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Sensitive Replicate Real-Time Quantitative PCR of *BCR-ABL* Shows Deep Molecular Responses in Long-Term Post–Allogeneic Stem Cell Transplantation Chronic Myeloid Leukemia Patients

Maya Koren-Michowitz [1,2,*], Avichai Shimoni [1], Filomena Daraio [3], Francesca Crasto [3], Roberta Lorenzatti [3], Yulia Volchek [1], Ninette Amariglio [1], Enrico Gottardi [3], Giuseppe Saglio [3], Arnon Nagler [1,2]

[1] *Division of Hematology, Chaim Sheba Medical Center Tel Hashomer, Ramat-Gan, Israel*
[2] *Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel*
[3] *Division of Hematology and Internal Medicine, Department of Clinical and Biological Sciences of the University of Turin, San Luigi Gonzaga University Hospital, Turin, Italy*

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Real-time quantitative PCR (RT-qPCR) is commonly used for follow-up of chronic myeloid leukemia (CML) patients treated with tyrosine kinase inhibitors, but its current sensitivity does not allow detection of very low *BCR-ABL* levels. Therefore RT-qPCR negativity is not synonymous with complete molecular response. Replicate RT-qPCR had shown increased sensitivity in tyrosine kinase inhibitor–treated patients and was, therefore, used here to evaluate whether RT-qPCR–negative post–allogeneic stem cell transplantation (SCT) patients harbor detectable disease. Samples from 12 patients were tested at 2 time points using 82 replicates of *BCR-ABL* RT-qPCR. One patient (38 months after SCT) had detectable transcripts at baseline and none at the follow-up test, done at a median of 107 months after SCT. This suggests cure from CML in the majority of allogeneic SCT patients who have no transcripts detectable by replicate RT-qPCR for *BCR-ABL*.

INTRODUCTION

Treatment with ABL tyrosine kinase inhibitors (TKI) has become the cornerstone of chronic myeloid leukemia (CML) therapy in recent years. Chronic phase CML patients achieving deep molecular responses (MR) on long-term TKI therapy may be thought of as having a “functional” cure and withholding TKI treatment in patients with continuous deep responses is currently under intensive investigation [1,2]. Nevertheless, allogeneic (Allo) stem cell transplantation (SCT) is still the only modality with proven efficacy in advanced phase cases and is a valid option in TKI-resistant cases. Absence of *BCR-ABL* transcripts after SCT with sensitive methods (ie, achieving PCR negativity) correlates with long-term disease-free survival, whereas re-emergence of *BCR-ABL* transcripts has been shown to predict disease relapse and is, therefore, used to trigger therapeutic interventions, such as the addition of a TKI or donor lymphocyte infusion [3-6]. With the widespread use of PCR to detect *BCR-ABL* transcripts, it has become clear that achieving PCR negativity is not synonymous with molecular complete response but rather depends on the sensitivity of the method used and technical variables, such as the RNA quality in each specific sample [7]. Real-time quantitative PCR (RT-qPCR) is the most commonly used method for follow-up of CML

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* Correspondence and reprint requests: Maya Koren-Michowitz, Division of Hematology, The Chaim Sheba Medical Center, Tel-Hashomer, Ramat-Gan 52621, Israel.
E-mail address: m.koren.michowitz@gmail.com (M. Koren-Michowitz).
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Table 1
Characteristics of Study Patients

Patient No.	Age, yr	Disease Status at SCT	Time from Diagnosis to SCT, mo	Conditioning	Donor	Chronic GVHD
1	57	AP	97	RIC	MUD	+
2	30	CP2	6	MA	Sibling	+
3	48	CP2	273	MA	MUD	+
4	52	CP1	4	RIC	Sibling	+
5	34	CP1	16	MA	MUD	+
6	30	CP1	39	MA	Sibling	−
7	40	CP1	6	MA	Sibling	+
8	68	CP1	61	RIC	MUD	+
9	46	CP1	33	RIC	MUD	+
10	56	CP1	18	RIC	Sibling	+
11	38	AP	6	RIC	Sibling	+
12	23	CP2	3	MA	Sibling	+

GVHD indicates graft-versus-host disease; AP, accelerated phase; RIC, reduced-intensity conditioning; MUD, matched unrelated donor; CP, chronic phase; MA, myeloablative.

patients during TKI therapy. Guidelines have been published on when and how to perform it, as well as how to internationally standardize the PCR results [8]. With a sensitivity of approximately 10^{-4} for the commonly used RT-qPCR, progressive reductions in *BCR-ABL* transcript levels correlate well with long-term outcomes with TKI therapy but may not be sensitive enough to detect low-level transcript changes after Allo-SCT. Replicate RT-qPCR (rRT-qPCR) utilizes regular RT-qPCR methods but has the advantage of repeating the test using many more replicates. Studies have confirmed that the number of measurement repetitions is relevant in the detection of rare transcripts, and rRT-qPCR was found to be more sensitive than conventional RT-qPCR [9]. We, therefore, utilized this method to study whether *BCR-ABL* transcripts can still be detected in RT-qPCR–negative post-SCT CML patients after long-term follow-up.

