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Clinical value of quantitative long-term assessment of bcr-abl chimeric transcript in chronic myelogenous leukemia patients after allogeneic bone marrow transplantation

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ABSTRACT

Background and Objectives. **For purposes of therapeutic decision making, we used quantitative polymerase chain reaction (PCR) for molecular follow-up of 55 patients with chronic myeloid leukemia (CML) in complete remission (CR) after allogeneic bone marrow transplantation (BMT) from HLA compatible donors.**

Design and Methods. **A total of 402 bone marrow samples from 40 patients transplanted in chronic phase (group 1) and 15 in accelerated/blastic phase (group 2) were analyzed by qualitative and quantitative PCR.**

Results. **Regarding clinical outcome, 34/40 (85%) group 1 vs. 8/15 (54%) group 2 patients are alive. Only 1/40 (2.5%) group 1 patient relapsed, as against 6/15 (40%) in group 2 (p = 0.0002). At qualitative PCR, 8/40 (19%) group 1 vs. 9/15 (60%) group 2 patients were positive, with a significantly greater total number of positive samples in group 2 (33/129, 27% vs. 16/273, 5%; p<0.001). The probability of qualitative PCR positivity >1 year after BMT was significantly lower in group 1 patients (4/40 pts, 10% vs. 9/15 pts, 60%; p = 0.01). At quantitative PCR, 4/8 (50%) group 1 patients were positive only once (< 400 transcripts/µg RNA). In group 2, 9/15 (60%) patients had 3 or more positive samples (always with >4,000 copies/mg RNA); therapeutic interventions (cyclosporin A discontinuation, temporary α-interferon or donor lymphocyte infusion) restored molecular remission in 4/9 (44%) cases.**

Interpretation and Conclusions. **This study indicates that quantitative PCR could provide practical indications capable of directing therapeutic interventions for transplanted CML patients, especially those transplanted in accelerated/blastic phase, for whom intensive monitoring is required.**

Key words: chronic myelogenous leukemia, minimal residual disease, Q-PCR, DLI

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The presence of the Philadelphia (Ph+) chromosome in human hematologic malignancies is associated with specific rearrangements between the BCR and the ABL genes.[1] The bcr-abl hybrid gene transcribes mRNA, which provides a marker for both molecular diagnosis of chronic myelogenous leukemia (CML) and detection of minimal residual disease (MRD) in CML patients who have undergone treatments such as an allogeneic bone marrow transplantation (BMT)[2-5] aimed at eradicating the leukemic clone.[6,7]

After an unmanipulated BMT from an HLA-identical sibling, a CML patient in first chronic phase has only 10-20% probability of relapsing within 5 years,[8] but patients who receive a transplant in an advanced phase of the disease have a much higher percentage of relapse (50-70%).[9,10] This difference cannot be explained solely on the basis of different degrees of effectiveness of pre-transplant conditioning regimens: other factors, such as a graft-versus-leukemia (GVL) effect, could play an important role in the process of eradicating of the Ph+ clone. Several cytogenetic studies have shown that disappearance of the Ph+ clone occurs only several months after transplant.[11,12] and that in some cases of long-term complete remission (CR), occasional Ph+ mitosis and molecular detection of the bcr-abl transcript by reverse transcription (RT) polymerase chain reaction (PCR) can be found even years after the transplant.[11] Approximately 80% of patients examined during the first few months after transplant are PCR-positive and become negative only during the course of the following year, and some patients remain constantly or intermittently PCR-positive even after several years. Monitoring of the amount of MRD by means of quantitative PCR is thought to be particularly useful for predicting clinical relapses.[13,14]

Taken as a whole, these observations suggest that the use of radio and/or chemotherapy as a conditioning system before the transplantation is not in itself capable of eliminating the leukemic clone, and that in general CML can only be definitively cured by exploiting the GVL mechanism. It is thus very important to learn how to modulate the GVL effect without greatly increasing graft-versus-host disease (GvHD).[15,16] To investigate whether quantification of MRD can provide useful indications for the implementation of therapeutic strategies based on the GVL effect, we utilized

Table 1. Patient characteristics.

