Autologous

Multigene Measurable Residual Disease Assessment Improves Acute Myeloid Leukemia Relapse Risk Stratification in Autologous Hematopoietic Cell Transplantation

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A B S T R A C T

We report here the largest study to date of adult patients with acute myeloid leukemia (AML) tested for measurable residual disease (MRD) at the time of autologous hematopoietic cell transplantation (auto-HCT). Seventy-two adult patients who underwent transplantation between 2004 and 2013 at a single academic medical center (University of California San Francisco) were eligible for this retrospective study based on availability of cryopreserved granulocyte colony–stimulating factor (GCSF)–mobilized autologous peripheral blood progenitor cell (PBPC) leukapheresis specimens ("autografts"). Autograft MRD was assessed by molecular methods (real-time quantitative PCR [RQ-PCR] for Wilms tumor 1 (WT1) alone or a multigene panel) and by multiparameter flow cytometry (MPFC). WT1 RQ-PCR testing of the autograft had low sensitivity for relapse prediction (14%) and a negative predictive value of 51%. MPFC failed to identify MRD in any of 34 autografts tested. Combinations of molecular MRD assays, however, improved prediction of post–auto-HCT relapse. In multivariate analysis of clinical variables, including age, gender, race, cytogenetic risk category, and CD34+ cell dose, only autograft multigene MRD as assessed by RQ-PCR was statistically significantly associated with relapse. One year after transplantation, only 28% patients with detectable autograft MRD were relapse free, compared with 67% in the MRD-negative cohort. Multigene MRD, while an improvement on other methods tested, was however suboptimal for relapse prediction in unselected patients, with specificity of 83% and sensitivity of 46%. In patients with known chromosomal abnormalities or mutations, however, better predictive value was observed with no relapses observed in MRD-negative patients in the first year after auto-HCT compared with 83% incidence of relapse in the MRD-positive patients (hazard ratio, 12.45; *P* = .0016). In summary, increased personalization of MRD monitoring by use of a multigene panel improved the ability to risk stratify patients for post–auto-HCT relapse. WT1 RQ-PCR and flow cytometric assessment for AML MRD in autograft samples had limited value for predicting relapse after auto-HCT. We demonstrate that cryopreserved autograft material presents unique challenges for AML MRD testing because of the masking effects of previous GCSF exposure on gene expression and flow cytometry signatures. In the absence of information regarding diagnostic characteristics, sources other than GCSF-stimulated PBSC leukapheresis specimens should be considered as alternatives for MRD testing in AML patients undergoing auto-HCT.

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INTRODUCTION

The role of autologous hematopoietic cell transplantation (auto-HCT) in acute myeloid leukemia (AML) remains

controversial [1-16]. Although commonly used in other hematological malignancies, auto-HCT has not been widely adopted in AML because of concerns regarding high post-transplantation relapse rates. These relapses may be due, in part, to autograft contamination with AML. High-sensitivity methods to detect residual AML after treatment are now available and have demonstrated the ability to identify those patients in morphological complete remission but at increased risk of relapse [17-28]. Such minimal, or more

correctly measurable [29], residual disease (MRD) assessments are increasingly recognized as an important factor in risk-adapted AML therapy and may be particularly important for predicting which patients achieve long-term disease-free survival after auto-HCT. Early trials evaluating the efficacy of auto-HCT as postremission therapy in AML did not, however, include assessment of MRD [30-33].

Measurement of Wilms tumor 1 (WT1) transcript levels using real-time quantitative PCR (RQ-PCR) is a common method for detecting MRD in AML. However, because of heterogeneous expression levels in AML, such testing only has utility for the subset of patients with sufficient leukemia-associated WT1 overexpression [34]. Messina et al. recently assessed WT1 levels in the leukapheresis product (LP) of granulocyte colony–stimulating factor (GCSF)–stimulated peripheral blood ("autografts") from 30 consecutive AML patients undergoing auto-HCT [35]. They reported that those with higher levels of WT1 in the autograft (n = 9) had, as a group, inferior relapse-free survival and overall survival than those with lower levels. The authors correctly noted that the size of the cohort was insufficient to provide a strong recommendation about postremission consolidation strategy. It has recently been suggested that MRD status might be used to help select AML patients who may achieve long-term freedom from relapse using auto-HCT [4].

In this study, we performed MRD analysis on the largest cohort of AML auto-HCT autograft samples (n = 72) to date using not only WT1 but also multigene RQ-PCR testing, including WT1, MSLN, PRAME, PRTN3, CCNA1, t(8;21), inv(16), t(15;17), and mutated NPM1. The heterogeneity of AML makes a multitarget approach for MRD attractive; we recently showed that a multigene panel for MRD assessment significantly improved sensitivity to predict relapse compared with using WT1 expression alone in patients undergoing allogeneic (allo) HCT at the National Institutes of Health (NIH) [19]. In addition, as there is no consensus as to the superiority of PCR-based or multiparameter flow cytometry (MPFC)–based MRD detection, we evaluated a subset of these autografts via both MPFC and RQ-PCR.

METHODS
Auto-HCT
All patients ages ≥18 with AML who underwent auto-HCT at the University of California, San Francisco (UCSF) Medical Center between June 1988 and July 2013 with cryopreserved autologous stem cell aliquots were considered eligible for this study. All samples were collected on institutional review board–approved research protocols. Disease classification was determined by the refined Medical Research Council cytogenetic classification system [36]. Patients received peripheral blood progenitor cell (PBPC) grafts and underwent an in vivo purge as part of stem cell mobilization and collection, which included i.v. cytarabine 16 g/m^2 cumulative dose and i.v. etoposide 40 mg/kg cumulative dose over 4 days, as described previously [37,38]. The target CD34-positive hematopoietic progenitor cell dose was 5×10^6/kg to 10×10^6/kg, with a minimum dose of 2×10^6/kg required to proceed to auto-HCT. The preparative regimen consisted of busulfan .8 mg/kg i.v. for 16 doses over 4 days, as well as a single dose of etoposide 60 mg/kg i.v. given 3 days before stem cell infusion. Patients were treated with auto-HCT initially as part of an institutional research protocol and then as standard institutional practice starting in the early 2000s, with the majority of patients receiving auto-HCT because of lack of an appropriate allo-HCT donor. Five of the 72 patients were treated on cooperative group research studies (CALGB 10503 and CALGB 19808). Nine patients received targeted dose-escalated busulfan on an institutional research protocol [39,40].