SUBJECTS AND METHODS

Consecutive post-SCT CML patients undergoing follow-up in the SCT day care unit of the Sheba Medical Center in Israel were recruited to the study. Patients had to have at least 2 sequential RT-qPCR negative results before inclusion in the study, although they were not excluded from analysis if a concurrent RT-qPCR done at the same date of rRT-qPCR reported positive results. The study was approved by the local institutional review board, and all patients gave their informed consent. Peripheral blood was collected into EDTA-containing tubes at 2 separate time points. Buffy coats were immediately prepared and white blood cell pellets homogenized in the Trizol reagents were frozen at −80°C until further analysis.

rRT-qPCR

rRT-qPCR was done in the hematological molecular laboratory in Orbassano, Italy, as previously described [10-12]. Briefly, RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). cDNA synthesis was achieved by incubating at 25°C for 10 minutes and at 42°C for 60 minutes, and then the reaction was inactivated by heating at 99°C for 5 minutes. The qPCR reaction mix for each well included 2 uL of cDNA, 300 nM of each primer (*BCR-ABL* F-gatgctgaccaactcgtgtg; *BCR-ABL* R-aacgaaaaggttggggtcat; *ABL* F- gaagccgctcgttggaact; *ABL* R-cctaagacccggagcttttca), and 200 nM probe (*BCR-ABL* agaccctgaggctcaaagtcagatgctact; ABL-agagtgttatctccactggccacaaaatca). The assay was carried out in 82 wells for each patient's sample in custom-made plates (Applied Biosystems, Foster City, CA) using a LightCycler 480 analyzer (Roche) under the following conditions: 95°C for 10 minutes, 50 cycles including 95°C for 10 seconds, 60°C for 1 minutes for 50 cycles, and a cooling step at 45°C for 5 minutes.

All samples from each time point were studied at the same time. Results were reported as the mean percentage of *BCR-ABL* relative to *ABL* transcripts and normalized according to the international scale [8,13]. Blood from 14 healthy controls was used to determine the test background, determining the cutoff of 2.7 (mean + 2 SD) positive wells. Results were considered positive when above the background [14]. PCR sensitivity in each reaction was determined by calculating the total number of *ABL* transcripts detected in the specific test sample.

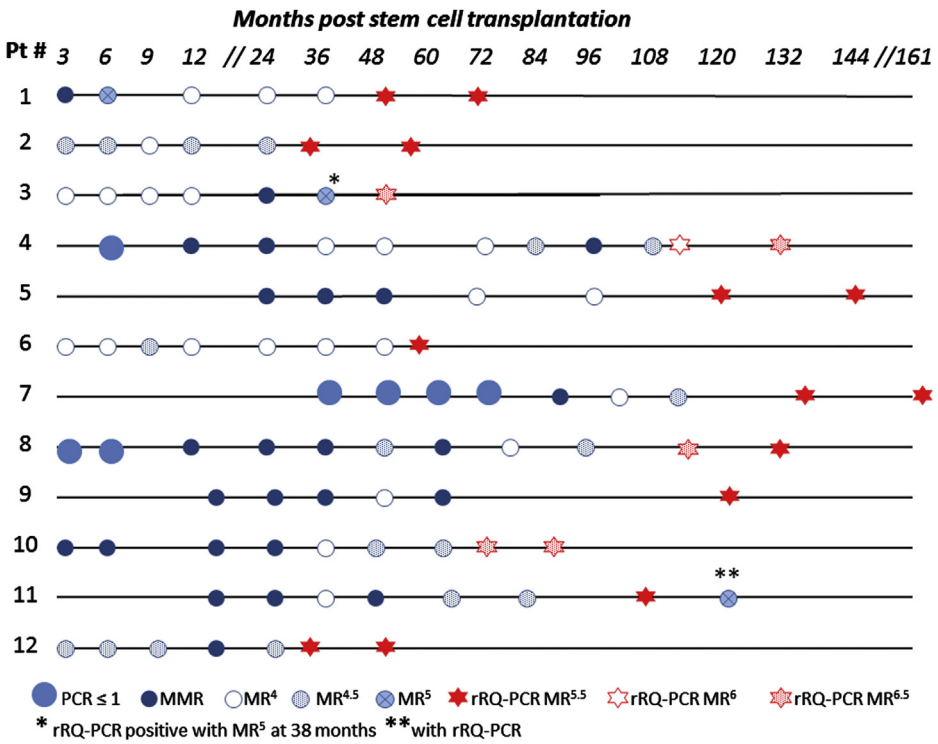

Figure 1. Individual patient RT-qPCR and rRT-qPCR results after Allo-SCT. Levels of molecular response for each patient at different time points after SCT using RT-qPCR (in *blue*) and rRT-qPCR (in *red*). MMR indicates major molecular response. (This figure is available in color online at www.bbmt.org).