Characteristics	*Group 1 Chronic Phase (n. 40) (73%)*	*Group 2 Accelerated/blastic Phase (n. 15) (27%)*
Age (years) mean (range)	35.21 (16 to 53)	37.8 (23-53)
Sex (F/M)	15 F (40%) 25 M (60%)	10 F (67%) 5 M (33%)
Ablative regimen		
Cy/TBI/DAUNO	14 (34%)	1 (7%)
Bu/CY	21 (52%)	5 (33%)
Bu/Cy/ATG	4 (10%)	0 (0%)
Cy/TBI	1 (2%)	0 (0%)
Cy/TBI/VP16	0 (0%)	9 (60%)
GvHD prophylaxis		
CsA alone	8 (20%)	2 (13%)
CD3 depletion	1 (2%)	0 (0%)
CsA/MTX	31 (77%)	13 (87%)
Post BMT follow-up (months)		
mean (range)	71;8 (3-153)	37;2 (2-84)
Interval before BMT (months)		
mean (range)	25;74 (7-78)	22 (5-108)
Type of donor		
HLA-sibling match	39	14
HLA-sibling mismatch	1	0
HLA-compatible unrelated donor	1	0
Sex of patient/sex of donor		
M/M	12	3
M/F	13	2
F/M	7	5
F/F	8	5
GvHD post BMT	20 (50%)	8 (53%)
acute grade I	11 (27%)	5 (33%)
acute grade II	5 (12%)	1 (7%)
acute grade III	1 (2%)	2 (13%)
acute grade IV	3 (8%)	0 (0%)
acute grade V	0 (0%)	0 (0%)
BM samples analyzed	273	129
mean	6;6	8;6
range	from 3 to 10	from 3 to 10

Abbreviations: CsA, cyclosporine A; F/M, female/male

competitive, quantitative RT-PCR in 55 patients in CR after allogeneic BMT.

Design and Methods

Fifty-five CML patients (25 female, 30 males) in hematological CR after allogeneic BMT (performed between September 1983 and May 1995) and who survived at least 6 months entered this retrospective study up to October 1997. For patients transplanted before 1991, bcr-abl analysis was made on frozen samples; after 1992 the study was prospectively conducted and bcr-abl analysis was performed on fresh samples. At the time of BMT 40 (73%) patients were in first chronic phase (group 1), and 15 (27%) were in accelerated phase or blastic phase (group 2) (Table 1). The mean age was 35.1 years (range 16-53) in group 1, and 37.8 years (range 23-53) in group 2. All patients were treated with α-interferon (α-IFN), hydroxyurea (HU) or busulphan (Bu) before BMT. The median interval between diagnosis and BMT was 26.7 months (range 7-708 months) and 22.0 months (range 5-180 months) in groups 1 and 2, respectively. In group 1, 39 patients were transplanted from an HLA identical sibling, 1 from an HLA mismatched sibling and 1 from an unrelated HLA identical typed donor. In group 2, 14 patients were transplanted from an HLA identical related donor. The remaining patient was transplanted from an HLA identical unrelated donor. There were 27 male and 28 female donors (M/M = 15, M/F = 15, F/M = 12, F/F = 13) (Table 1). Five different preparative regimens were used: Bu plus cyclophosphamide (Bu/Cy) alone (26 pts; 46%) or with anti-T lymphocyte globulin (ATG) (4 pts; 10%); Cy and total body irradiation (Cy/TBI) either alone (1 pt; 2%) or with daunoblastine (15 pts; 26%) (Table 1). The remaining 9 patients (all in group 2) were conditioned with Cy/TBI plus VP16. Two different protocols for prevention of GVHD were used: cyclosporin A alone (CsA) (10 pts; 17%) or, subsequently, with methotrexate (CsA/Mtx) (44 pts; 80%), or plus T-cell depletion (TcD) (1 pt; 1%) (Table 1). The molecular study was based on a total of 402 bone marrow samples analyzed: 273 from group 1 (mean samples/patient = 6.6) and 129 from group 2 (mean samples/patient = 8.6). Most of the patients transplanted in chronic phase before 1985 had the first PCR performed several months after transplantation: this was due to the absence of frozen material before this date suitable for retrospective PCR studies. Qualitative RT-PCR was performed as described elsewhere.[17] cDNAs were prepared from 1 μg RNA templates in 50 μL RT reaction. The RT reaction was carried out by incubation at 37°C for 1 h. The PCR amplification was performed using 10 μL of the RT product, plus 100 μL final volume with the 2.5 U DNA polymerase from Thermus Acquaticus (Taq polymerase, Perkin-Elmer, Milan, Italy) and AZ-EA122 primer.[17]