MRD Detection by MPFC
Flow cytometry was performed at Fred Hutchinson Cancer Research Center. Immunophenotypic data from initial disease presentation were not available; therefore, attempts to detect MRD were made by identification of populations showing deviation from the normal patterns of antigen expression seen on specific cell lineages at specific stages of maturation as compared with either normal or regenerating marrow. When identified, the abnormal population was quantified as a percentage of the total CD45$^+$ white cell events. MRD was assessed on cryopreserved autograft PBPC aliquots with up to 1 million events per tube.

MRD Detection by RQ-PCR
RQ-PCR was performed at the NIH. RNA was isolated from up to 10 million cells using the Qiagen Allprep kit (Qiagen, Valencia, CA). RNA quantity and integrity was assessed using a Nanodrop 1000 Spectrophotometer (Wilmington, DE) and an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit (Santa Clara, CA). The RT2 Firststrand kit (Qiagen) was used for gDNA elimination and cDNA synthesis from 1 µg of total RNA.

Previously validated assays for WT1 [34], NPM1 mutations (type A, B, D) [22], and chromosomal translocations t(8;21), inv(16), and t(15;17) [41] were utilized and thermocycled in a RotorgeneQ thermocycler (Qiagen). Cycling conditions described in detail elsewhere (see Supplemental Methods) were used with 100 ng RNA equivalent cDNA in a 25 µL reaction volume using Taqman Universal MasterMix (Qiagen) [22,34,41]. Overexpression of AML associated gene transcripts in the multigene MRD array were measured using custom-built RQ-PCR arrays as previously reported [19] with SYBR Green Mastermix (Qiagen) and an ABI7900 thermocycler (Applied Biosystems, Foster City, CA).

Further details on methods and analysis can be found in the Supplemental Methods.

RESULTS
Patient Characteristics and Outcomes
Of 334 consecutive adult AML patients who underwent auto-HCT for AML at UCSF between June 1988 and July 2013, 72 (22%) were eligible for this study based on availability of viably cryopreserved PBPC aliquots. An additional 2 samples were identified but rejected because of insufficient RNA quality (Figure 1). Baseline patient characteristics are shown in Table 1. The median age was 48 (range, 24 to 69) and 99% were in complete remission at the time of transplantation.

Relapse free survival at 1 year after auto-HCT was 50%, with 31 of 37 observed relapses occurring in the first year after transplantation. Three- and 5-year overall survival rates were 66% and 56%, respectively. These outcomes are representative of the overall clinical experience at this center. The majority of patients in this cohort (65%) had intermediate cytogenetic risk, of whom 54% remained alive and 39% remained

Table 1
Patient Characteristics

Characteristic	Value
No. of patients	72 (100)
Male sex	33 (46)
Age at autoHCT, yr	48 (24-69)
Cytogenetic risk classification	
Favorable	17 (24)
Intermediate	47 (65)
Poor/secondary	4 (5)
Unknown	4 (5)
Remission status at autoHCT	
CR1	64 (89)
CR2	6 (8)
CR3	1 (1)
PIF	1 (1)
CD34$^+$ cell dose, x10^6/kg	
<5	8 (11)
≥5-10	29 (40)
≥10	27 (38)
Unknown, n (%)	8 (11)
Treatment era	
Pre-2005	6 (8)
2005-2009	42 (58)
2010-2013	24 (33)
Length of post-autoHCT follow-up, yr	2.6 (.3-10)

Data presented are n (%), unless otherwise indicated.
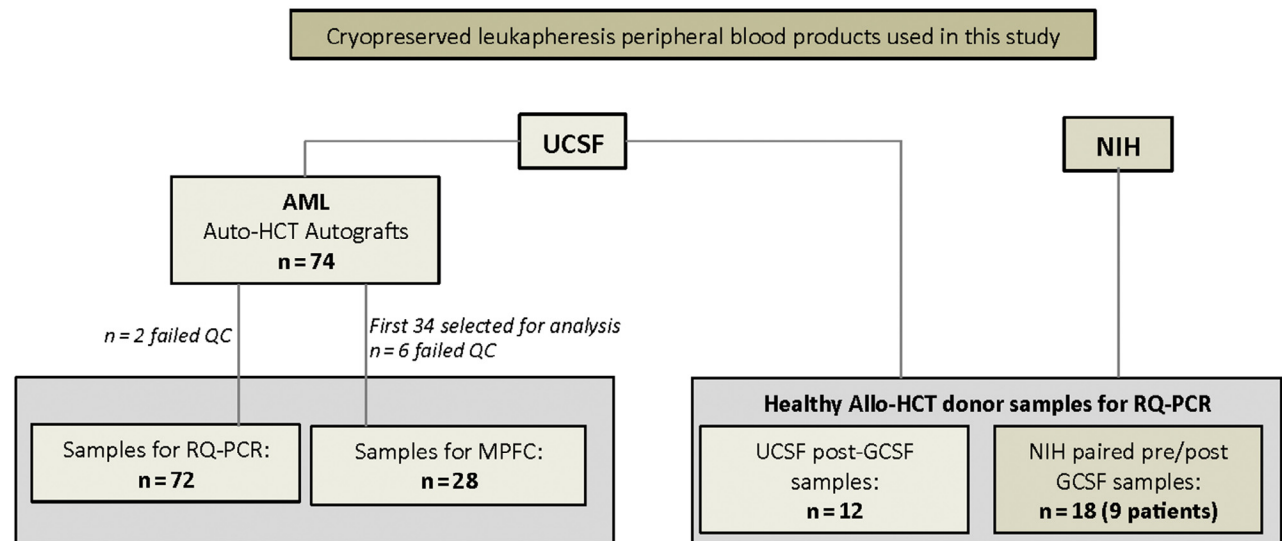CR indicates complete remission; PIF, Primary induction failure.

