–2270
Blasts† (%)		
Mean ± S.E.M.	5 ± 2	6 ± 1
Median	2	3
Range	0–19	0–8

* $P = 0.039$ (Student's t-test). † Peripheral myeloblasts and promyelocytes.

6 CML patients underwent BMT (Figure 2) and they were all Ph- and PCR-positive for the *BCR/ABL* transcript immediately before BMT. They all had the same conditioning regimen and all were serially monitored after BMT with PCR and cytogenetic analyses. A representative PCR analysis is shown in Figure 3. 3 of the 6 patients were PCR-positive soon after BMT, but became PCR-negative within 6 months of transplantation (Figure 2).

Patient 1 became PCR- and Ph-positive in the 4th month. The cyclosporin treatment was discontinued, a chronic GVHD ensued and 1 month later the PCR and Ph became negative and remained so, along with the chronic GVHD. Patient 3 had a cytogenetic relapse 10 months after BMT; cyclosporin treatment was discontinued but the patient progressed to the accelerated phase. The PCR of patient 5 alternated between positive and negative for 6 months postBMT, but remained negative thereafter. Patient 2 was PCR-negative at 4 months, had a molecular and cytogenetic relapse at 6 months and died in blastic crisis. Patients 4 and 6 were PCR-negative throughout the observation period.

DISCUSSION

The *BCR/ABL* chimeric transcript characterising the Ph chromosome abnormality encodes a 210-kD protein in CML cells. This protein plays a fundamental role in the pathogenesis of CML [9]. Furthermore, it has been hypothesised that this leukaemia-specific protein stimulates an immune response to CML cells [23], which is particularly important in patients with transplants. PCR has become a powerful and less time-consuming tool [10] for detecting the *BCR/ABL* transcript, especially in treated CML patients [12–17], taking the place of cytogenetic or Southern blot analysis of genomic DNA, both of equivalent use when assessing minimal residual disease. The technical problems encountered when PCR is used to amplify rare cDNA are well known [19], and a series of precautions were taken to minimise these.

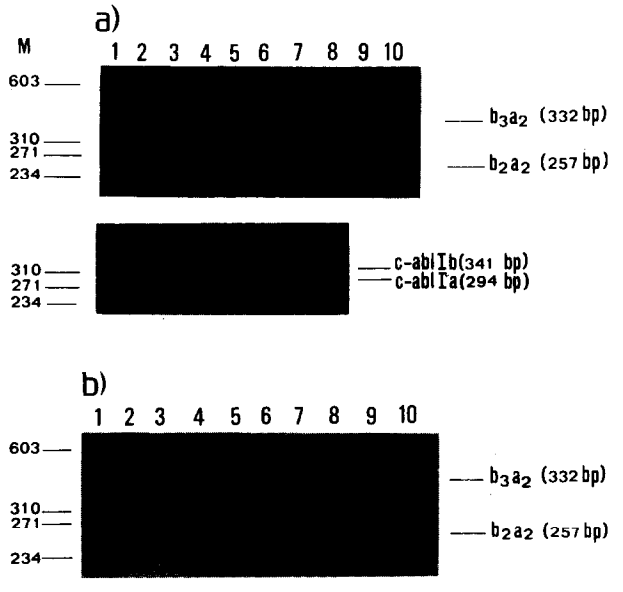

Figure 3. A representative PCR of the minimal residual *BCR/ABL* transcripts in CML marrow mRNA samples after allograft. (a) Gel fractionated PCR products of *BCR/ABL* (b3a2 and b2a2 type; nested PCR, primers A–C) and c-abl Ia/Ib (primers Ia, Ib and D) transcripts. (b) Southern blot hybridisation with ^{32}P end-labelled primer B (3-h exposure). M: ΦX174/Hae III DNA molecular weight markers. Lane 1, patient 1, 5 months after BMT. Lane 2, patient 2, 6 months after BMT. Lane 3, patient 3, 7 months after BMT. Lane 4, patient 4, 5 months after BMT. Lane 5, patient 5, 5 months after BMT. Lane 6, patient 6, 6 months after BMT. Lane 7, normal mRNA. Lane 8, all the PCR components except RNA. Lane 9, K562 cell mRNA. Lane 10, CML cells expressing b2a2 transcript.