Quantitative RT-PCR was done by the capillary electrophoresis, as reported in detail elsewhere.[18] Briefly, after determination of bcr-abl type of junction by qualitative RT-PCR, serial dilutions with a number of $TA210b_3$, $TA210b_2$ competitor molecules were amplified with a fixed amount of cDNA from the patients' samples.[18]

Results

Clinical outcome

All patients engrafted. The median time for polymorphonuclear cells (PMN) recovery was 20 days to reach > 0.5×10^9/L PMN (range 10-31 days). The median time to reach > 50×10^9/L platelets (PLT) was 28 days (range 20-112 days). In group 1 chronic phase (CP), 34 patients (85%) are alive and in CR defined as complete cytogenetic absence of Ph+ metaphases and clinical signs of disease; 6 (15%) patients died, 2 (5%) from acute GVHD, 1 (2.5%) from chronic GVHD, 1 (2.5%) after relapse (UPN 193) and 2 (5%) from infection (Table 1). In group 2 accelerated/blastic phase, 8 (54%) patients are alive and in CR; 7 patients (46%)

Table 2. Clinical status, outcome and molecular analyses either by qualitative and quantitative PCR.

Patient status		*GROUP 1 Chronic Phase*		*GROUP 2 Accelerated/blastic Phase*		*GROUP 1 vs. GROUP 2*
		Pts	*%*	*Pts*	*%*	
Alive/well		34/40	85	8/15	54	p < 0.02
Died		6/40	15	7/15	46	
Cause of death:	aGVHD	2/40	5	/	/	
	cGVHD	1/40	2.5	/	/	
	relapse	1/40	2.5	6/15	40	p < 0.02
	infection	2/40	5	1/15	6	n.s.
Qualitative PCR+ (no. of patients)		8/40	19	9/15	60	p < 0.004
	Single PCR	4/40	10	0/15	0	
	Repeatedly PCR+	4/40	10	9/15	60	p < 0.004
	No. of samples PCR+	16/273	5	33/129	27	p < 0.000001
	PCR+ after 1 year beyond BMT	4/20	20	9/15	60	p < 0.01
Quantitative PCR (Q-PCR)						
	no. of pts PCR-	32/40	80	6/15	40	p < 0.02
	no. of pts PCR+	8/40	20	9/15	60	p < 0.02
	no. of pts PCR+ samples	8/71	11	39/61	64	p < 0.001
	no. of PCR samples resulted < 400 bcr-abl transcript/µg RNA	4/40	10	0/15	0	
	no. of PCR samples resulted < 4000 bcr-abl transcript/µg RNA	3/40	7.5	/	/	
	no. of PCR samples resulted < 40000 bcr-abl transcript/µg RNA	1/40	2.5	9/15	60	p < 0.001