Figure 1. Cryopreserved leukapheresis peripheral blood products used in this study. All samples were collected on institutional review board–approved research protocols. (Left) 74 autograft samples sent to National Heart Lung Blood Institute from UCSF, from patients with AML who underwent auto-HCT at the UCSF Medical Center between June 1988 and July 2013. Two samples did not meet quality control criterion set by ABL1 housekeeping gene copy number values and were rejected. The remaining 72 samples (Table 1 and Supplemental Table S1) were used for RQ-PCR analysis at the NIH. The first 34 samples from this the RQ-PCR cohort were also analyzed at the Fred Hutchinson Cancer Research Center using multiparameter flow cytometry. Six of these 34 samples were judged insufficient quality for full analysis. (Right) In addition, GCSF-mobilized leukapheresis peripheral blood stem cell products from healthy donor samples (collected for allo-HCT) were used as controls in this study. Twelve healthy donor samples (after GCSF) were sent from UCSF to NIH for evaluation. To study the effect of GCSF on peripheral blood cell composition in healthy individuals, paired cryopreserved leukapheresis samples from before and after GCSF stimulation were obtained from 9 patients.

relapse-free 5 years after transplantation. These outcomes compare well with the 59% alive and 43% relapse-free outcomes recently reported for all 113 intermediate-risk AML patients who underwent transplantation with auto-HCT between 1998 and 2013 at this center [8].

For additional clinical information see Supplemental Table S1 and Supplemental Figure S1.

Auto-HCT Relapse Is Poorly Predicted by WT1 in Leukapheresis PBPC Autograft

It has previously been reported that WT1 transcript detection in autograft samples could predict post-transplantation relapse in patients undergoing auto-HCT [35]. Using the same

assay [34,35] on all 72 autograft samples, we found WT1 levels were not significantly greater in patients who relapsed (Figure 2A) or in those who relapsed within the first year ($P = .26$, data not shown). WT1 MRD positivity, defined either by using the established threshold described by Cilloni et al. [34] (Figure 2B) or by the modified threshold used by Messina et al. [35] (Figure 2C), failed to significantly discriminate patients for relapse risk after auto-HCT. WT1 transcript as a single MRD marker was a poor predictor of relapse within 1 year after (sensitivity, 16%; specificity, 95%; positive predictive value [PPV], 71%, negative predictive value [NPV], 60%) or any time after auto-HCT (sensitivity, 14%; specificity, 94%; PPV, 71%; NPV, 51%).
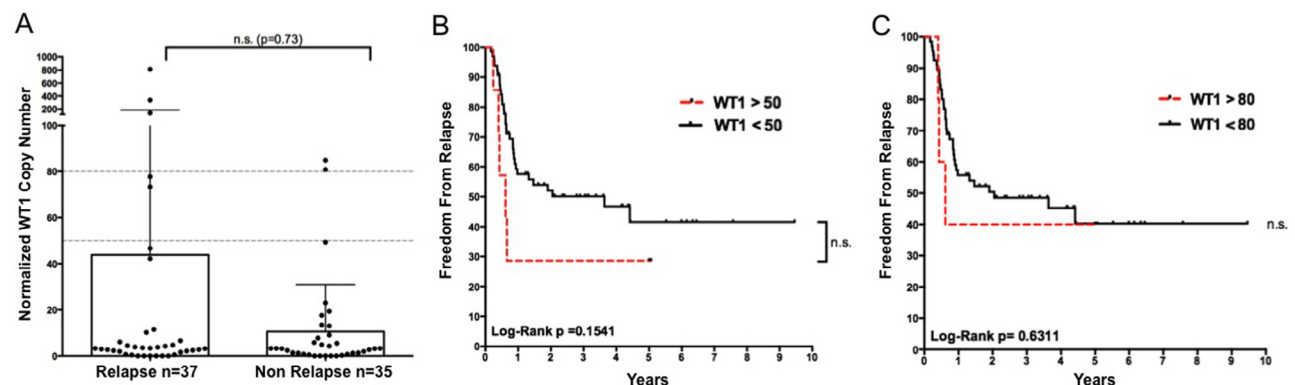
Figure 2. WT1 RQ-PCR of autograft is not sufficient for MRD detection in auto-HCT. (A) WT1 copy number in patients experiencing relapse after auto-HCT did not differ significantly from those who did not relapse during this period ($P = .73$ Mann Whitney test). Two nonrelapsing patients (patients 8 and 45) experienced relapse-free survival greater than 5 years after transplantation despite high levels of autograft WT1 transcript. Kaplan-Meier survival estimates of relapse versus MRD status as assigned by WT1 copy number above (B) the standard ELN threshold (50 WT1 copies/10^4 ABL1) and (C) the threshold used by Messina et al. (80 WT1 copies/10^4 ABL1).

Autologous Transplant Samples Have WT1 and PRTN3 Gene Expression Signatures Suboptimal for MRD Determination

We previously reported multigene MRD detection using expression of WT1, CCNA1, MSLN, PRAME, and PRTN3 in the context of AML patients receiving allo-HCT improved relapse risk prediction compared with the use of WT1 RQ-PCR alone. This work used thresholds based on gene expression in LP from a cohort of 50 unmanipulated healthy donors [19]. As autograft samples in the current study were from patients who had received a course of GCSF immediately before collection, we performed additional testing to confirm that previously established thresholds remained appropriate. Twelve healthy donor PBPC LP samples collected after GCSF administration from UCSF were tested using the multigene panel. Of note, 2 of 12 GCSF-mobilized PBPC LP from healthy donors had WT1 levels above the 98th percentile seen for healthy donors in the prior study, with 1 healthy donor being found to have WT1 expression that would have been falsely described as AML MRD (Figure 3A).

