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Evaluation of Minimal Residual Disease by Real-Time Quantitative PCR of Wilms' Tumor 1 Expression in Patients with Acute Myelogenous Leukemia after Allogeneic Stem Cell Transplantation: Correlation with Flow Cytometry and Chimerism

Mi Kwon,[1,2] Carolina Martínez-Laperche,[1,2] María Infante,[2] Fernando Carretero,[2] Pascual Balsalobre,[1,2] David Serrano,[1,2] Jorge Gayoso,[1,2] Ana Pérez-Corral,[2] Javier Anguita,[1,2] Jose Luis Díez-Martín,[1,2] Ismael Buño[1,2]

Relapse remains the main cause of treatment failure in patients with acute myelogeous leukemia (AML) after allogeneic hemopoietic stem cell transplantation (SCT). The Wilms' tumor 1 gene (WT1) is reportedly overexpressed in >90% of patients with AML and thus can be useful for minimal residual disease (MRD) monitoring. The aim of this study was to evaluate the usefulness of WT1 expression as a relapse predictor marker in patients with AML after SCT and compare it with flow cytometry (FC) and chimerism studies. WT1 expression was assessed retrospectively using quantitative RT-PCR in bone marrow and peripheral blood from 21 patients. Patients were classified according to WT1 dynamics posttransplantation. Eleven of the 21 patients had low and stable WT1 levels. All of these 11 patients showed complete chimerism and negative MRD by FC and remained in complete remission with a median follow-up of 27 months (range, 18-98 months). In contrast, 10 of 21 patients showed WT1 overexpression after SCT, and 9 of these 10 patients relapsed. The incidence of relapse differed significantly between the 2 groups of patients according to WT1 expression post-SCT ($P = .00003$). Relapse in the 9 patients occurred at a median of 314 days (range, 50-560 days). Interestingly, in these patients, relapse was first predicted by WT1 (with negative FC and complete chimerism) in 7 patients. WT1 overexpression was correlated with disease burden in patients with AML before and after allogeneic SCT. In patients who relapsed, both medullary and extramedullary relapse were better anticipated by WT1 overexpression compared with FC and chimerism.

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KEY WORDS: Molecular follow-up, Relapse, Early detection, Aberrant gene expression

INTRODUCTION

Relapse and progression remain the main causes of treatment failure in patients with acute myelogenous leukemia (AML) after undergoing allogeneic hemopoietic stem cell transplantation (SCT) and often presents as refractory disease. Thus, quantitative follow-up of minimal residual disease (MRD) after allogeneic SCT is of key importance for the early detection of relapse and the subsequent selection of patients who may benefit from modulation of immunosuppression or donor lymphocyte infusion (DLI). Various methods with different sensitivities, including flow cytometry (FC), chimerism, cytogenetics, and molecular analysis, have been used in this setting [1]. FC has become the gold standard for evaluating MRD in patients with acute lymphoblastic leukemia. However, up to 20% of patients with AML lack appropriate markers for MRD follow-up at diagnosis, and changes in the original immunophenotype might occur in relapse after SCT. Moreover, approximately 65% of patients with AML lack a specific fusion transcript that can serve as a molecular target for residual disease monitoring. Chimerism analysis by RT-PCR, although universally applicable in the postallogeneic

From the [1]Gregorio Marañon Institute for Health Research; and [2]Department of Hematology, Hospital General Universitario Gregorio Marañón, Madrid, Spain.

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Correspondence and reprint requests: Mi Kwon, MD, Department of Hematology, Hospital General Universitario Gregorio Marañón, Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain (e-mail: mkwon.hgugm@salud.madrid.org).