died, 1 (6%) from infection and 6 (40%) after relapse defined as reappearance of cytogenetic Ph+ metaphases and clinical signs of disease (Table 2). None of the patients developed venous occlusive disease, despite the absence of prophylaxis. Patients with grade I-II GVHD were treated with methylprednisone at a dose of 1-2 mg/kg (and the patients with grade III or/and IV also received ATG). The two patients with grade IV GVHD died on days +360 and +390. At the time of writing, 34/40 (85%) patients are alive in group 1 vs 8/15 (54%) in group 2 ($p = 0.0002$). Only 1/40 (2.5%) patients relapsed in group 1, as against 6/15 (40%) in group 2 ($p = 0.0002$) (Table 2).

Qualitative PCR analysis of MRD

Qualitative PCR revealed bcr-abl in 8/40 (20%) group 1 patients: 4/40 (10%) were positive only once, and 4/40 (10%) were repeatedly (at least three times) positive during the follow-up (Table 2). In group 2, there were 9/15 (60%) positive patients, all of whom remained persistently positive, a significant increase with respect to group 1 ($p<0.004$). Patient UPN 211 died of infection and was excluded from further statistical analysis. Furthermore, significantly less positive samples were found in group 1: 16/273 (5%) vs 33/129 (27%); $p<0.001$. The probability of finding persistent disease 1 yr after transplantation was also significantly lower among group 1 patients: 4/40 (10%), vs. 9/15 (60%); $p = 0.01$. The conditioning regimen was very heterogeneous, in particularly in the 5 patients who received T-cell depleted (with or without ATG) transplants. We speculate that this could influence the bcr-abl status. A number of variables were investigated in order to try to establish what factors could be associated with longer persistence of the hybrid transcripts. Qualitative PCR positivity showed a strong trend to correlation only with disease status at the time of BMT ($p=0.01$). Sex, age, time to transplantation, the different types of GVHD prophylaxis (CsA, CsA/Mtx, etc.) were not found to be relevant.

Quantitative PCR

Thirty-two (80%) group 1 patients had no evidence of disease in any of their 202 samples. The remaining 8 group 1 patients turned out to be PCR positive in 18/71 (25%) samples: 4 patients (UPN 49, 102, 164, 194) had only one positive assay, with < 400 transcripts/µg RNA; 3 patients (UPN 132, 143, 193) had repeatedly positive assays with >4,000 transcripts/µg RNA but without a rising number of transcripts; the remaining patient (UPN 122) was PCR positive three times, once with >40,000 transcripts/µg RNA (Figure 1). Patient UPN 193 died of infection in the first 6 months after transplantation and was censored from further quantitative and statistical analysis.

In group 2, we confirmed that 6 patients (UPN 111, 113, 140, 141, 154, 192) had no evidence of disease in a total of 68 samples; the remaining 9 patients (UPN 98, 131, 145, 185, 187, 190, 199, 211, 214), who had been positive at qualitative PCR, showed the presence of bcr-abl in 39/61 (64%) samples, all of them having two or three positive samples with ≥4,000 transcripts/µg RNA. Of these a patient with positive PCR UPN 211 died early of infection; patient four (UPN 131, 185, 187, 190) have rising numbers of transcripts, two (UPN 98, 199) have decreasing, but high amounts of bcr-abl transcript and one patient (UPN 145) has a stable amount of bcr-abl transcript. There was olny patient UPN 214 available for a single quantitative determination of bcr-abl transcript before