PRTN3 expression in autograft materials was not a useful marker of AML MRD. One-third of GCSF-mobilized healthy donors and 80% of patients on this study would have been considered MRD-positive using our previously established threshold (Figure 3B). CCNA1 was also not useful for autograft AML MRD, with no patient showing expression above the level seen in healthy donors after GCSF (Supplemental Figure S3).

G-CSF Mobilization Enriches Transcripts from Leukemia-Associated Biomarkers WT1 and PRTN3 in Matched Case Control Healthy Donors

To determine the impact of GCSF on gene expression based AML MRD RQ-PCR, we tested paired peripheral blood leukapheresis samples from healthy donors at the NIH taken before and immediately after GCSF administration. WT1 levels
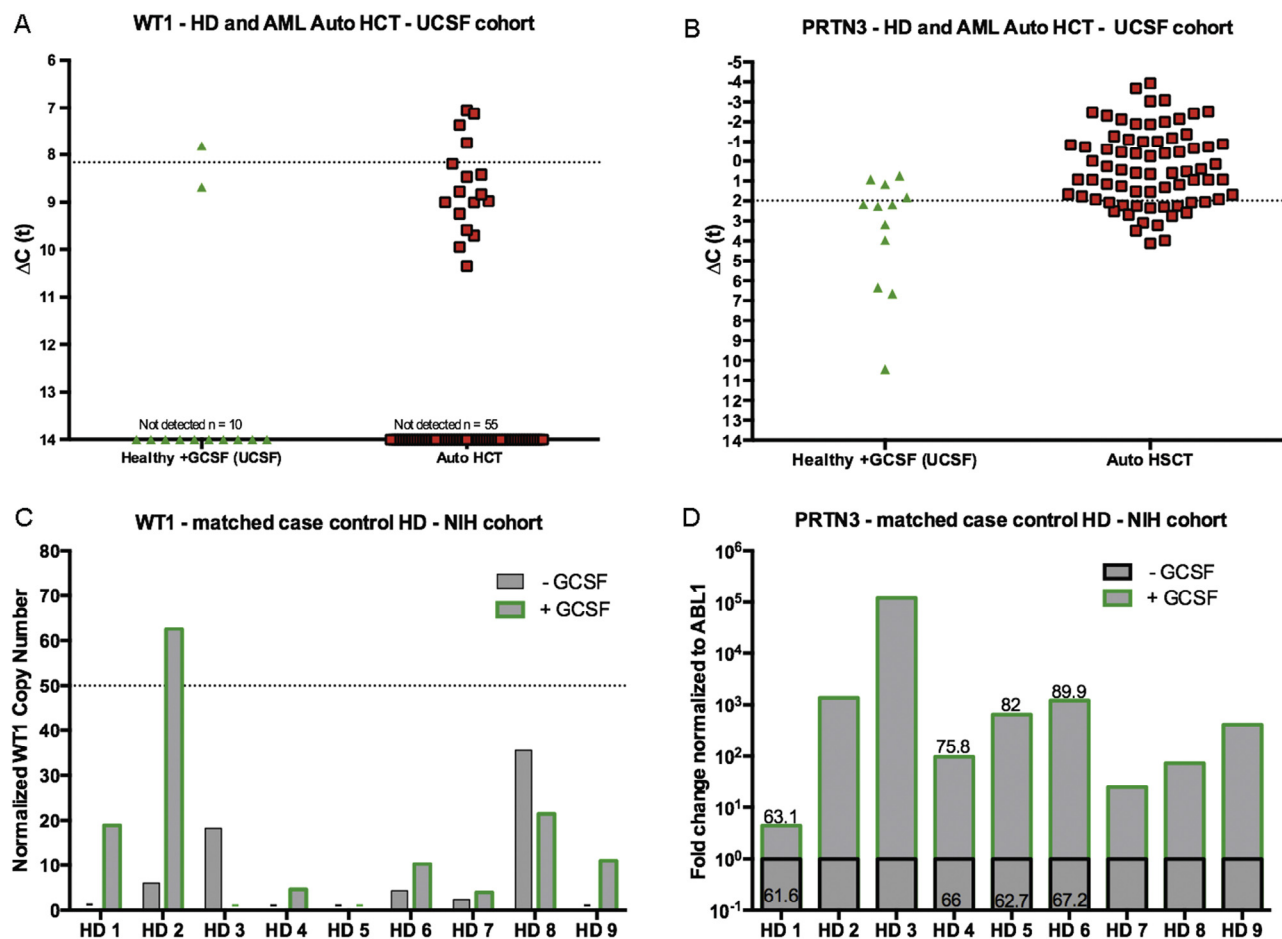
Figure 3. GCSF treatment results in MRD-positive levels of elevated gene expression in healthy donors. Analysis on healthy donor (HD) allo-HCT and AML auto-HCT samples from UCSF: (A) WT1 and (B) PRTN3 mRNA expression found in 12 healthy allo-HCT donors after GCSF administration treated at UCSF (grey triangle), and postchemomobilization GCSF-stimulated PBPC AML patient autograft samples (red and black square). The dotted line indicates the previously calculated MRD-positive threshold established using non-GCSF stimulated healthy leukapheresis samples as a baseline. Expression is normalized to expression of the ABL1 control gene. A ΔCt value of 14 indicates gene expression levels below the limit of detection of the assay (Ct >35). Analysis on paired samples from NIH healthy donors before and after GCSF: (C) WT1 mRNA measured with the ELN Ipsogen WT1 Profile Quant kit reveals reveals increased WT1 levels after GCSF administration including in 1 healthy donor above the validated ELN WT1 MRD threshold (50 copies/1×10^4 copies ABL). (D) Multigene Array RQ-PCR normalized fold change expression of PRTN3 relative to baseline in match case control healthy donors before and after GCSF shows increased expression after GCSF stimulation. Correlation with absolute neutrophil counts in match case control samples is shown as the percent neutrophil count on the bar from available complete blood count data.

assessed with the European LeukemiaNet ELN assay [34] increased after GCSF in more than one-half of cases (Figure 3C). One healthy donor with initially undetectable WT1 had transcript enrichment to a level consistent with AML MRD after GCSF. Increased PRTN3 expression was also seen after GCSF stimulation and was associated with increased neutrophil percentage in peripheral blood (Figure 3D) and CD34 dose. These findings highlight the difficulty in distinguishing residual leukemic clones from normal hematopoietic cells in the GCSF-mobilized auto-HCT graft.