Table 2
Results of Replicate RT-QPCR

Patient No.	Baseline				Follow-Up			
	Time from SCT, mo	*BCR- ABL/ ABL** (IS)	Total *ABL* Transcripts	Level of MR	Time from SCT, mo	*BCR- ABL/ ABL** (IS)	Total *ABL* Transcripts	Level of MR
1	49	0	575035	$MR^{5.5}$	71	0	597913	$MR^{5.5}$
2	35	.0000862†	705622	$MR^{5.5}$	55	0	646660	$MR^{5.5}$
3	38	.0007393‡	563238		47	0	1387143	$MR^{6.5}$
4	110	0	997523	MR^{6}	131	0	1263341	$MR^{6.5}$
5	123	0	555769	$MR^{5.5}$	145	0	690461	$MR^{5.5}$
6	58	0	700630	$MR^{5.5}$		NA		
7	139	0	874248	$MR^{5.5}$	161	0	950068	$MR^{5.5}$
8	109	.0000484†	1067353	$MR^{6.5}$	132	0	514529	$MR^{5.5}$
9	122	0	938046	$MR^{5.5}$		NA		
10	68	0	1467748	$MR^{6.5}$	89	0	505766	$MR^{5.5}$
11	104	0	776272	$MR^{5.5}$	125	0	271026	MR^{5}
12	29	0	860351	$MR^{5.5}$	51	0	532937	$MR^{5.5}$

IS indicates international scale; NA, not available.
* Mean result of positive wells normalized to the international scale.
† PCR positive in 1 and 2 wells of 82 tested, below the background cut-off.
‡ PCR positive in 6 wells of 82 tested.

RESULTS

Twelve CML patients were included in the study. Indications for SCT included advanced phase (accelerated phase -2, blast crisis -3) and TKI intolerance in chronic phase in 5 and 3 patients, respectively. Four patients underwent SCT as first-line therapy in the pre-imatinib era. Additional characteristics of the study patients are shown in Table 1. Results of regular RT-qPCR before inclusion in the study are given in Figure 1.

rRT-qPCR Results

At the first time point analysis, patients were at a median of 68 (range, 29 to 123) months after transplantation; 110 and 38 months for patients in chronic and advanced, respectively. *BCR-ABL* transcripts were detected in 3 of 12 patients but were above the background and, therefore, considered true positive in only 1 (Table 2). This test was in a patient 38 months after a myeloablative SCT for second chronic phase after a myeloid blast crisis. Interestingly, this patient had the longest disease duration before SCT and was not treated with a TKI until after the blast crisis had occurred (12 years after CML diagnosis).

Follow-up samples were available in 10 patients, drawn at a median of 22 months from baseline (median of 107 months after SCT). No *BCR-ABL* transcripts were detected in all samples, including the previously positive patient, at this time point.

Assay Sensitivity

By increasing RT-qPCR replicates number, the absolute number of *ABL* copies detected in 21 of the 22 assays done was above 320,000, supporting at least an $MR^{5.5}$. Of those, assay sensitivity has allowed for the confirmation of MR^{6} and $MR^{6.5}$ in 4 and 1 of the PCR negative patients, respectively. MR^{5} was confirmed in the last PCR-negative patient.

DISCUSSION

Molecular monitoring in *BCR-ABL*–positive leukemia has become the standard of care, with well-established associations between milestone response and long-term outcome, in particular for chronic phase CML patients [15]. Treatment with TKI is not curative according to traditional definitions; therefore, most TKI-treated patients have detectable *BCR-ABL* transcripts even after long-term therapy. Treatment cessation trials have shown increased RT-qPCR levels in the majority of those patients [1,2,16]. The graft-versus-leukemia effect of Allo-SCT, in contrast, could result in true cure, and no evidence of *BCR-ABL* transcripts should be found in long-term post-SCT CML patients. Indeed, using a highly sensitive RT-qPCR, all patients tested negative for *BCR-ABL* with a sensitivity of $10^{-5.5}$ to $10^{-6.5}$ when tested at least 47 months after SCT. Encouragingly, 5 of the 12 patients in the current study underwent transplantation because of advanced phase CML, suggesting that cure is feasible even in this poor prognosis subgroup.

rRT-qPCR is a highly sensitive method that can be used to determine *BCR-ABL* transcript levels, even when present at extremely low levels. Using this method to follow Allo-SCT patients could assist in determining post-SCT interventions; however, further studies are needed to clarify the exact clinical role of this method. Indeed, several highly sensitive PCR methods to detect *BCR-ABL* were recently described, including digital PCR for mRNA and patient-specific genomic DNA–based PCR [12,17,18]. A prospective trial using these different sensitive methods after Allo-SCT could be informative in this regard. Furthermore, follow-up with sensitive methods could also assist in clinical decisions regarding the addition of TKI in the post-SCT period as either consolidation/preemptive therapy or at early signs of molecular relapse. Finally, rRT-qPCR negativity in the SCT setting could probably be very near the definition of molecular disease cure.

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