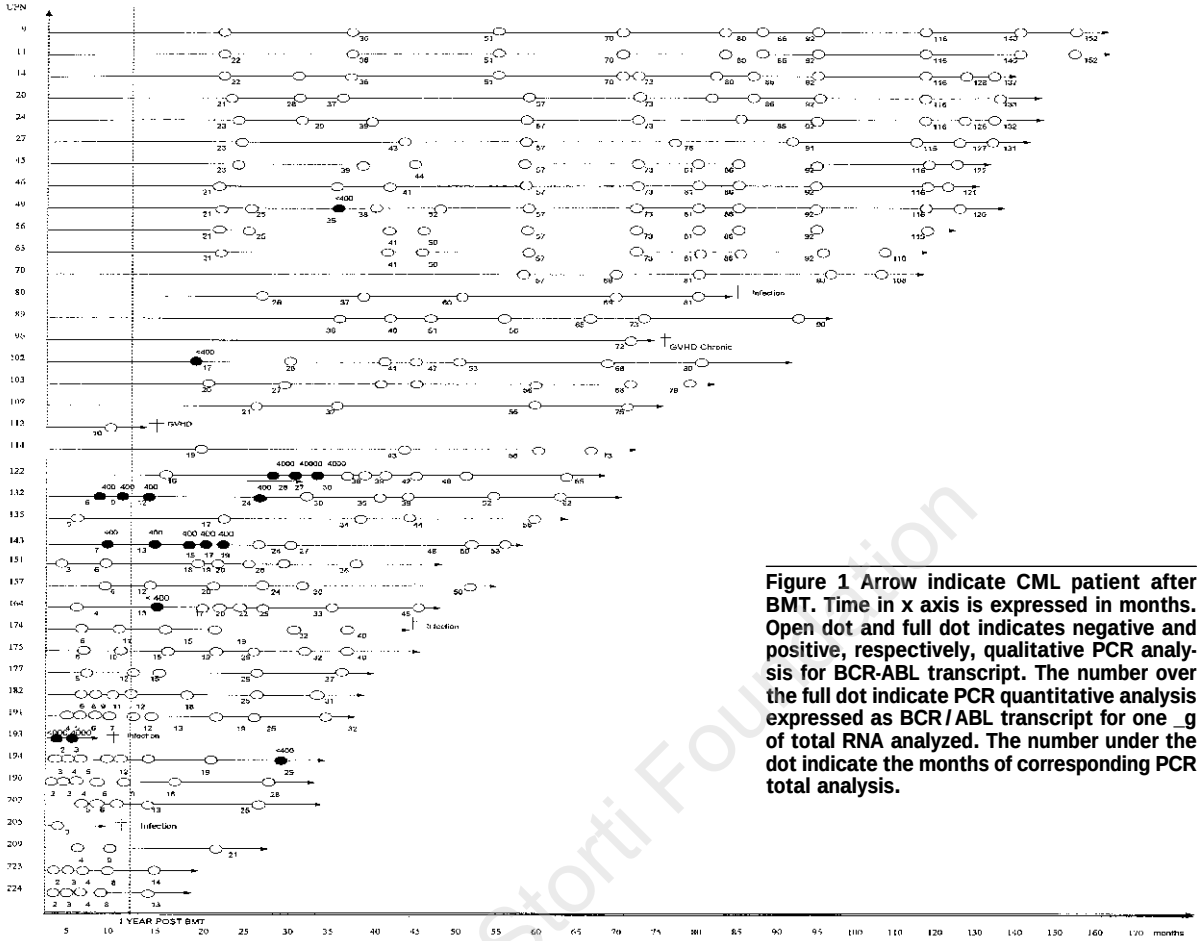

Figure 1 Arrow indicate CML patient after BMT. Time in x axis is expressed in months. Open dot and full dot indicates negative and positive, respectively, qualitative PCR analysis for BCR-ABL transcript. The number over the full dot indicate PCR quantitative analysis expressed as BCR/ABL transcript for one _g of total RNA analyzed. The number under the dot indicate the months of corresponding PCR total analysis.

therapeutic intervention (CsA discontinuation).

Quantitative PCR permitted identification of a subset of patients destined to relapse. In particular, none of the 6 group 1 patients (excluding UPN 193) with at least one positive assay but without a rising number of transcripts patients relapsed, and all them spontaneously returned to being PCR negative. The remaining chronic phase patient (UPN 122), who showed increasing expression of bcr-abl, subsequently obtained PCR negativity after α-IFN therapy (signed as an arrow in Figure 1).