Somatic Mutation Detection Improves Prediction of Relapse in Auto-HCT

Given observed differences in WT1 and PRTN3 gene expression after GCSF stimulation, we next assayed leukemia-specific targets in the form of recurrent somatic mutations and chromosomal translocations in all patients. In total, 21 these patients had known leukemia-specific mutations at diagnosis that were amenable to detection using previously validated RQ-PCR assays (Figure 4A) [22,41]. Of 7 patients with translocation (8;21) at diagnosis, 3 were found to have t(8;21) transcript in the autograft, of whom 2 relapsed within the first year after transplantation. Six patients had a known NPM1 mutation at diagnosis, which was detected in 2 patients who relapsed after 2.5 and 10 months. A patient of unknown NPM1 status, screened for somatic mutations because of Inv(16) AML diagnosis, was also positive for NPM1 mutation autograft MRD and relapsed 7.5 months after transplantation. Inv(16) (n = 7) and PML-RARA (n = 1) were not detected in any graft samples. Together, when 21 patients harboring a known mutation or chromosomal abnormality amenable to detection with RQ-PCR were screened for these mutations and gene expression biomarkers, MRD positivity effectively enriched a group of patients at high risk of relapse. In the first year, no MRD-negative patients in this group relapsed, whereas 84% of MRD-positive patients relapsed (hazard ratio, 12.45; log-rank $P = .0016$) (Figure 4B).

Predictors of Relapse after Auto-HCT

MRD detected with gene expression tests not affected by GCSF exposure, together with somatic mutation detection, was highly complementary in nature when used in the full cohort (Figure 5). Only 1 patient was "double positive" for any of the MRD markers tested (patient 14). Using multigene detection on autograft samples with WT1, PRAME, MSLN, inv(16), t(8;21), and mutated NPM1 tests, we detected MRD in 16 patients who relapsed within the first year after auto-HCT and in an additional patient who relapsed after 1 year. Autograft MRD positivity significantly enriched for a group of patients at risk of early post-transplantation relapse. One year after transplantation, only 28% of MRD-positive patients had not relapsed compared with 67% in the MRD-negative cohort (Figure 5B). Of 31 patients relapsing within 1 year of auto-HCT, multigene MRD was detectable in the autograft of 16 (sensitivity, 52%; specificity, 83%; PPV, 70%; NPV, 69%) (Figure 5D). In contrast, and consistent with our previous findings in allo-SCT [19], MRD at the time of transplantation was poorly predictive of late relapse (only 1 of 6 had an MRD-positive autograft) resulting in marginally inferior ability to predict relapse at any time after auto-HCT (sensitivity, 46%; specificity, 83%; PPV, 74%; NPV, 59%). Use of multigene MRD without the use of WT1 allowed similar stratification between cohorts (Figure 5C) but with fewer patients correctly classified in the MRD-positive group (sensitivity, 39% for 1-year post-transplantation relapses).

In a multivariate analysis with variables including sex, race, age at transplantation, cytogenetic risk category, CD34-postive cell dose, and MRD status, the only significant predictor of freedom from relapse after auto-HCT was the absence of MRD in the autograft (Figure 5E).

Patient	Test Result	Target Present at Diagnosis	MRD Positive	Relapse	RFS (years)
1	True Positive	t(8;21)	WT1	YES	0.24
71	True Positive	inv(16)	WT1	YES	0.62
28	True Positive	t(8;21)	t(8;21)	YES	0.98
42	True Positive	t(8;21)	t(8;21)	YES	0.56
3	True Positive	NPM1mut	MSLN	YES	0.44
10	True Positive	NPM1mut	NPM1	YES	0.84
11	True Positive	NPM1mut	WT1	YES	0.66
29	True Positive	NPM1mut	NPM1	YES	0.22
30	True Positive	inv(16)	MSLN	YES	0.52
46	True Positive	inv(16)	NPM1	YES	0.62
25	True Negative	t(8;21)	NO	NO	2.12
73	True Negative	inv(16)	NO	NO	0.27
12	True Negative	t(8;21)	NO	NO	1.8
52	True Negative	t(8;21)	NO	NO	2.36
50	True Negative	NPM1mut	NO	NO	6.46
49	True Negative	inv(16)	NO	NO	3.38
51	True Negative	inv(16)	NO	NO	0.71
58	True Negative	inv(16)	NO	NO	2.89
45	False Positive	NPM1mut	WT1	NO	5
32	False Positive*	t(8;21)	t(8;21)	NO	3.13
72	False Negative	t(15;17)	NO	YES	1.9

MRD indicates Measurable Residual Disease, RFS, Relapse Free Survival
*Patient had 20/20 metaphases positive for t(8;21) at diagnosis

Figure 4. Personalization of MRD monitoring allows for improved relapse risk stratification. (A) MRD analysis of the 21 cases in which diagnostic information on known somatic mutations amenable to RQ-PCR detection (NPM1 mutation, chromosomal translocations inv(16), t(8;21), or t(15;17)) was available. Samples are listed sorted by test result with the third column showing the target detected at diagnosis and the fourth column showing the marker for which the patient was MRD-positive. (B) Kaplan-Meier survival estimates of the 21 patients with a known mutation at diagnosis demonstrate significant survival benefits for multigene MRD negativity log-rank $P = .0016$ and hazard ratio = 12.45. One case of early nonrelapse mortality [49] was included in all analyses with censoring at time of death.