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Table 1. Characteristics of 21 Patients with AML

Case	Age, Years	Sex	FAB	Karyotype	FLT3/NPM1	Pre-SCT Status	TPH	Conditioning	Follow-Up, Months
1	44	F	M1	+4, +8	NA	Relapse		Bu/Flu	40
2	30	F	M5b	Normal	NA	CR1		Bu/CY	60
3	41	M	M4	+8	NA	CR1		Bu/CY	98
4	30	M	M1	Normal	Positive/negative	CR, MRD$^+$		TBI/CY	5
5	39	M	RAEB-II	Normal	Negative/negative	CR1, MRD$^+$			16
6	61	F	M5	Complex		Relapse			7.5
7	19	M	M1	NA	Negative/negative	CR1	HLA-identical sibling		15
8	41	M	M2	NA	Positive/positive	CR1			32
9	36	F	M0	NA	Positive/negative	CR1			11
10	31	M	M1	MLL+	Negative/negative	CR1		Bu/Flu	23
11	44	M	M1	Normal	Negative/negative	CR1			27
12	42	F	TD	Normal	Negative/negative	CR1, MRD$^+$			21
13	51	F	M6	Normal	Negative/negative	CR1			21
14	35	F	M4	Normal	NA	CR1			72
15	51	F	M4	Normal	Positive/positive	CR1	MUD		19
16	39	F	M1	Normal	Negative/NA	Relapse			5.6
17	44	M	M2	Complex		CR, MRD$^+$		Bu/CY	14
18	24	F	M4	Complex		Relapse			47.5
19	45	M	M1	Normal	Positive/negative	CR1	UCB/second-degree donor (dual)	Bu/Flu/CY/ATG	26
20	34	F	M4	Normal	Negative/positive	CR1			19
21	48	F	M2	Iso(17q)	NA	CR1	Haploidentical	Bu/Flu (RIC)	16

ATG indicates antithymocyte globulin; Bu, busulfan; CY, cyclophosphamide; Flu, fludarabine; NA, not available; UCB, unrelated cord blood; RIC, reduced-intensity conditioning; TBI, total body irradiation.

SCT setting, has a sensitivity of only 0.1% to 1%. In this context, new markers are needed to further contribute to the spectrum of follow-up markers in the posttransplantation period.

The Wilms' tumor 1 gene (WT1), located on chromosome 11p13, encodes a transcription factor with both oncogene and tumor suppressor functions [2]. WT1 is reportedly overexpressed in >90% of patients with AML and thus can be used for MRD monitoring by quantitative RT-PCR [2-7]. Normal hemopoietic cells have low WT1 expression, which can be readily distinguished from expression in AML cells by RT-PCR methods [5-7]. Several studies have analyzed the role of WTI as a marker for MRD in AML after chemotherapy and also as a predictor of relapse after induction and/or consolidation therapy [2,5,7-9]. A limited number of studies have focused on MRD monitoring by WT1 transcript expression in adults with AML after SCT [10-14]. The aim of this study was to evaluate the usefulness of WT1 expression as a marker predictive of relapse in patients with AML after SCT and to correlate the results with those from FC and chimerism studies.

PATIENTS AND METHODS

Patients

This study included 21 patients (12 females and 9 males) with AML who underwent allogeneic SCT in our bone marrow transplantation unit between 2003 and 2010 and had ARN samples available for WT1 analysis. Demographic and clinical data at the time of diagnosis and at transplantation are presented in Table 1. All patients had WT1 overexpression at diagnosis or during follow-up before transplantation.

The median patient age at transplantation was 41 years (range, 19-61 years). All patients had poor-risk AML with one or more of the following adverse features: AML secondary to myelodysplastic syndrome, high-risk cytogenetics, presence of FLT3-ITD, partial remission (PR), more than one cycle to achieve complete remission (CR), or disease status beyond first CR. Only 3 patients had a specific molecular marker for MRD monitoring (NPM1 mutation). Seventeen patients (80%) underwent myeloablative SCT (13 from a sibling donor and 4 from a matched unrelated donor), 3 underwent dual transplantation (a single cord blood unit with coinfusion of selected CD34$^+$ cells from a non–HLA-identical related donor), and 1 patient underwent a reduced-intensity conditioning SCT with a haploidentical related donor (Table 1). Response to therapy before and after transplantation was assessed based on National Cancer Institute criteria, as revised by the International Working Group in AML [15].