On the other hand, in group 2, 3/4 (75%) patients (UPN 131, 185 and 187) who had repeated positive assays with rising numbers of transcripts later relapsed cytogenetically and clinically despite various forms of therapeutic intervention such as prophylactic CsA discontinuation, temporary α-IFN administration, or donor lymphocyte infusion (DLI) (Figure 2): the fourth patient (UPN 190) with increasing expression of bcr-abl, subsequently obtained PCR negativity after CsA discontinuation plus α-IFN therapy.

One patient (UPN 145) with a stable amount of bcr-abl transcript underwent a second transplant but died of sepsis 3 months later. The amount of bcr-abl seemed to decrease in this patient after second transplantation.

In the remaining 3 patients (UPN 98, 199 and 214) of group 2 with a high number of transcripts, the amount of transcript fell persistently (UPN 98, and 214: these patients are both currently in CR) or temporarily (UPN 199: relapse and died) to undetectable or reduced levels, respectively, after therapeutic intervention in the form of CsA discontinuation (UPN 199 and 214) or α-IFN therapy (UPN 98).

Discussion

The present study, in which quantitative PCR was used on a large series of Italian CML patients, highlights the need for different types of molecular monitoring for patients transplanted either in chronic or accelerated/blastic phases. After allogeneic BMT in the chronic phase, the vast majority of patients (97.5% in our series), including those who display sporadic PCR positivity, survive for many years. About 90% of these patients remained without detectable levels of MRD either by qualitative or quantitative PCR. In contrast, those patients transplanted in accelerated/blastic phase face a much poorer prognosis, with a relapse rate of about 40% in our series. We found that the patients who relapsed had more PCR^+ samples and higher levels of expression of the bcr-abl gene at quantitative PCR. The much higher levels of bcr-abl expres-