Figure 5 (A) — Comparison between post–auto-HCT relapse and detectable MRD. Red-shaded cells (MRD-positive) are marked with "+".

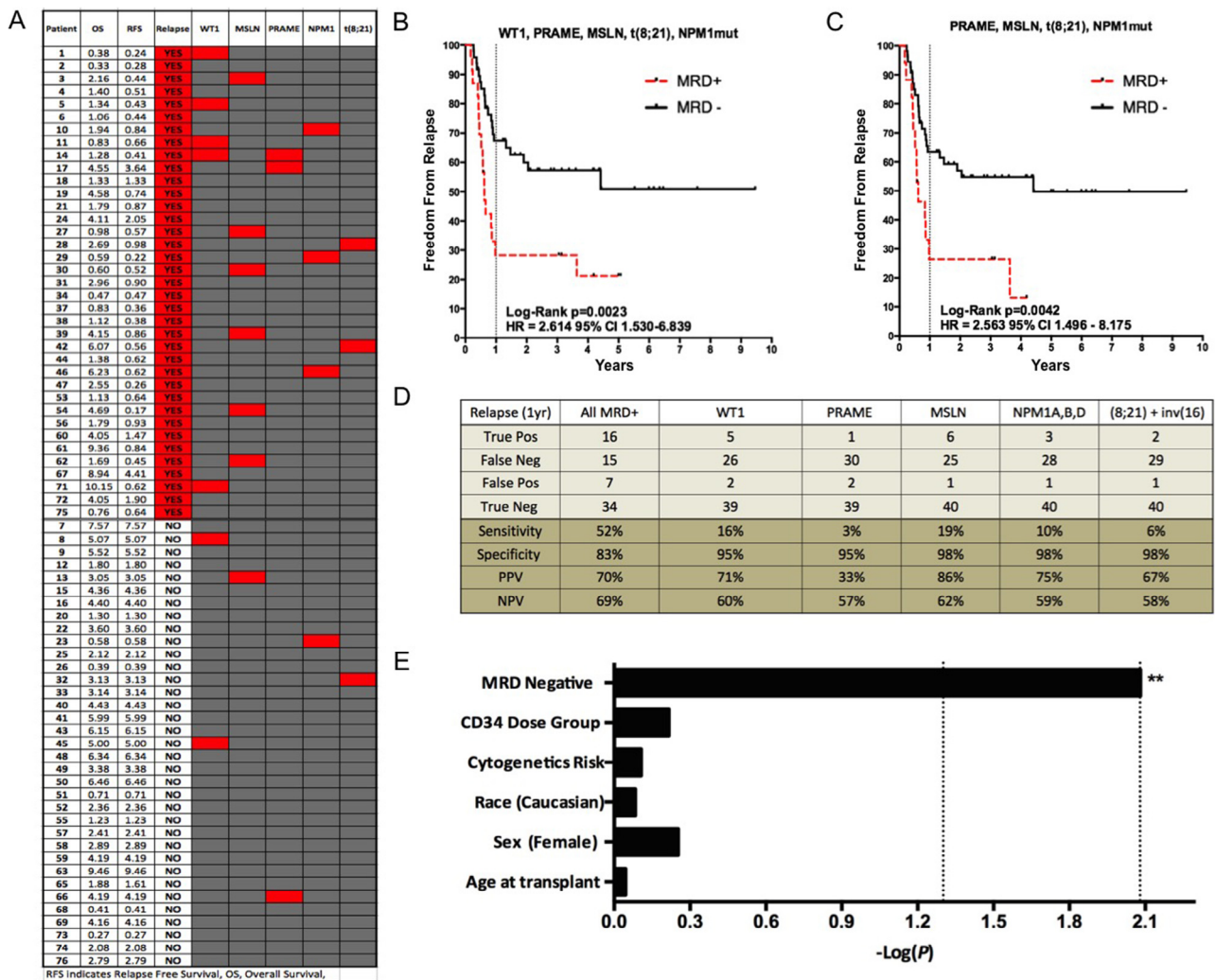| Patient	OS	RFS	Relapse	WT1	MSLN	PRAME	NPM1	t(8;21)
1	0.38	0.24	YES	+				
2	0.33	0.28	YES					
3	2.16	0.44	YES		+			
4	1.40	0.51	YES					
5	1.34	0.43	YES					
6	1.06	0.44	YES					
10	1.94	0.84	YES				+	
11	0.83	0.66	YES	+				
14	1.28	0.41	YES	+		+		
17	4.55	3.64	YES			+		
18	1.33	1.33	YES					
19	4.58	0.74	YES					
21	1.79	0.87	YES					
24	4.11	2.05	YES					
27	0.98	0.57	YES		+			
28	2.69	0.98	YES					+
29	0.59	0.22	YES		+			
30	0.60	0.52	YES	+				
31	2.96	0.90	YES					
34	0.47	0.47	YES					
37	0.83	0.36	YES					
38	1.12	0.38	YES					
39	4.15	0.86	YES		+			
42	6.07	0.56	YES					+
44	1.38	0.62	YES					
46	6.23	0.62	YES				+	
47	2.55	0.26	YES					
53	1.13	0.64	YES					
54	4.69	0.17	YES		+			
56	1.79	0.93	YES					
60	4.05	1.47	YES					
61	9.36	0.84	YES					
62	1.69	0.45	YES		+			
67	8.94	4.41	YES					
71	10.15	0.62	YES	+				
72	4.05	1.90	YES					
75	0.76	0.64	YES					
7	7.57	7.57	NO					
8	5.07	5.07	NO	+				
9	5.52	5.52	NO					
12	1.80	1.80	NO					
13	3.05	3.05	NO					
15	4.36	4.36	NO					
16	4.40	4.40	NO					
20	1.30	1.30	NO					
22	3.60	3.60	NO					
23	0.58	0.58	NO		+			
25	2.12	2.12	NO					
26	0.39	0.39	NO					
32	3.13	3.13	NO					+
33	3.14	3.14	NO					
40	4.43	4.43	NO					
41	5.99	5.99	NO					
43	6.15	6.15	NO					
45	5.00	5.00	NO	+				
48	6.34	6.34	NO					
49	3.38	3.38	NO					
50	6.46	6.46	NO					
51	0.71	0.71	NO					
52	2.36	2.36	NO					
55	1.23	1.23	NO					
57	2.41	2.41	NO					
58	2.89	2.89	NO					
59	4.19	4.19	NO					
63	9.46	9.46	NO					
65	1.88	1.61	NO					
66	4.19	4.19	NO			+		
68	0.41	0.41	NO					
69	4.16	4.16	NO					
73	0.27	0.27	NO					
74	2.08	2.08	NO					
76	2.79	2.79	NO					

