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WT1 Measurable Residual Disease Assay in Patients With Acute Myeloid Leukemia Who Underwent Allogeneic Hematopoietic Stem Cell Transplantation: Optimal Time Points, Thresholds, and Candidates

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A B S T R A C T

The absence of relevant guidelines for *Wilms tumor 1* (*WT1*) gene quantification as a measurable residual disease (MRD) assessment for patients with acute myeloid leukemia (AML) undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT) has limited the widespread use in practice. We investigated optimal time points, thresholds, and candidates for the bone marrow *WT1* MRD assay in 425 consecutive patients with AML who underwent allo-HSCT. *WT1* expression kinetics before allo-HSCT and at 1 or 3 months after allo-HSCT were determined by real-time PCR using the European LeukemiaNet (ELN) normalized method. Relapsed patients had significantly higher *WT1* levels before allo-HSCT and at 3 months after allo-HSCT. The best time point for the *WT1* MRD assay was before allo-HSCT by the receiver operating characteristic curve. Among various thresholds, 250 copies recommended from ELN researchers were mostly predictive of post-transplant relapse. In multivariate analysis, *WT1* MRD positivity independently predicted relapse, resulting in inferior survival. In subgroup analyses, pre-transplant *WT1* MRD positivity was predictive of post-transplant relapse in the intermediate group, whereas *WT1* MRD positivity occurred at 3 months after allo-HSCT in favorable and adverse risk groups. Among MRD-positive patients before allo-HSCT, all patients who were MRD positive at 3 months relapsed within 6 months. The *WT1* MRD assay before allo-HSCT or 3 months after allo-HSCT is useful for predicting post-transplant relapse with a different significance in each risk group by time points, showing the benefit of multiple tests over time. Such monitoring is particularly available in patients with AML without specific molecular targets.

INTRODUCTION

Mounting evidence indicates that measurable residual disease (MRD) is an independent, prognostic indicator in acute myeloid leukemia (AML). MRD assessment is important for risk stratification and treatment planning in conjunction with other well-established clinical, cytogenetic, and molecular data assessed at diagnosis [1,2]. The European LeukemiaNet (ELN) proposed MRD-negative complete remission (CR) as a new response criterion in 2017 [3]. In the absence of guidelines or recommendations despite the use of a variety of multiparameter flow cytometry (MFC) and molecular protocols for MRD assessment in various clinical settings, the ELN reported a consensus document for MRD in AML. The ELN guidelines recommend real-time quantitative PCR (RQ-PCR) as a gold standard for AML with suitable abnormalities, including *PML-RARA, RUNX1-RUNX1T1, CBFB-MYH11,* or *NPM1*, limited to 40% of patients with AML [1]. For other patients without suitable abnormalities, MFC, which focuses on the phenotype of the leukemic cells, is initially recommended based on its applicability for almost all patients [4,5], and numerous studies in various clinical settings, including chemotherapy [6-9] and

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Table 1
Patients' Characteristics

Variables	Study Cohort (n = 425)	*WT1* MRD Positive before Allo-HSCT (n = 62)	*WT1* MRD Negative before Allo-HSCT (n = 363)	P Value*
Age at allo-HSCT, median (range), yr	47 (18-70)	47 (18-70)	48 (18-69)	.848
Age <60 yr/≥60 yr, n (%)	360 (85)/65 (15)	53 (86)/9 (14)	302 (83)/61 (17)	.653
Sex, male/female, n (%)	228 (54)/197 (46)	33 (53)/29 (47)	195 (54)/168 (46)	.943
WBC count ($\times 10^9$/L) at diagnosis, <50/≥50, n (%)	325 (77)/100 (23)	44 (71)/18 (29)	281 (77)/82 (23)	.269
Modified NCCN criteria, n (%)**				
Favorable/intermediate/adverse	120 (28)/206 (49)/99 (23)	10 (16)/30 (48)/22 (36)	110 (30)/176 (49)/77 (21)	.015
CN-AML, n (%)	188 (44)	30 (48)	158 (44)	.476
NPM1+/FLT3−ITD−	46	5 (17)	41 (26)	
NPM1+/FLT3−ITD+	20	1 (3)	19 (12)	
NPM1−/FLT3−ITD+	14	8 (27)	6 (4)	
NPM1−/FLT3−ITD−	103	15 (50)	88 (56)	
ND	5	1 (3)	4 (3)	
CBF-AML, n (%)				
RUNX1-RUNX1T1/CBFB-MYH11	74 (73)/28 (27)	4 (50)/4 (50)	70 (75)/24 (25)	.210
C-KIT, mutated/nonmutated/ND	37 (36)/53 (52)/12 (12)	4 (50)/3 (38)/1 (12)	49 (52)/34 (36)/11 (12)	.993
Donor age, median (range), yr	40 (11-68)	40 (11-68)	35 (6-70)	.167
<40 yr/≥40 yr, n (%)	245 (58)/180 (42)	31 (50)/31 (50)	214 (59)/149 (41)	.187
Donor sex, male/female, n (%)	281 (66)/144 (34)	39 (63)/23 (37)	242 (67)/121 (33)	.563
Female to male, yes/no, n (%)	81 (19)/344 (81)	15 (24)/47 (76)	66 (18)/297 (82)	.265
HCT-CI, <3/≥3, n (%)	265 (62)/160 (38)	15 (63)/9 (37)	125 (66)/65 (34)	.750
Donor type, MSD/MUD/haplo, n (%)	199 (47)/117 (28)/109 (26)	36 (58)/12 (19)/14 (23)	163 (45)/105 (29)/95 (26)	.136
Stem cell source, PB/BM, n (%)	361 (85)/64 (15)	54 (87)/8 (13)	307 (85)/56 (15)	.912
Disease status at transplantation, CR1/CR2, n (%)	400 (94)/25 (6)	59 (95)/3 (5)	341 (94)/22 (6)	1.000
Conditioning intensity, MAC/RIC, n (%)	230 (54)/195 (46)	39 (63)/23 (37)	191 (53)/172 (47)	.133
GVHD prophylaxis, CS + MTX/FK + MTX, n (%)	199 (47)/226 (53)	36 (58)/26 (42)	163 (45)/200 (55)	.055
ATG use, no/yes, n (%)	199 (47)/226 (53)	37 (60)/25 (40)	162 (45)/201 (55)	.028
$CD34^+$ cell dose ($\times 10^6$/kg), median (range)	5.3 (0.9-14.7)	5.3 (0.9-14.7)	5.2 (1.1-14.5)	.682