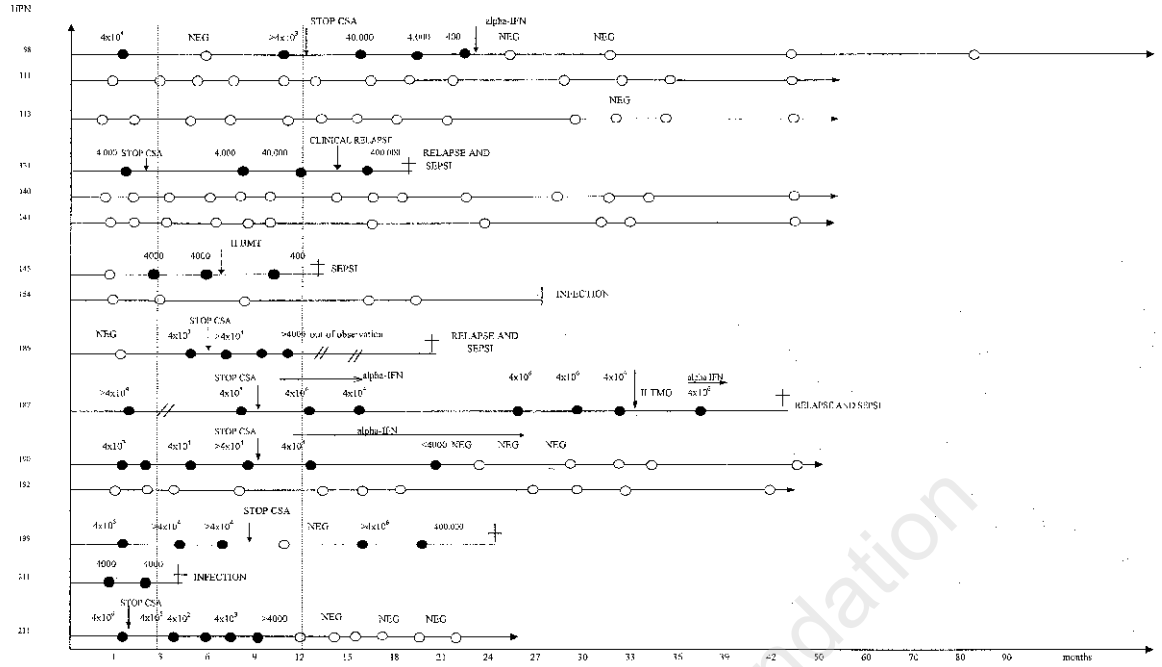

Figure 2 Arrow indicate CML patient after BMT. Time in x axis is expressed in months. Open dot and full dot indicates negative and positive, respectively, qualitative PCR analysis for BCR-ABL transcript. The number over the full dot indicate PCR quantitative analysis expressed as BCR/ABL transcript for one _g of total RNA analyzed. The number under the dot indicate the months of corresponding PCR total analysis.

sion (over 40,000 transcripts/μg RNA) and subsequent relapse in this group very likely reflects differences in the genotype of the blastic cells. On clinical grounds, it seems possible that quantitative PCR may be used in these higher risk patients to provide a warning of likely relapse and guide modifications of maintenance therapy.

Despite different molecular observations[13,19] we suggest that patients transplanted during chronic phase should receive molecular monitoring with quantitative PCR particularly for the first two years after BMT while in the subsequent years it could be necessary less frequently. In this group of patients, quantitative PCR can probably indicate the most suitable moment for suspending immunosuppressive therapy. On the other hand, our clinical findings and molecular data and those of others[13,19] strongly reinforce the concept that patients transplanted in accelerated or blastic phase must always continue to be considered at risk of relapse. We are in complete agreement with Goldman[19] that at least in the first 4 or 5 years after BMT this group of patients should receive frequent quantitative PCR analyses (eg. every 2-3 months). It has been suggested that this intensive form of monitoring could be efficiently performed on peripheral blood samples, thereby saving patients a great deal of discomfort.[20] In addition, and despite not many of the relapsing patients transplanted in advanced phase having responded to the different treatments, the data presented here seem to suggest that molecular monitoring, although not indispensable, could help the clinician to make early therapeutic decisions.

In conclusion, our findings provide further important evidence that frequent quantitative PCR, especially for patients transplanted in accelerated/blastic phase, does indeed provide practical indications capable of directing subsequent therapeutic interventions, such as suspension of CsA, administration of temporary α-IFN therapy and reinfusion of donor lymphocytes.

Potential implications for clinical practice

- Ourclinical findings and molecular data strongly underline the concept that patients transplanted in accelerated or blastic phase must always continue to be considered at risk of relapse.
- At least in the first 4 or 5 years after BMT this group of patients should receive frequent quantitative PCR analyses (eg. every 2-3 months).This intensive form of monitoring could be efficiently performed on peripheral blood samples, thereby saving patients a great deal of discomfort.
- The data presented here seem to suggest that molecular monitoring, although not indispensable, could help the clinician to make early therapeutic decisions.

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Contributions and Acknowledgments

GM was the principal investigator: he designed the study and was responsible for ethical approval of the program, was responsible for funding and direct supervision. VM developed and carried out cryopreservation procedures and flow cytometry assay. MA and CT set up PCR procedures and drafted the paper. NT and EO were responsible for cytogenetic. FB, GR and GB were responsible for clinical management of patients and clinical response of the project. GS and ST revised critically the manuscript and gave the final approval for publication.