RFS indicates Relapse Free Survival, OS, Overall Survival,

(B) WT1, PRAME, MSLN, t(8;21), NPM1mut — Freedom From Relapse vs Years; MRD+ / MRD–. Log-Rank p=0.0023; HR = 2.614 95% CI 1.530-6.839

(C) PRAME, MSLN, t(8;21), NPM1mut — Freedom From Relapse vs Years; MRD+ / MRD–. Log-Rank p=0.0042; HR = 2.563 95% CI 1.496 - 8.175

(D)

Relapse (1yr)	All MRD+	WT1	PRAME	MSLN	NPM1A,B,D	(8;21) + inv(16)
True Pos	16	5	1	6	3	2
False Neg	15	26	30	25	28	29
False Pos	7	2	2	1	1	1
True Neg	34	39	39	40	40	40
Sensitivity	52%	16%	3%	19%	10%	6%
Specificity	83%	95%	95%	98%	98%	98%
PPV	70%	71%	33%	86%	75%	67%
NPV	69%	60%	57%	62%	59%	58%

(E) Multivariate analysis, -Log(P): MRD Negative (**), CD34 Dose Group, Cytogenetics Risk, Race (Caucasian), Sex (Female), Age at transplant.

Figure 5. Multigene MRD allows enrichment of AML patients with high risk of post–auto-HCT relapse. (A) Comparison between post–auto-HCT relapse and detectable MRD in the autograft shows lack of redundancy between tests in the multigene panel. Red shaded squares signify MRD-positive for the corresponding test (column). (B) Kaplan-Meier survival estimates for patients with residual disease detected with WT1, PRAME, MSLN, NPM1mut (types A, B, and D) and t(8;21). MRD positivity was associated with decreased freedom from survival, log-rank *P* = .0023; hazard ratio (HR), 2.614; 95% confidence interval [CI], 1.530 to 6.839). (C) If WT1 were removed from this analysis, patients are still effectively risk stratified but at the expense of fewer relapsing patients (n = 13 versus n = 17) being correctly assigned to the high-risk MRD-positive group (log-rank *P* = .0042; HR, 2.563; 95% CI, 1.496 to 8.175). Two cases of early nonrelapse mortality [23,49] were included in all analyses with censoring at time of death. (D) Statistical performance characteristics of each MRD assay reveals minimal efficacy for each test used on the entire cohort individually but complementarity when used in tandem (sensitivity, 52%; specificity, 83% for 1 year after auto-HCT relapse when all markers used to detect MRD in autograft). (E) Multivariate maximum likelihood estimate reveals MRD negativity as only significant variable associated with post–autoHCT relapse (protective factor) in a multivariate analysis of cytogenetic risk group, CD34 dose, age at time of transplantation, sex, and race. The first and second dotted lines represent significance at the .05 and .0083 (Bonferroni corrected) levels, respectively. MRD negativity was protective from relapse (HR, .349; 95% CI, .160 to .763). CD34 dose (HR, 1.157; 95% CI, .662 to 2.020), cytogenetic risk group (HR, .907; 95% CI, .455 to 1.810), race (HR, .909; 95% CI, .389 to 2.122), sex (HR, 1.273; 95% CI, .606 to 2.524), and age at transplantation (HR, 1.002; 95% CI, .973 to 1.031) were not predictive of relapse.

Leukemia Immunophenotype Is Indistinguishable from Healthy Cells in the Autograft

Finally, replicate autograft aliquot samples from the first 34 patients on this study were also sent to Fred Hutchinson Cancer Research Center for MPFC analysis. In total, 20 of these patients experienced relapse after auto-HCT (59%), with most occurring in the first year after transplantation (17 of 20). MPFC was performed in a laboratory with significant experience according to protocols previously described [42]. The majority of frozen samples (82%) were evaluable by MPFC and consisted of a mixture of lymphocytes, mature monocytes, and few plasmacytoid dendritic cells with a variable number of CD34-positive progenitors having a stem cell–like immunophenotype (bright CD34+, low-to-absent CD38) without maturation to lineage committed forms. Findings were typical of leukapheresis stem cell products of this type (Supplemental Figure S2). The median percentage of CD34-positive cells among PBPC mononuclear cells was .42% (range, .05 to 12.5). The findings across the all samples were similar overall and no specimen contained definitive evidence of immunophenotypic MRD. The combination of regenerative or induced antigenic changes that overlap with leukemic immunophenotypes, limited internal control populations to judge relative intensity changes and variable sample quality were considered likely to account for these false-negative results.

DISCUSSION

For 3 decades, there has been debate regarding the possibility that leukemic contamination of autologous stem cell grafts could contribute to the high post-transplantation relapse rates observed in AML patients [43-45]. The advent of highly sensitive methods for detection of measurable residual disease made this hypothesis testable. Early reports that were limited by small cohort size (n = 7, n = 24) showed nonsignificant trends for patients relapsing after transplantation to have had higher WT1 levels in the autograft but with considerable overlap between the ranges observed in relapsing and nonrelapsing patients [46,47]. A recent well-performed study on 30 AML patients undergoing auto-HCT reported that the median WT1 transcript level in the autograft of relapsing patients was significantly higher than those who did not relapse [35]. Again, however, the median WT1 value of those relapsing (82 copies/10^4 ABL) was within the range observed of those not relapsing (4 copies/10^4 ABL to 109 copies/10^4 ABL), making the benefit of such testing to any individual patient questionable.