Samples

WT1 expression was assessed retrospectively in a total of 300 samples, including 184 peripheral blood (PB) samples and 116 bone marrow (BM) samples, from the 21 patients (median, 15 samples per patient). BM samples for chimerism and MRD analysis were obtained before SCT and on days +30, +100, +180, and +365 post-SCT, and then once a year thereafter. PB samples were obtained for chimerism determination starting on day +14 post-SCT. Patients with mixed chimerism (MC) were studied in PB every other week until complete chimerism (CC). Once CC was

achieved, studies were performed monthly during the first year post-SCT, and every other month thereafter.

In addition, 12 BM samples and 11 PB samples from healthy volunteer donors were tested as negative controls, to define cutoff values of overexpression. All samples were obtained after the patients and the donors provided signed informed consent.

Quantitative Assessment of WT1 Transcript Expression

Quantitative assessment of the WT1 transcript amount was performed by quantitative RT-PCR. Total RNA was purified from PB and BM samples using TRIzol (Life Technologies, Carlsbad, CA), following the manufacturer's instructions. cDNA (30 μL) was synthesized by reverse-transcription from 1 μg of total RNA using Applied Biosystems High-Capacity RNA-to-c-DNA Master Mix (Life Technologies) following the manufacturer's instructions and stored at −20°C until use. RT-PCR was performed in a Light Cycler 1.5 (Roche Applied Science, Mannheim, Germany) using GUS as a reference gene and the K562 cell line as a calibrator. The RT-PCR primers and Taqman probes (TIB Molbiol, Berlin, Germany) for WT1 transcripts [16] were as follows: forward, 5′-ACAG GGTACGAGAGCGATAACCA-3′ (exon 6, nt position: 1205-1227, N,M_024426); reverse, 5′-CACACG TCGCACATCCTGAAT-3′ (exon 6/7, nt position: 1289-1309, NM_024426), probe 5′-6FAM-CAACGC CCATCCTCTGCGGAGCCCA-TAMRA-3′ (exon 6, nt position: 1230-1254, NM_024426). The RT-PCR primers and Taqman probes for GUS [17] were as follows: forward, 5′-GAAAATATGTGGTTGGAGAGC TCATT- 3′ (nt position: 1759-1785; GenBank accession no. M15182); reverse, 5′-CCGAGTGAAG ATCCCCTTTTTA-3′ (nt position: 1833-1859; GeneBank accession no. M15182), and probe 5′-6FAM-CCAGCACTCTCGTCGGTGACTGTTCA-TAM RA-3′ (nt position: 1859-1881; GenBank accession no. M15182). The PCR reactions were carried out in a total volume of 10 μL containing 1 × TaqMan Master Mix (Roche Diagnostics), 0.5 μmol/L of each primer, 0.2 μmol/L of probe, and 3 μL of cDNA. The reaction conditions were 10 minutes at 95°C and 45 cycles of 94°C for 10 seconds, then 60°C for 30 seconds. In all experiments, the K562 cell line was used as a positive control, and sterilized water was used as a nontemplate control.

All experiments were performed in duplicate, and the mean value was recorded for further calculations. If the results showed a discrepancy of >1 Ct between duplicates, then the assay was repeated. Results were analyzed with LightCycler SW 3.5 software package (Roche Diagnostics). *WT1* gene expression was calculated by relative quantification [18] using the normalized ratio of the target gene (*WT1*) in relation to a reference gene (*GUS*) and taking cell line K562 as the control (calibrator) sample. Results for each sample were expressed as the percentage of the control, which was arbitrarily assumed to have 100% WT1 expression.