The order of authorship has been made according to the substantial contribution given to the study.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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case of the month

Hemostasis & Thrombosis

Tacrolimus (FK 506) induced thrombotic thrombocytopenic purpura after ABO mismatched second liver transplantation: salvage with plasmapheresis and prostacyclin

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ABSTRACT

We report the course of thrombotic thrombocytopenic purpura (TTP) in a patient receiving tacrolimus (FK506) immunosuppression for an ABO mismatched second liver graft. A Chinese woman with fulminant hepatitis-B reactivation failed a living-related orthotopic liver transplantation (OLT) due to portal vein thrombosis. An ABO mismatched cadaveric OLT (group A to O) was performed, with peri-operative plasmapheresis to reduce anti-A hemagglutinin titers. On day 30, she developed fever, hemolysis, thrombocytopenia and neurologic dulling. Prominent microangiopathic features in peripheral blood film, and characteristic brain lesions on magnetic resonance imaging confirmed TTP. She responded initially to intensive plasmapheresis with cryosupernatant replacement, and withdrawal of FK506. An attempted reintroduction of FK506 for threatened rejection led to TTP exacerbation. This was controlled with prolonged plasmapheresis and a ten-day infusion of prostacyclin. Immunosuppression was changed to mycophenolate mofetil. By day 53, the peripheral film and lactate dehydrogenase level had returned to baseline and plasmapheresis was stopped.

Key words: tacrolimus, TTP

Acquired thrombotic thrombocytopenic purpura (TTP) is a rare microvascular hemolytic disease. The central feature in the pathogenesis of TTP is abnormal platelet aggregation in the microcirculation, secondary to abnormal platelet endothelial interaction.[1] Recent evidence has shown that many primary cases are of immune-mediated etiology.[2,3] However, in secondary cases, an immune etiology may or may not be implied.[4,5] A large number of drugs have been implicated as causative agents.[6] Some, such as cyclosporin and alkylating agents, have both been implicated as cause and also used for treatment. TTP has rarely been reported in the setting of orthotopic liver transplantation (OLT).[7,8] The prognosis of TTP is often poor, partly due to delayed diagnosis and definitive therapy, and partly due to the serious nature of the underlying conditions.[9] We report a case of tacrolimus (FK506) induced TTP after ABO mismatched second OLT. The disease ran a protracted clinical course, but the patients was successfully salvaged with intensive plasmapheresis, and prostacyclin infusion after FK506 withdrawal.

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Report of a case

A 44-year old Chinese woman (blood group O) underwent OLT from an ABO matched living sibling donor because of a fulminant flare-up of chronic hepatitis B virus (HBV) infection, under lamivudine coverage.[10,11] Graft failure occurred due to portal vein thrombosis. She underwent a second emergency OLT 17 days later from a cadaveric donor (blood group A). Peri-operative plasmapheresis (one plasma volume exchange: four liters), with fresh frozen plasma replacement was performed, immediately before her operation, and four and seven days after it. Serial monitoring of the anti-A hemagglutinin titers was performed, and levels were kept below 1 in 8.[6] For both OLTs, the immunosuppression used was prednisolone (20mg daily) and FK506 (target trough level 10-15 ng/mL),[12] with the addition of OKT3 induction (5 mg $\times$ 5 days) for the second OLT. One month after the second OLT, she was admitted to the intensive care unit with swinging fever, pancytopenia and a grossly elevated lactate dehydrogenase (LDH) level (506U/L normal: 200-360) (Figure 1). Her complete blood picture showed hemoglobin (Hb): 4.9g/L, white cell count (WCC): 5.9×10^9/L and platelet count (Plt): 15×10^9/L (uncorrected for red blood cell (RBC) fragments). A peripheral blood film showed severe red cell fragmentation, polychromasia, nucleated RBC and very severe thrombocytopenia. The coagulation profile was normal. Rapid clinical deterioration followed, with renal failure, sepsis and seizures. A magnetic resonance imaging (MRI) scan showed hyperintense white matter infiltrates in bilateral cerebellar hemispheres and occipital lobes, characteristic of TTP[13](Figure 2). Extensive screening for auto-immune antibodies, direct antiglobulin test (DAT), lupus anticoagulant and viral studies were negative, and her anti-A hemagglutinin titer was not