Our study represents a larger patient cohort than all 3 of these previous studies combined and shows no statistically significant difference between autograft WT1 transcript levels in patients who relapsed or did not relapse after auto-HCT. Although we observed a nonsignificant trend for WT1 levels in the autograft to be, on average, higher in those patients who relapsed, there was considerable overlap between the ranges observed. Seven patients in our study had WT1 levels above the ELN threshold [34], 5 of whom relapsed within 1 year (sensitivity, 16%; specificity, 95%). The differences in the performance of WT1 as a MRD assay in auto-HCT between our study and previous work are likely because of stochastic factors associated with differences in sample size. In addition to sample size effects, differences in numbers of cycles of consolidation chemotherapy and the use of in vivo purging in this cohort may have also decreased detectable WT1 expression levels in our study. False-positive results were seen; however, in both the recent study by Messina et al. [35] and our study, using the WT1 level of >50 copies/10^4 ABL threshold established by the ELN [34], 2 of 12 WT1 positive patients were false-positives in that study (17%) as were 2 of 7 WT1-positive patients in our study (29%).

The poor performance of traditional AML MRD methodologies, with proven utility in the conventional allo-HCT setting, was likely due to the unique circumstance of the effects of GCSF mobilization in these auto-HCT patients. By assessing transcript levels in a cohort of 9 NIH healthy donors before and after GCSF administration, we show that WT1 transcripts can increase in the peripheral blood LP after GCSF, even in healthy subjects, to a level compatible with AML MRD, thus potentially leading to false-positive results. Similarly, the inability of MPFC to detect AML MRD was likely multifactorial but was due in part to the altered cell composition in the autograft in patients who had received GCSF. The use of a multigene panel of molecular markers not influenced by GCSF expression mitigated some of the weaknesses of using WT1 testing alone on autografts to predict relapse risk.

This study has limitations. Despite the fact that in this study 97% of samples had sufficient RNA integrity to be used for RQ-PCR testing, cryopreserved autograft material may not represent an ideal source for AML MRD testing for other reasons. In addition to effects of GCSF on RQ-PCR and MPFC signatures, it is not clear that an aliquot of the autograft is a fully representative sample of the total AML burden of a patient undergoing auto-HCT. Inadequate sampling rather than

insufficient sensitivity of MRD testing may be the most important factor in detection of low levels of residual AML [48]. MRD assessment of the bone marrow of these patients would have likely provided important additional information but, unfortunately, was not possible in this historical cohort. Unlike previous studies [49-53], we did not observe an association between CD34+ cell dose and post–auto-HCT relapse risk. Our study may have been insufficiently powered to detect this effect, though we did observe a trend in univariate analysis for lower rates of freedom from relapse in those receiving CD34+ doses greater than 10 million per kilogram.

We also observed several false-positive events when using a multigene testing approach. The PPV for multigene MRD in predicting 1-year post–auto-HCT relapse was 70%, with 7 false-positive events. In addition to the 2 false-positive WT1 tests described above, 1 patient with detectable NPM1 mutation had not relapsed at the time of follow-up, at only 7 months, and so was assigned as a "false-positive," which unfortunately may be a premature classification given the other 3 patients with NPM1 mutation–positive MRD ultimately all relapsed within 1 year of auto-HCT. A fourth "false-positive" patient had detectable t(8;21) in the autograft but did not relapse over 3 years of follow-up (Figure 5, patient 32). Bone marrow cytogenetics for this patient at diagnosis showed 20 out of 20 metaphases with a 45,X,-X,t(8;21)(q22;q22) karotype, suggesting the t(8;21) anomaly may have been present in the majority of bone marrow cells and not just the leukemic clone, further suggesting presence of preleukemic clonal hematopoiesis. Such persistence after therapy has been described previously [54,55]. Finally, 2 positives were observed for PRAME in patients who did not relapse with 1 year (1 of whom did relapse, but after 1 year) and 1 for MSLN. Finally, MRD analysis by flow cytometry found no interpretable signature in the first 34 autograft samples examined. It is likely that leukemia-associated immunophenotype information from initial diagnosis would have improved the performance of MPFC.

Although the detection of residual disease in the autograft product before transplantation to reduce relapse risk is a logical and highly appealing strategy [4,35,46,47,56], unfortunately the autograft, due to changes in peripheral blood composition after GCSF stimulation, is not optimal for AML MRD detection using gene expression RQ-PCR or MPFC-based methods. In summary, we show here that testing of autograft samples using WT1 RQ-PCR alone is insufficient for optimal stratification for post-transplantation relapse risk. Second, we show a mechanism for the observed overlapping ranges for WT1 levels in the auto-HCT setting, due to the effects of GCSF increasing baseline WT1 expression levels. The use of a multigene MRD panel mitigates some of these deficiencies and could identify a high-risk group of patients, with only 28% chance of remaining in remission 1 year after auto-HCT. Finally, the proof of principle demonstration that patients could be highly effectively stratified into groups with high and low risks of relapse after auto-HCT when a known mutation target was available for tracking was remarkable. Given the intrinsic heterogeneity of AML, such personalization may represent the future for MRD monitoring in this disease [57-59] and should be validated with additional studies. Ultimately, however, only randomized trials of transplantation versus nontransplantation therapy in cohorts selected based on MRD status will clarify if such testing can identify AML patients who may benefit from auto-HCT [60]. We show that GCSF-stimulated PBSC leukapheresis specimens have limitations for use in the detection of MRD

and prediction of relapse risk after auto-HCT. Evaluation of alternative sample sources other than autografts for MRD testing, and/or novel technologies for detecting residual disease, in AML patients undergoing auto-HCT appears warranted.

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APPENDIX. SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2016.08.014.

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