FC Analysis of MRD

Leukemic aberrant phenotypes at diagnosis were identified by 4-color combinations of monoclonal antibodies and used for subsequent MRD detection by multiparametric FC with a Cytomics FC-500 flow cytometer (Beckman-Coulter, Brea, CA) with the following combinations of monoclonal antibodies (mAbs): FITC, PE, PE-Texas Red, and PE-Cy5: CD13/CD33/CD45/CD34, CD38/CD56/CD45/CD34, CD15/CD14/CD45/CD34, CD15/CD13/CD34/CD33, CD64/CD11B/CD45/CD34, HLA-DR/CD117/CD34/CD33, CD5/CD117/CD34/CD33, CD7/CD34/CD45/CD2, CD20/CD19/CD34/CD45, CD41/CD235a/CD45/CD34, and CD45/CD4/CD8/CD3. All mAbs were purchased from Beckman-Coulter. To enhance the sensitivity of the analysis, data acquisition in the flow cytometer was performed in 2 consecutive steps with a live gate, and information was collected for at least 10^6 bone marrow-nucleated cells. The CXP acquisition program (Beckman Coulter) was used for acquisition. INFINICYT software (Cytognos SL, Salamanca, Spain) was used for further data analysis [19]. A percentage of leukemic cells exceeding 0.1% was considered a positive result.

Chimerism Analysis

Chimerism analysis was performed by short tandem repeat PCR (STR-PCR). Total genomic DNA was purified from BM and PB samples using the Maxwell 16 Blood DNA Purification Kit (Promega, Madison, WI) following the manufacturer's instruction. Multiplex STR-PCR was performed with 2 ng of genomic DNA using the AmpFlSTR SGM Plus Kit (Life Technologies), which contains 10 STR loci plus the X-Y homolgous amelogenin gene labeled on 3 different colors (blue, 5-FAM; green, JOE; yellow, NED). Amplified PCR products were subjected to fragment analysis by capillary electrophoresis in an ABI Prism 3100 automated DNA sequencer (Life Technologies) under conditions recommended by the manufacturer. Chimerism was quantified with Genemapper 4.0 (Life Technologies), using the area in pixels under diagnostic allele peaks to estimate the amounts of donor and recipient DNA [20].

Statistical Analysis

Cutoff values of overexpression were defined as median + 2 SD of the WT1 expression values found in control PB and BM samples from healthy donors. Clinically relevant positive levels were considered after 2 consecutive measurements above the cutoff value. Fisher's exact test was used to evaluate the association between WT1 overexpression and post-SCT relapse.

RESULTS

WT1 Transcript Expression in Normal PB and BM Samples

To determine the cutoff value for pathological overexpression of WT1 in our laboratory, WT1 transcript expression was examined in 12 BM samples and 11 PB samples from healthy volunteer controls. Varying levels of WT1 expression were detected in the PB (mean, 0.007%; range, 0.001%-0.019%) and BM (mean, 0.26%; range, 0.004%-0.42%) samples. According to the mean + 2 SD cutoff, values >0.025% in PB and >0.55% in BM were considered positive results (Figure 1).

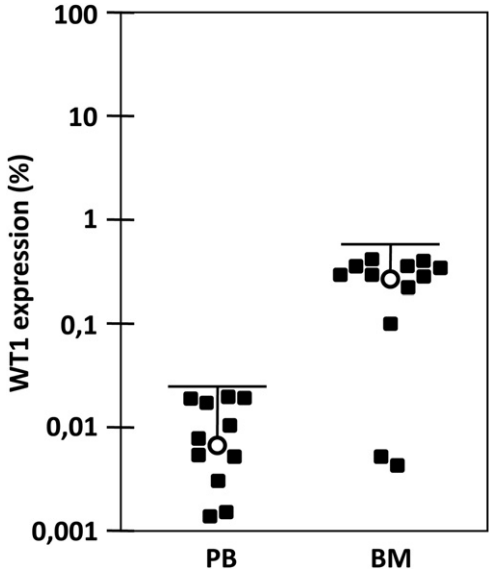

Figure 1. Results of WT1 expression in normal PB and BM samples to calculate the cutoff level (mean + 2 SD) for positivity in patient samples (PB, 0.025%; BM, 0.55%).