NCCN indicates National Comprehensive Cancer Network; CN-AML, cytogenetically normal acute myeloid leukemia; ND, not determined; CBF-AML, core binding factor acute myeloid leukemia; HCT-CI, hematopoietic stem cell transplantation-comorbidity index; MSD, matched sibling donor; MUD, matched unrelated donor; PB, peripheral blood; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; GVHD, graft-versus-host disease; CS, cyclosporine; MTX, methotrexate; FK, FK506; ATG, antithymocyte globulin.

* Statistical comparison between *WT1* MRD positive and *WT1* MRD negative.

** Modified NCCN criteria means 2017 NCCN risk stratification with the exception of *TP53* mutations.

allogeneic hematopoietic stem cell transplantation (allo-HSCT) [10-16].

Wilms tumor gene 1 (*WT1*) encodes a transcription factor highly expressed in several hematopoietic tumors, including AML, and normal marrow cells [17,18]. *WT1* transcript levels in normal, regenerating, and AML marrow cells could be possibly differentiated [19,20]. The usefulness of molecular monitoring of *WT1* transcripts for prediction and management of relapse after allo-HSCT was suggested in 2003 [21]. In 2009, ELN researchers used an optimized and standardized *WT1* assay and established reference ranges for *WT1* expression in normal blood and bone marrow (BM), with a large number of control samples, and transcript levels indicative of residual leukemia distinguished from background levels [22]. Despite several reports demonstrating the promising role of *WT1* in MRD assessment under chemotherapy [22,23] and allo-HSCT settings [24-31], the *WT1* MRD assay is not widely used in AML because of the lack of large-scale, controlled studies, particularly for allo-HSCT. Indeed, recent ELN guidelines for MRD in AML recommend the *WT1* MRD assay only if other MRD assays, including flow cytometric ones, are unavailable as the condition to use a validated *WT1* MRD assay by ELN researchers [22]. Moreover, there are no specific guidelines for clinically relevant time points, thresholds, or candidates for the *WT1* MRD assay compared with those for MFC. Thus, in the current study, we retrospectively evaluated a large cohort consisting of 425 consecutive adult patients diagnosed with AML who underwent allo-HSCT in remission. We investigated the optimal time points, thresholds, and candidates for normalized BM *WT1* levels by analyzing the *WT1* expression kinetics before and after allo-HSCT.

MATERIALS AND METHODS

Patients

We retrospectively evaluated 527 consecutive patients diagnosed with de novo AML who underwent unmanipulated allo-HSCT at the Catholic Seoul St. Mary's Hematology Hospital between 2012 and 2016. Among 481 patients undergoing the first allo-HSCT, 425 patients with CR at allo-HSCT were analyzed. Patients without *WT1* overexpression at diagnosis were excluded from this study (Supplementary Figure S1). Demographic characteristics of the enrolled patients are summarized in Table 1. The median age of patients was 48 years (range, 18 to 70 years). The study included 228 male patients (54%). Cytogenetic and molecular data at diagnosis facilitated risk stratification according to 2017 National Comprehensive Cancer Network guidelines with the exception of *TP53* mutations (modified National Comprehensive Cancer Network risk stratification). Of the total 425 patients, 120 (28%), 206 (49%), and 99 (23%) were assigned to favorable, intermediate, and adverse risk groups, respectively. Most patients (n = 400, 94%) were in CR1, whereas 25 patients (6%) were in CR2 at allo-HSCT. Transplant donors included matched siblings (47%), unrelated donors (28%), and haploidentical-related donors (26%). Of all patients, 54% received myeloablative conditioning, whereas 46% received reduced-intensity conditioning. Stem cell sources included BM (15%) and peripheral blood (85%). Antithymocyte globulin (thymoblogulin, Genzyme, Cambridge, MA) was used in graft-versus-host disease prophylaxis for patients who underwent transplantation from unrelated or haploidentical-related donors [32]. Treatment courses and transplantation procedures were performed as previously described [26]. The Institutional Review Board