PCR and cytogenetic analyses of patient samples at diagnosis showed highly congruent results (Table 1). These data indicate that PCR studies are not strictly necessary for CML patients at time of diagnosis, and should be carried out only for clinical investigations and to confirm the lack of chimeric transcript in Ph-negative CML patients, or to identify the subset of Ph-negative *BCR/ABL*-positive patients.

Our data show significant differences in the platelet counts ($P = 0.039$) (Table 2) of patients carrying the b3a2 or b2a2 transcripts, with the b3a2 transcript associated with a higher platelet count. The platelets in our b3a2 group did not reach the levels detected by Inokuchi and Nomura [21]. Instead, in our b2a2 group, the platelet counts were slightly lower than those observed by others ([21] and references therein). Marked thrombocytosis did not occur in our cases: only 2 patients, expressing the b3a2 type mRNA, had platelet counts over 900×10^9/l at diagnosis, and when these 2 patients from the b3a2 group were omitted (platelets mean ± S.E.M. 475 ± 54.3), the difference in platelet count between the two groups was still significant ($P = 0.006$). It is of note that the b3a2 type mRNA has also been observed more frequently than the b2a2 type in the rare Ph-positive essential thrombocythaemia [24]. The molecular and biological significance of the correlation between the b3a2 type *BCR/ABL* transcript and increased thrombopoiesis is unknown, and further investigations are necessary to elucidate the biological interactions between the b3a2 transcript and the megacaryopoietic and thrombopoietic activity. Our data plus those of Inokuchi and Nomura [21] and Lee and associates [22] are, so far, the only ones reporting a high platelet count significantly associated with the b3a2 transcript. Other groups have not succeeded in evidencing any correlation, either by analysing the rearranged mRNA [25, 26] or by analysing the breakpoint site on genomic DNA ([21] and references therein). Some unknown environmental factors may, in part, explain discrepancies between studies carried out in different geographical areas. We also evaluated the relationship between haemoglobin, white blood cells, peripheral blast findings and the two types of transcript, but we found no significant differences, in contrast with the data of Lee and colleagues [22], who found higher white blood cell counts in the b3a2 group.

The fact that 3 of 6 patients show positive PCR soon after BMT (Figure 2) suggests that the ablative regimen does not effectively eliminate all the leukaemic cells. Our patients became PCR-negative within 6 months after BMT, and this is in agreement with a general pattern observed in others' studies [15, 16]. Alternating negative and positive PCR in a group of patients identifies a group with lower risk of relapse [13, 14, 16], but in individual patients this pattern does not predict either lack of relapse or early relapse [16]: cases 2, 3 and 5 are in line with this observation. There is evidence that patients with transplants with persistently negative PCR have no haematological relapses [14, 16]. In addition, a late molecular and cytogenetic relapse is considered a high-risk event [13, 15]. For our cases, we need longer follow-up to see whether or not, even in the cases with persistent negative PCR, some late positives will appear, as has been described by others [16]. However, for early detection of impending relapse by PCR, detection of an expanding leukaemic clone by a more feasible quantitative PCR

is probably necessary [27, 28], especially if consecutive PCR-positive results appear. The cyclosporin treatment was reduced in the 6 BMT patients and then stopped between 6 and 12 months postBMT. Patient 1 is noteworthy: cyclosporin was discontinued abruptly 4 months post-transplant and the Ph/PCR became negative shortly thereafter (Figure 2). The patient developed a chronic GVHD and reverted to Ph/PCR-negative for the entire follow-up. Patient 4, always PCR-negative after the graft, developed an early limited GVHD. The anti-leukaemic effect of GVHD is well established [23, 29–31], and seems to play a role in suppressing the malignant clones, probably involving a complicated system of co-operation between different cellular types, such as MHC-restricted T-lymphocytes, and MHC-unrestricted lympholkine-activated killer cells and natural killer cells [23]. In this regard, PCR may be useful for evaluating the effectiveness of discontinuation of post-transplant immunosuppression and graft versus leukaemia effects *in vivo*.

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