WT1 Expression Pre-SCT

WT1 expression was correlated with the presence of disease before SCT (Table 2). In fact, 8 patients exhibited disease at the time of transplantation (4 relapsed and 4 MRD-positive by FC). All 7 of these patients studied were positive for WT1. The remaining 13 patients underwent SCT while in CR with negative MRD by FC, and none of 10 patients studied showed WT1 overexpression. Of note, 3 of the 4 patients in CR with positive MRD by FC and WT1 experienced relapse after SCT.

WT1 Expression Post-SCT

Chimerism determination was available in all of the 116 post-SCT BM samples, and MRD assessment by FC was available in 77 of these samples. After SCT, patients were classified according to WT1 dynamics (Figure 2). Eleven of the 21 patients showed negative and stable WT1 levels in PB and BM on follow-up assessment (Figure 2A and B). All of these 11 patients showed CC and negative MRD by FC (Table 2) and remained in CR with a median follow-up of 27 months (range, 18-98 months). In 3 patients, NPM1 mutation was available for molecular MRD monitoring and also remained negative throughout the follow-up. In this group of patients, significant (up to 1 log) increases

Table 2. Correlation of the Results Obtained from the Follow-Up of MRD by WT1 Expression, Chimerism, and FC

Case	Pre-SCT Status	WT1 Pre-SCT	Day of Positive WT1 Post-SCT[a]	Days from Positive WT1 to Relapse	Day of Appearance of MC	Day of Positive FC	Evolution/Status at Last Follow-Up
1	Relapse	NA	No	-	-	-	CR, alive
2	CR	NA	No	-	-	-	CR, alive
3	CR	Negative	No	-	-	-	CR, alive
4	CR, MRD$^+$	Positive	Continuous (PB, BM)	140	+104	+153	Relapse day +153, exitus
5	CRI, MRD$^+$	Positive	+118 (PB)	149	Continuous	+267	Relapse day +267, exitus
6	Relapse	Positive	+50 (PB)	0	+50	-	Relapse day +50, exitus
7	CR1	NA	+147 (BM)	-	-	-	CR, alive
8	CR1	Negative	No	-	-	-	CR, alive
9	CR1	NA	+260 (PB)	54	+314	+314	Relapse day +314, alive
10	CR1	Negative	+192 (PB)	283	-	+475	Relapse day +475, alive
11	CR1	Negative	No	-	-	-	CR, alive
12	CRI, MRD$^+$	Positive	No	-	-	-	CR, alive
13	CR1	Negative	No	-	-	-	CR, alive
14	CR1	Negative	No	-	-	-	CR, alive
15	CR1	Negative	No	-	-	-	CR, alive
16	Relapse	Positive	+43	57	+100	NA	Relapse day +100, exitus
17	CR, MRD$^+$	Positive	+285 (BM)	52	+337	+285	Relapse day +337, exitus
18	Relapse	Positive	Continuous	134	+314	NA	Relapse day +314, alive
19	CR1	Negative	No	-	-	-	CR, alive
20	CR1	Negative	No	-	-	-	CR, alive
21	CR1	Negative	+100	460	+292	NA	Relapse day +560, exitus

NA indicates not available.
To show the ability to anticipate relapse from WT1 expression follow-up, days from positive WT1 expression and hematologic relapse are shown.
[a]Positive WT1 was defined as 2 consecutive evaluations with values above the cutoff level for either PB or BM.