Table 2
WT1 Levels at Diagnosis, before, and 1 or 3 Months after allo-HSCT

Time Points for *WT1* Assay/*WT1* Levels/10^4 *ABL1* $\times$ 10^4 (Copies)	Time Range of the Determinations	Nonevaluable Patients	Range	25%	50%	75%
Diagnosis (n = 425)	NA	NA	273-110,830	1540	4545	10,080
Before (n = 425)	Between days 49 and 10 before allo-HSCT	NA	0-9630	20.1	60.7	152
1 month (n = 333)	Between days 26 and 35 after all-HSCT	NRM <1 month (n = 5)	3.2-1800	24.4	49.8	111
		Relapse <1 month (n = 2)				
		MRD not performed (n = 85)				
3 months (n = 346)	Between days 82 and 100 after allo-HSCT	NRM <3 months (n = 17)	0-1670	31.5	55.5	119
		Relapse <3 months (n = 13)				
		MRD not performed (n = 49)				

WT1 levels were normalized with respect to the number of *ABL1* transcripts and expressed as copy numbers per 10^4 copies of *ABL1*. *WT1* levels/10^4 *ABL1* $\times$ 10^4 (copies).
NA indicates nonapplicable.

of the Catholic Medical Center approved this single-center study. All analyses were performed according to guidelines provided by Institutional Review Board guidelines and the tenets of the Declaration of Helsinki.

Molecular Markers and MRD Assessment With WT1

Diagnostic BM samples obtained from all patients were analyzed for mutations involving *NPM1, FLT3, CEBPA,* and *C-KIT* using well-established protocols [26,33]. The levels of BM *WT1* at diagnosis, before allo-HSCT, and at 1 or 3 months after allo-HSCT were determined by RQ-PCR using the WT1 ProfileQuant kit (Ipsogen, Marseille, France). *WT1* gene transcripts generated by RQ-PCR were normalized with respect to the number of *ABL1* transcripts and expressed as copy numbers per 10^4 copies of *ABL1*. Assays were performed in replicate for greater accuracy for comparative results. When *ABL1* quantification was inappropriately low, we repeated the assay up to three times (supplementary methods). Receiver operating characteristic (ROC) curve analysis was used to determine the optimal time points for MRD assessment with *WT1*.

Statistical Analysis

Categorical variables were compared using the chi-square test or Fisher exact test, whereas continuous variables were analyzed with the Student *t* test or Wilcoxon rank-sum test. Overall survival and disease-free survival curves were plotted using the Kaplan-Meier method and analyzed with the log-rank test. The cumulative incidence was used to estimate the probability of the cumulative incidence of relapse (CIR) and nonrelapse mortality (NRM), treating nonrelapse death and relapse as competing risk factors for relapse and NRM, respectively, and compared using the Gray test. For multivariate analysis, variables with a *P* value of <.10, as determined by univariate analysis, were considered for entry into the model selection procedure based on the Cox proportional hazards model or a proportional hazards model for a