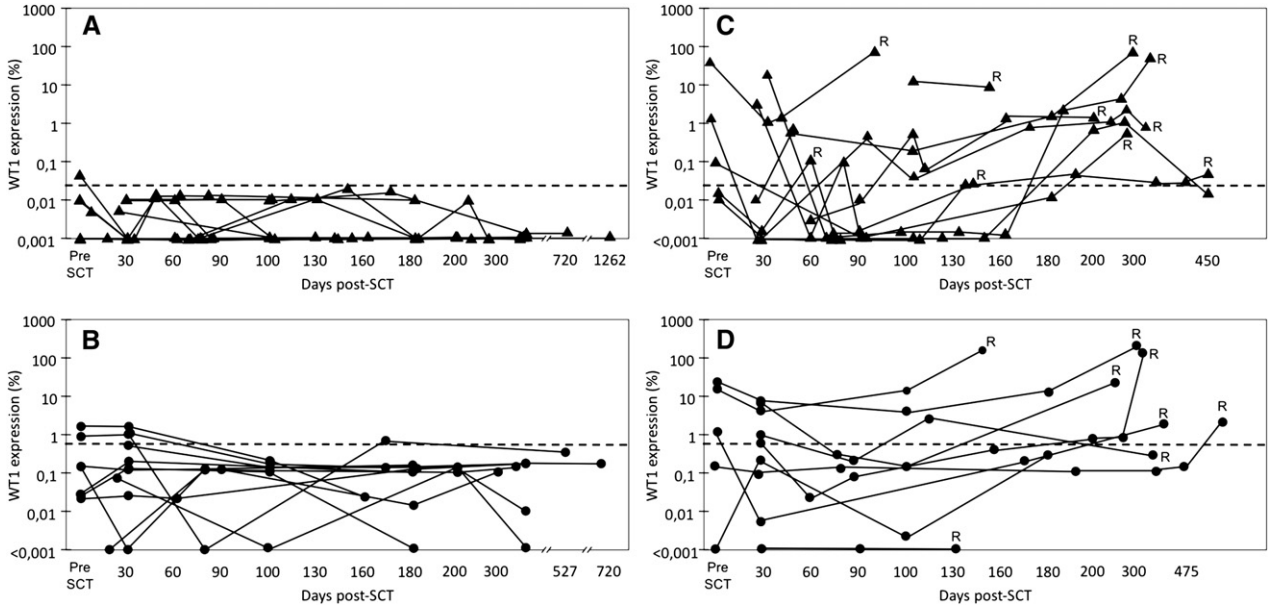

Figure 2. Evolution of WT1 expression in PB (upper panels, triangles) and BM (lower panels, circles) in patients with low and stable WT1 levels (left panels) and patients with WT1 overexpression (right panels) after SCT. R, relapse post-SCT.

in WT1 expression were observed below the cutoff value, followed by a further decrease in or stabilization of the WT1 expression level. Only 1 patient (patient 8) showed a significant increase in BM above the cutoff value with a further decrease below the positivity level while remaining in CR (Table 2 and Figure 2).

In contrast, 10 of the 21 patients exhibited WT1 overexpression after SCT (Table 2 and Figure 2C and D). Nine of these 10 patients relapsed (all except patient 7) (Figures 3 and S1). Among the 9 patients who relapsed, 6 had initial negative values followed by a significant increase in WT1 expression, reaching positive levels in a median of 155 days (range, 50-285 days) after SCT and had positive values thereafter (Figures 3 and S1). In contrast, 3 of the patients who relapsed exhibited positive high values continuously throughout the follow-up. Among the 9 relapsed patients, 2 had positive levels only in PB samples, 1 had a positive level only in a BM sample, and 6 had positive levels in both BM and PB samples (Figure 3). Three patients with extramedullary relapse had WT1 overexpression in PB only in 2 cases and in both PB and BM in 1 case.

Relapse in the 9 patients occurred within a median of 314 days (range, 50-560 days) after SCT. In one case, WT1 overexpression together with MC occurred at the time of hematologic relapse, whereas in the other 8 patients, WT1 turned positive preceding relapse at a median of 137 days (range, 52-462 days) (Table 2). Interestingly, in 7 of these 8 relapsed patients, relapse was first predicted by positive WT1 (with negative FC and CC), whereas the remaining patient had MC together with positive WT1 (FC data not available) (Figure 3).

The incidence of relapse differed significantly ($P = .00003$) between the 2 groups of patients according to WT1 expression post-SCT. None of the 11 patients without WT1 overexpression after SCT relapsed, whereas 9 of 10 patients with WT1 overexpression relapsed (Table 2 and Figure 2).