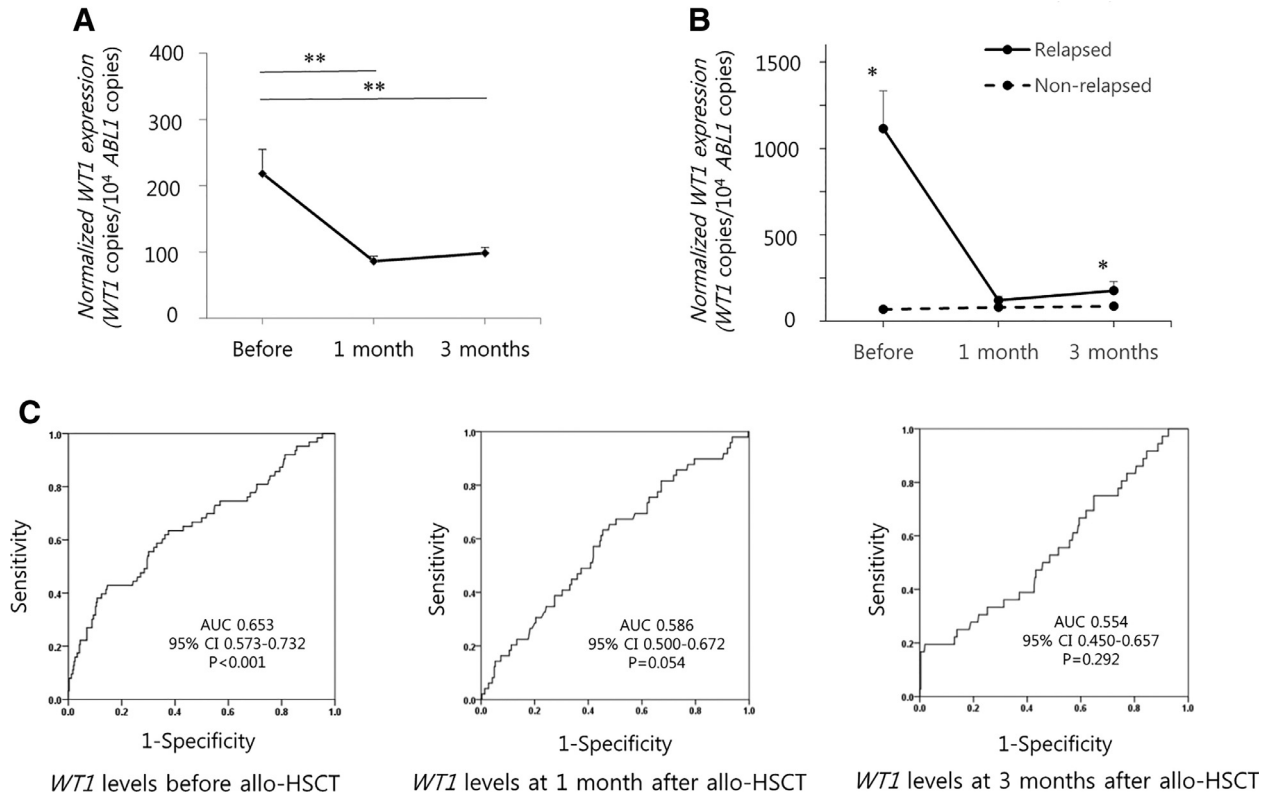

Figure 1. *WT1* expression kinetics during peritransplantation period and optimal time points for *WT1* MRD assay. (A) *WT1* expression kinetics before allo-HSCT and at 1 or 3 months after allo-HSCT in all patients (before, n = 425; 1 month, n = 333; 3 months, n = 346). (B) *WT1* expression kinetics according to the occurrence of relapse after allo-HSCT (numbers of relapsed patients versus nonrelapsed patients; before, 63 versus 326; 1 month, 49 versus 284; 3 months, 36 versus 310). (C) ROC curve analysis at each time point of the *WT1* assay. *WT1* levels were normalized with respect to the number of *ABL1* transcripts and expressed as copy numbers per 10^4 copies of *ABL1*. Results are expressed as the mean ± SEM. $^*P < .05$. $^{**}P < .01$. AUC indicates area under curve.

subdistribution of the competing risk factors. [34] Statistical significance was determined as a *P* value less than or equal to .05 (2-tailed). For estimating serum ferritin dynamics over time, a repeated-measures analysis of variance was used. All statistics were conducted using SPSS, version 13.0 (SPSS, Inc, Chicago, IL), and R software (version 3.2.3; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

WT1 *Expression Kinetics and Optimal Time Points for the* WT1 *MRD Assay*

At a median follow-up of 39 months for survivors, 4-year overall survival, disease-free survival, CIR, and NRM were 64% ± 2.6%, 62% ±2.6%, 16% ± 2.1%, and 22% ± 2.5%, respectively. We serially monitored MRD by *WT1* in the post-transplant BM samples during routine disease assessment at 1 and 3 months after allo-HSCT. Serial RQ-PCR assays of *WT1* expression at each time point were presented by quartile (Table 2). The median *WT1* level at diagnosis was 4545 copies (range, 273 to 110,830 copies). Chemotherapies significantly reduced the *WT1* levels at remission before allo-HSCT. *WT1* levels were further decreased after allo-HSCT. Figure 1A shows *WT1* expression kinetics with a significant decrease after allo-HSCT.

Among 425 patients at remission before transplantation, 63 relapsed between 52 and 898 days after allo-HSCT. These relapsed patients had significantly higher *WT1* levels before allo-HSCT and at 3 months after allo-HSCT but not at 1 month after allo-HSCT (Supplementary Table 1, Figure 1B). ROC curve analysis revealed that the optimal time point for *WT1* quantification to predict relapse was before allo-HSCT compared with after allo-HSCT (at 1 or 3 months) (Figure 1C). The performance was increased when limited to patients with a higher degree of *WT1* overexpression at initial diagnosis (ie, upper 50% of WT1 expression; Supplementary Figure S2).

Optimal Thresholds for the WT1 *MRD Assay*

ELN researchers have defined the upper limit of normal as 250 copies of *WT1* for BM samples derived from healthy volunteers (n = 204) to distinguish residual leukemia from background amplification [22]. Some reports have suggested 100 copies as the threshold for *WT1* quantification in the setting of allo-HSCT [24,25]. To elucidate the optimal thresholds of *WT1* levels for predicting relapse after allo-HSCT, we compared various thresholds, including median, 100 copies, top 25%, 250 copies based on ELN cutoff, and 300 copies. Figure 2 shows the CIR of each cutoff level before allo-HSCT and at 1 or 3 months after allo-HSCT. As shown by the ROC curve analysis (Figure 1C), every cutoff level before allo-HSCT was effective in identifying patients at high risk of relapse, and the threshold of 250 copies (n = 62) appeared to be the most effective considering the small number of patients expressing *WT1* exceeding 300 copies (n = 7). *WT1* levels at 3 months after allo-HSCT showed that only higher cutoff levels (250 and 300 copies) were effective in predicting the CIR, whereas *WT1* levels at 1 month were not useful for such prediction. Multivariate analysis of significant factors in univariate analysis (Supplementary Table S2) adjusted for age revealed that MRD positivity based on more than 250 copies of *WT1* before allo-HSCT and at 3 months after allo-HSCT independently predicted the CIR with hazard ratios of 3.4 (95% confidence interval, 2.0 to 5.8; $P < .001$) and 9.5 (3.8 to 24.0; $P < .001$), respectively (Table 3). Taken together, these data indicate that the optimal threshold for MRD assessment using the *WT1* assay is 250 copies, consistent with the upper limit of normal demonstrated by ELN researchers [22]. Among evaluable patients, 15% and 6% were *WT1* MRD positive before allo-HSCT and at 3 months after allo-HSCT, respectively, with relapse occurring at a median of 239 days (range, 51 to 898 days) and 112 days (range, 59 to 165 days), respectively, from MRD assessment (Supplementary Table S3). Patients with MRD positivity based on the *WT1* assay defined as greater than 250 copies before or 3 months after allo-HSCT showed significantly inferior survival (Supplementary Figure S3).

Optimal Candidates for the WT1 *MRD Assay*

Subgroup analyses were performed to reveal specific groups indicated for the *WT1* MRD assay. Cytogenetic and molecular risk stratification was another significant factor

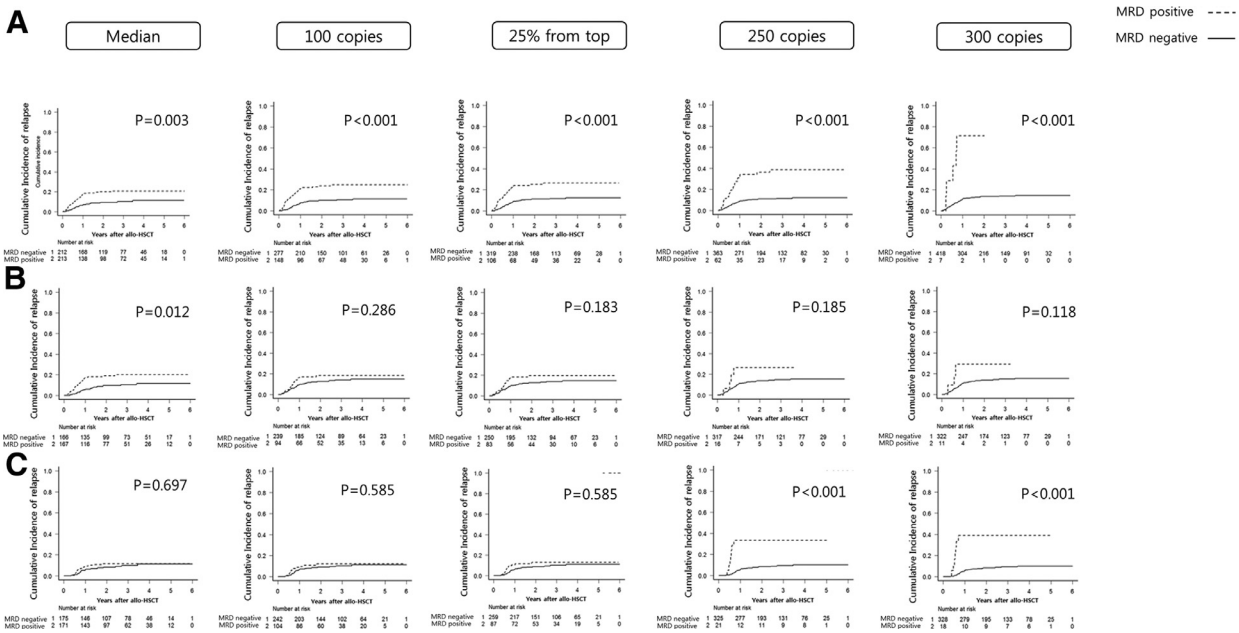

Figure 2. Cumulative incidence of relapse by *WT1* MRD assay with various thresholds at each time point. Cumulative incidence of relapse according to various thresholds of the *WT1* MRD assay before allo-HSCT (A) and at 1 month (B) or 3 months (C) after allo-HSCT.

Table 3
Multivariate Analysis for the Cumulative Incidence of Relapse after Allo-HSCT

	Before (n = 425)			1 Month (n = 333)			3 Months (n = 346)		
Variable	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age	1.0	0.98-1.03	.680	1.0	0.99-1.04	.225	1.03	1.0-1.07	.031
WBC count at diagnosis ($\times 10^9$/L)									
<50	1.0			1.0			1.0		
≥50	1.8	1.1-3.0	.027	1.6	0.9-3.0	.107	2.3	1.1-4.7	.024
Modified NCCN criteria*			.074						.001
Favorable	1.0			1.0		.004	1.0		
Intermediate	1.8	0.9-3.9	.112	2.3	0.9-5.8	.067	3.8	1.1-13.2	.038
Adverse	2.5	1.1-5.5	.023	4.4	1.7-10.9	.002	9.2	2.6-32.1	<.001
WT1 levels									
<250 copies	1.0			1.0			1.0		
≥250 copies	3.4	2.0-5.8	<.001	2.6	0.9-7.5	.071	9.5	3.8-24.0	<.001

HR indicates hazard ratio; CI, confidence interval.
* Modified NCCN criteria means 2017 NCCN risk stratification with the exception of the *TP53* mutation.

predicting the CIR after allo-HSCT (Table 3 and Supplementary Table S2). The *WT1* MRD assay conducted before allo-HSCT was significantly effective in predicting the CIR in the intermediate risk group (n = 206) based on every threshold, whereas it was not significant in favorable (n = 120) or adverse risk groups (n = 99) (Figure 3). In patients with normal cytogenetics without *NPM1* mutations (n = 117) as a part of the intermediate risk group, the *WT1* MRD assay was only significant in predicting the CIR in samples before allo-HSCT (Supplementary Figure S4). By contrast, the *WT1* MRD assay at 3 months after allo-HSCT predicted the CIR in patients included in both favorable and adverse risk groups with higher *WT1* cutoffs (250 or 300 copies), although it was insignificant in predicting the CIR in the intermediate risk group (Figure 4). Thus, using the optimal threshold defined as greater than 250 copies, *WT1* MRD positivity before allo-HSCT effectively identified patients at high risk of relapse in the intermediate risk group (57% versus 12%, $P < .001$), whereas *WT1* MRD positivity at 3 months after allo-HSCT was predictive of post-transplant relapse among patients included in favorable (29% versus 1%, $P < .001$) and adverse (60% versus 21%, $P = .001$) risk groups.