For 73 data points, both PB and BM were available. Results obtained in both sources were highly correlated (Figure S2). In fact, 19 data points/samples were positive, and 40 were negative in both PB and BM, whereas only 14 samples had discordant results. Nine samples from 6 patients were negative in PB but positive in BM. Five of these 6 patients did not relapse and had positive WT1 results in BM early after SCT, with negative values thereafter in PB and BM. One patient had positive WT1 in BM but negative WT1 in PB after hematologic relapse. On the other hand, 5 samples from 5 different patients showed positive WT1 results in PB but negative results in BM. Four of these patients relapsed. In 1 patient, the positive result in PB was detected immediately after SCT, whereas in the other 3 patients, PB turned positive while remained BM negative; these patients experienced extramedullary relapse.

DISCUSSION

Detection and monitoring of MRD in patients with AML after SCT together with chimerism analysis are important to assess remission and early relapse management [1,10-14]. Unfortunately, only 40% of AML cases show a specific molecular marker for MRD evaluation. Because more than 90% of patients with AML show WT1 overexpression, WT1 has

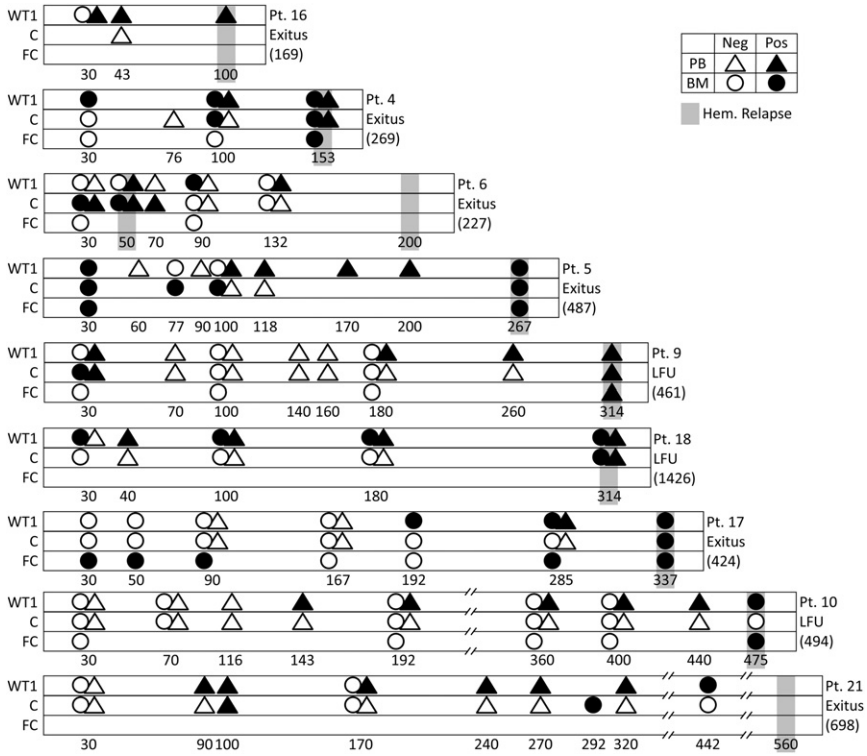

Figure 3. Comparison of the results obtained from WT1 expression, chimerism (C), and FC for the follow up of MRD in PB (triangles) and BM (circles) from patients who relapsed after SCT. Positive results are identified by solid black symbols (triangles or circles), and negative results appear as hollow white symbols. Contiguous symbols (triangles and circles) represent PB and BM samples obtained on the same day. Vertical gray bars indicate the time of hematologic relapse. Patient numbers are identical to those in Tables 1 and 2 and Figure S1.

been proposed as a useful marker for disease monitoring, including evaluation of MRD after chemotherapy or SCT, as long as a quantitative RT-PCR method is used [10-14].

The variable expression of WT1 in normal PB and BM must be addressed before WT1 can be used as an MRD marker [1-7,8,10]. In our laboratory, we determined the cutoff value for pathological overexpression of WT1 by evaluating BM and PB samples from healthy volunteer donors. Positive values were defined as the median + 2 SD of WT1 expression in normal samples.