In a separate group of patients with AML with core binding factors (n = 102), a main part of the favorable risk group, the *WT1* MRD assay was not significant in predicting the CIR except at a very high threshold (300 copies) at 3 months after allo-HSCT, whereas the presence of *C-KIT* mutations was associated with post-transplant relapse (Supplementary Figure S5 and Supplementary Table S2). In terms of conditioning intensity, the *WT1* MRD assay before allo-HSCT was applicable to patients who received myeloablative ($P < .001$) and reduced-intensity conditioning ($P = .003$).

Multiple WT1 *MRD Assays over Time*

The *WT1* MRD-positive patients (≥250 copies) before allo-HSCT (n = 62) were CR (76%) and CR with incomplete recovery (24%) before transplantation, including 74% who had BM blasts fewer than 2% (Supplementary Table S4). Among the *WT1* MRD-positive patients (≥250 copies) before allo-HSCT (n = 62),

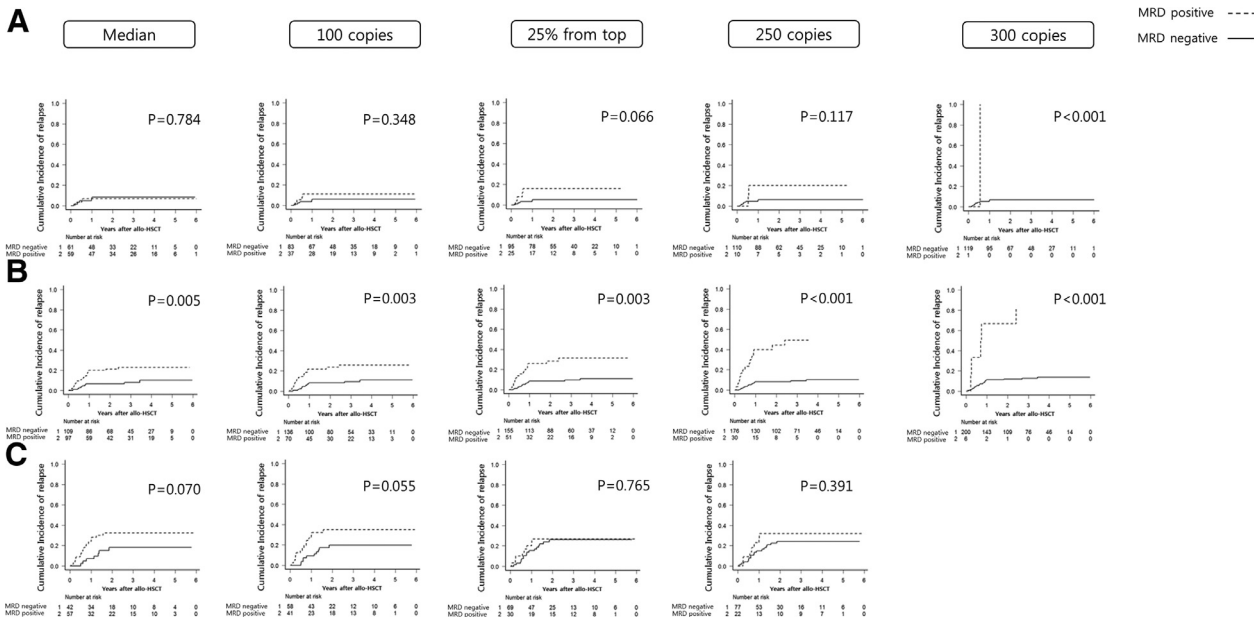

Figure 3. Cumulative incidence of relapse by the *WT1* MRD assay before allo-HSCT according to modified National Comprehensive Cancer Network (NCCN) risk groups. Cumulative incidence of relapse by the *WT1* MRD assay before allo-HSCT according to modified NCCN risk groups: (A) favorable, (B) intermediate, and (C) adverse.

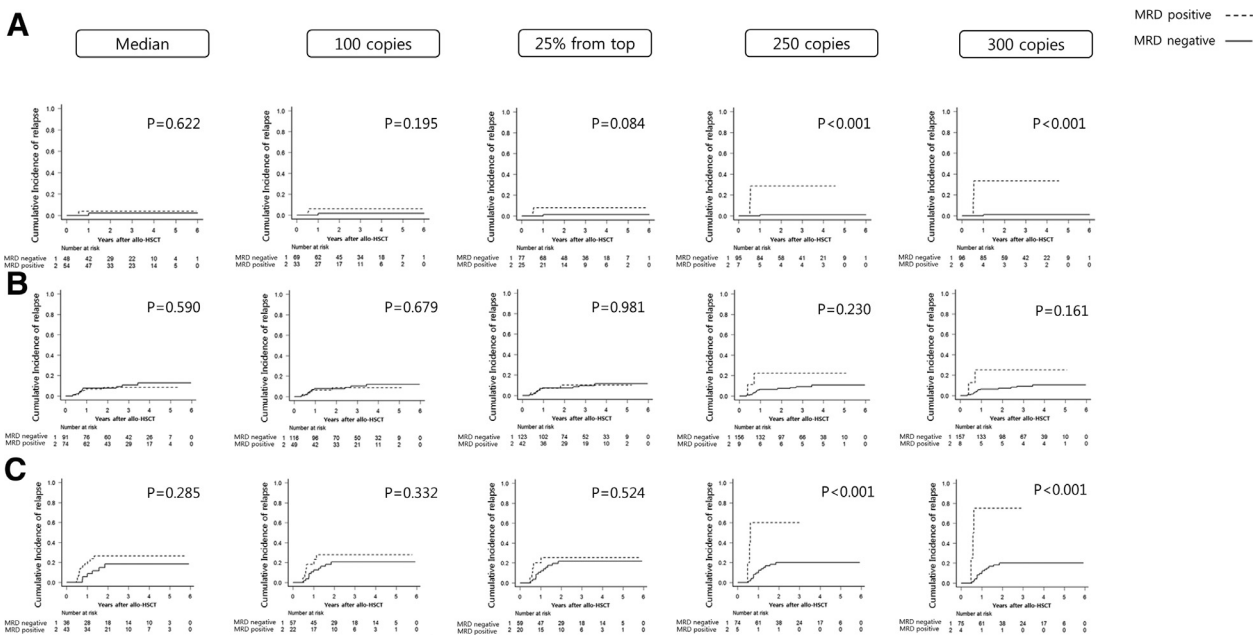

Figure 4. Cumulative incidence of relapse by the *WT1* MRD assay at 3 months after allo-HSCT according to modified National Comprehensive Cancer Network (NCCN) risk groups. Cumulative incidence of relapse by the WT1 MRD assay at 3 months after allo-HSCT according to modified NCCN risk groups: (A) favorable, (B) intermediate, and (C) adverse.

patients with relapse starting at 3 months after allo-HSCT (n = 14) had significantly higher *WT1* levels at 3 months after allo-HSCT compared with nonrelapsed patients (n = 30), whereas no significant difference in *WT1* level was detected at 1 month after allo-HSCT between relapsed and nonrelapsed patients. All MRD-positive patients at 3 months (n = 14) relapsed within 6 months (Figure 5).

DISCUSSION

Based on the diverse genetic etiology and clonal heterogeneity of AML [3], PQ-PCR of specific targets, such as *PML-RARA, RUNX1-RUNX1T1, CBFB-MYH11,* or *NPM1*, the current gold standard for MRD assessment, is useful only in 40% of patients with AML [1]. Although MFC-based AML MRD testing is applicable to most cases with a rapid turnaround, it also has a few limitations, including potential changes in phenotype over time, relatively less sensitivity, heterogeneity of leukemic phenotypes, operator-dependent bias because of the need for considerable expertise and experience, and subjective elements of analysis and data interpretation [2]. To identify another universal marker for MRD assessment, extensive efforts have been made to develop MRD tests targeting transcripts aberrantly expressed in AML [35,36], such as *WT1* [17-20,22-31]. Overexpression of the *WT1* gene in most patients with AML provides a target for novel immunotherapeutic approaches [37-39]. ELN researchers have introduced the standardized

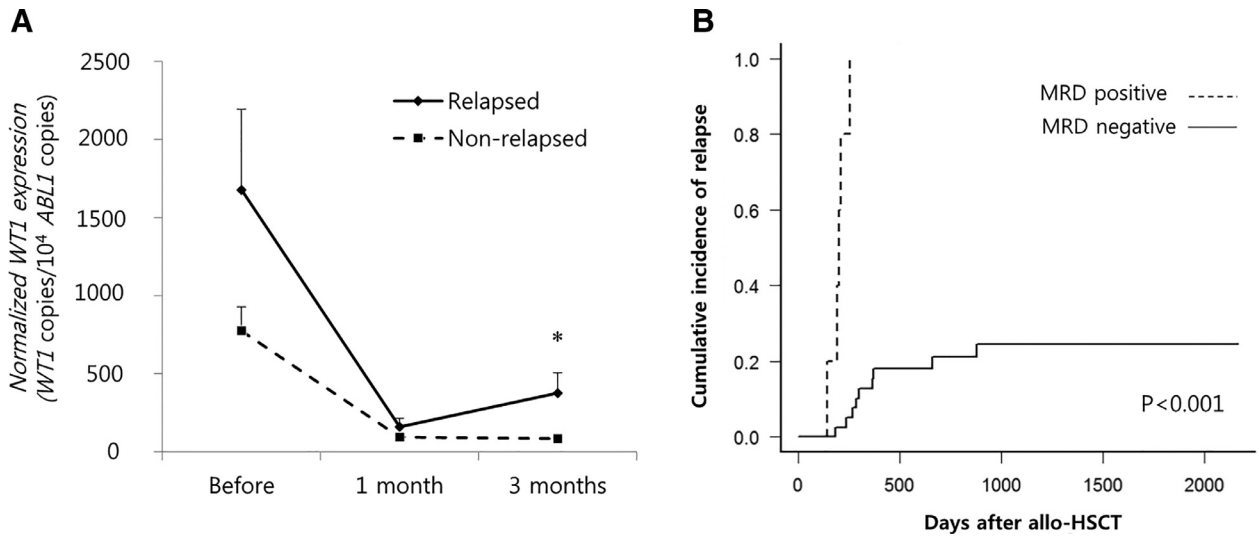

Figure 5. *WT1* expression kinetics in *WT1* MRD-positive patients before allo-HSCT. (A) *WT1* expression kinetics in *WT1* MRD-positive patients (over 250 copies) before allo-HSCT (n = 62). (B) Cumulative incidence of relapse by *WT1* MRD positivity at 3 months after allo-HSCT in patients who were MRD positive before allo-HSCT. WT1 levels were normalized with respect to the number of *ABL1* transcripts and expressed as copy numbers per 10^4 copies of *ABL1*. Results are expressed as the mean ± SEM. *P< .05.