We found a good correlation between WT1 gene expression and disease status before and after allogeneic SCT. Before transplantation, WT1 was positive in all patients with disease in a visible phase or with positive MRD by FC (7 of 7) and was negative in the patients with negative MRD as measured by FC (0 of 10) (Table 2). During the posttransplantation period, high concordance between normal WT1 expression level and remission status was seen in patients in CR. In all patients, WT1 levels remained below cutoff levels throughout the follow-up period and were consistent with morphological and immunophenotypic BM analysis, PB and BM chimerism testing, and detection of molecular-specific markers (when available).

Conversely, the majority of patients (9 of 10) with posttransplantation WT1 overexpression experienced disease recurrence (Figures 3 and S1). None of these patients had a low WT1 level at the time of hematologic relapse, confirming that WT1 overexpression is a marker of AML activity. WT1 positivity occurred significantly earlier than hematologic relapse in 8 of 9 patients and also earlier than conversion from full donor to MC or FC positivity in 7 of these 8 patients, demonstrating the predictive capacity of WT1 for hematologic relapse (Figure 3). In concordance with previous studies [12,13], variability in the kinetics of WT1 increase was seen in the patients who relapsed after allogeneic SCT; some of the patients displayed a low and steady rise of WT1 transcripts, whereas others had a rapid growth in gene expression level. However, the molecular diagnosis and morphological diagnosis of AML relapse were concomitant in only 1 patient (Figure 2). Therefore, patients in CR after SCT but with a progressive and slow increase in WT1 levels and without signs of graft-versus-host disease might be candidates for modulation of immune suppression either by suspension of drug therapy or, in selected cases, by preemptive DLI [11-14] to avoid disease relapse.

Good correlation of WT1 expression in PB and BM was observed (Figure S2). Six patients had a negative PB sample with a positive BM sample; however, this occurred early after SCT, with negative PB and

BM seen thereafter, and thus this observation has no clinical implications. Conversely, 5 patients had a positive PB sample with a negative BM sample. Four of these patients relapsed, 3 with extramedullary relapse. Interestingly, the patients with extramedullary disease, although not anticipated by WT1 level in BM, had WT1 overexpression in PB samples, which could allow appropriate anticipation of disease relapse (Figures 3 and S1). We hypothesize that the increased capability to detect AML cells in the PB in this context could be due to the higher sensitivity of the assay in PB compared with BM, as well as the probable detection of circulating leukemic cells derived from extramedullary sites. Considering the high correlation between the results in PB and BM, the higher sensitivity of the assay in PB, and the greater ability to detect extramedullary relapses, it can be argued that PB analysis is suitable for the follow-up of WT1 expression in patients with AML after SCT.

Our results show that absolute WT1 values are less clinically informative than WT1 expression dynamics in patient follow-up. In this scenario, the relative quantification approach used here is fully applicable for the follow-up of MRD in patients with AML on a routine clinical basis. Moreover, any control sample (K562 in our case) could be used to normalize WT1 expression values, provided that it is available for this purpose. Finally, being equally useful, this approach is faster, cheaper, and easier compared with techniques based on absolute quantifications.

In conclusion, our findings indicate that WT1 overexpression was correlated with disease burden in patients with AML before and after allogeneic SCT. In relapsed patients, both medullar and extramedullar relapse were significantly anticipated by WT1 overexpression compared with FC and chimerism. Because immunotherapy is clearly more effective for patients with a low tumor burden (ie, at molecular relapse) [21], monitoring of MRD by quantitative assessment of WT1 level may have a favorable impact on the prognosis of relapsed patients after allogeneic SCT. Therefore, quantification of WT1 overexpression by quantitative RT-PCR should be used for MRD detection during the post-SCT follow-up after SCT in patients with AML to facilitate immunosuppressive therapy and identify candidates for early DLI.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2012.01.012.

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