WT1 assay as a tool for enhancing risk stratification and assessing response to *WT1*-targeted therapies [22]. Since then, several studies have provided evidence supporting the clinical use of the *WT1* assay as the MRD tool in AML. However, the *WT1* assay is not used widely despite its prognostic value in relapse because of the lack of well-controlled and large-scale studies, particularly for allo-HSCT [24-31].

In the setting of allo-HSCT, previous studies evaluating the BM *WT1* assay using the ELN method only included a small number of patients with AML (<200). Thus, they were limited in their ability to identify optimal time points, thresholds, and specific groups using the *WT1* assay [24-31]. Indeed, each study suggested different cutoff levels, including 64, 100, 138, and 250 copies or upper 25% levels, at different time points without analysis of the risk. Furthermore, most studies even included patients without remission (7% to 33%) before allo-HSCT [24-26,28,31], which was inappropriate to evaluate the role of residual leukemia in patients with morphologic remission. In the absence of a prospective study, the current study provides relevant clinical guidelines for the BM *WT1* MRD assay based on evaluation of a large consecutive cohort of patients with AML at remission (n = 425) before allo-HSCT and comparing 3 different time points: before allo-HSCT and at 1 or 3 months after allo-HSCT. Based on the ROC curve analysis, the time point before allo-HSCT was recognized as optimal for the BM *WT1* MRD assay. The performance was enhanced in patients with a higher degree of *WT1* overexpression at diagnosis. Every threshold tested before allo-HSCT was significantly predictive of post-transplant relapse. Among them, MRD positivity exceeding 250 copies in BM recommended by ELN researches to distinguish residual leukemia from background amplification [22] was independently predictive of post-transplant relapse and translated into inferior survival outcomes, in accordance with recent studies involving a small number of cases [29,31].

In the current study, the large number of patients facilitated identification of specific groups indicated for the BM *WT1* MRD assay. Our data demonstrate that the BM *WT1* MRD assay before allo-HSCT was indicated for the intermediate risk group, particularly those without molecular targets, such as *NPM1* mutations, although it showed limited efficacy in both favorable and adverse risk groups. The MFC MRD assay also has been reported to be mostly useful for the intermediate risk group [6,8,9] with limited ability to predict post-transplant relapse in the adverse risk group characterized by a high probability of relapse even after allo-HSCT [10]. However, despite the decreased power of subgroup analysis caused by fewer patients in each group, our data suggest that *WT1* MRD assessment after allo-HSCT facilitated the detection of patients with a higher risk for relapse in the adverse risk group because of the efficacy of BM *WT1* MRD positivity (>250 copies) at 3 months after allo-HSCT. In addition, among *WT1* MRD-positive patients before allo-HSCT, all patients who were *WT1* MRD positive at 3 months after allo-HSCT had a relapse within 2 to 6 months. This suggests that multiple *WT1* MRD tests over time are more informative than a single test in the allo-HSCT setting. The useful time point for the BM *WT1* MRD assay after allo-HSCT is not 1 month but 3 months.

Based on the foregoing limitation associated with the MFC-based MRD assay, the analysis based on the leukemic phenotype shows suboptimal sensitivity because of scarce BM cellularity early after allo-HSCT. The accuracy and reproducibility of molecular monitoring of *WT1* transcripts are affected by the limited regulation of engraftment after allo-HSCT and hematopoietic recovery after intensive chemotherapy [21,22]. The advantages of the *WT1* assay include standardization, easy accessibility, and limited operator bias and subjective interpretation, with a sensitivity comparable to MFC in an allo-HSCT setting [15]. In particular, the *WT1* MRD assay directly facilitates adoptive transfer of *WT1*-specific cytotoxic T cells [40,41]. The current study provides relevant evidence and guidelines for clinical application to encourage the use of assay. The Spanish group has recently demonstrated the potential of the BM *WT1* assay in identifying patients with a high probability of relapse after allo-HSCT irrespective of the results from MFC [25]. Peripheral blood was tested to determine the *WT1* levels in a small series of patients [28,42], which needed to be confirmed in a large cohort.

Currently, no AML MRD assay displays perfect sensitivity and specificity to accurately predict leukemia relapse [2]. Therefore, multiple tests over time, combined with multiple approaches, such as MFC and *WT1* [29], and incorporation of novel methods, such as next-generation sequencing [43] or digital PCR, may increase the accuracy of relapse prediction [1,2]. Recent reports demonstrate the feasibility of next-generation sequencing-based MRD monitoring before [44] or after [45] allo-HSCT that is worthy of further validation in prospective independent cohorts. The use of a single-gene *WT1* MRD assay can be combined with assays for other aberrantly expressed transcripts using multigene RQ-PCR arrays [35] and multigene RNA-sequencing protocols [36]. Furthermore, the role of MRD assays should be established in well-designed trials of MRD-based intervention for improving outcomes, as shown in a small series of pre-emptive treatment with donor lymphocyte infusion based on *WT1* assays [24,27].

In conclusion, the current study of a large consecutive cohort provides relevant guidelines for BM *WT1* MRD monitoring in patients with AML undergoing allo-HSCT. Such monitoring is particularly indicated in patients with AML without specific molecular targets, such as *NPM1*, *RUNX1-RUNX1T1*, and *CBFB-MYH11*. Different significance by time points and benefits of more than 2 different time points should be considered for clinical applications of the *WT1* assay to identify high-risk patients with AML for relapse, potential candidates for hypomethylating agents, targeted therapies, and immune approaches based on persistently high *WT1* levels.

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SUPPLEMENTARY MATERIALS

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