Biol Blood Marrow Transplant 19 (2013) 958–966

Serial Measurement of *WT1* Expression and Decrement Ratio Until Hematopoietic Cell Transplantation as a Marker of Residual Disease in Patients with Cytogenetically Normal Acute Myelogenous Leukemia

Jae-Ho Yoon [1], Hee-Je Kim [1,*], Seung-Hwan Shin [1], Seung-Ah Yahng [1], Sung-Eun Lee [1], Byung-Sik Cho [1], Ki-Seong Eom [1], Yoo-Jin Kim [1], Seok Lee [1], Chang-Ki Min [1], Seok-Goo Cho [1], Dong-Wook Kim [1], Jong-Wook Lee [1], Woo-Sung Min [1], Chong-Won Park [1], Ji-Hyang Lim [2]

[1] *Department of Hematology, Catholic Blood and Marrow Transplantation Center, Seoul St. Mary's Hospital, The Catholic University of Korea, Seoul, Korea*

[2] *Department of Laboratory Medicine, College of Medicine, The Catholic University of Korea, Seoul, Korea*

Article history:
Received 31 January 2013
Accepted 22 March 2013

Key Words:
Acute myelogenous leukemia
Normal karyotype
WT1
FLT3-ITD
NPM1

A B S T R A C T

Using real-time quantitative PCR, we monitored Wilms tumor gene 1 (*WT1*) expression from diagnosis to hematopoietic stem cell transplantation (HSCT) in adult patients with cytogenetically normal acute myelogenous leukemia (CN-AML) and *FLT3-ITD* and *NPM1* mutations. The values at diagnosis were evaluated in 104 patients. Data collected after induction chemotherapy were available for all patients, but only 68 patients were treated with HSCT. Significant *WT1* expression cut-offs were determined by receiver operation characteristic curve analysis, and rates of overall survival (OS) and disease-free survival (DFS) were estimated. *WT1* decrement ratios (DR) at postinduction chemotherapy and at pre- and post-HSCT compared with the diagnostic level were calculated. Higher *WT1* expression at diagnosis, postinduction chemotherapy, and pre-HSCT showed inferior OS ($P = .015$, $<.001$, and .002) and DFS ($P = .006$, $<.001$, and .003). The cut-offs were determined at the median for diagnostic *WT1* expression and at the 25% level from the top for other time points excluding post-HSCT. The *WT1* DR $\geq$ 1-log after induction chemotherapy showed superior OS and DFS ($P = .009$ and .002) and *WT1* DR $\geq$ 1-log preceding HSCT also showed superior OS and DFS ($P = .009$ and .003). Results of *WT1* DR were consistently applicable in each subgroup with higher ($\geq$1.0) and lower ($<$1.0) *WT1* expression at diagnosis and also in *NPM1*-wild-type/*FLT3-ITD*–negative CN-AML. The *WT1* DR therefore predicted survival outcomes after HSCT more accurately than did the diagnostic *WT1* expression. *WT1* expression may serve as a reliable marker for residual disease and *WT1* DR as a prognostic indicator, particularly in *NPM1*-wild-type/*FLT3-ITD*–negative CN-AML. These measures may be applied throughout the course of treatment and even after HSCT.

INTRODUCTION

Most patients with acute myelogenous leukemia (AML) have at least 1 chromosomal aberration in their marrow blasts at diagnosis. Several recurrent structural and numeric cytogenetic aberrations have been identified, and many of them are shown already to independently predict the likelihood of complete remission (CR), relapse, and overall survival (OS) [1,2]. However, in 40% to 49% of adults with AML and in 25% of children with AML, no microscopically detectable chromosomal abnormality can be found. This cytogenetically normal AML (CN-AML) is the largest cytogenetic subgroup of adult AML [2,3]. Although this group is characterized as having intermediate risk, only 40% of patients are believed to be long-term survivors [4]. This has led to recognition of CN-AML as a highly heterogeneous subgroup of AML and to identification of several genetic abnormalities with prognostic value. These include mutations in *FLT3* [5,6], *NPM1* [7], *CEBPA* [8], and *MLL* genes and aberrant expression of *BAALC* [4,9], *ERG*, and *MN1*. Use of these markers may increase the accuracy in predicting response to current therapy and may lead to improved survival through development of risk-adaptive treatment strategies [10,11].

Wilms tumor gene 1 (*WT1*), located on chromosome 11p13, encodes a transcriptional regulator with both activator and repressor capabilities. *WT1* may behave as either a tumor suppressor gene [12] or an oncogene. However, the functional expression, protein isoforms, and target genes of *WT1* may be cell type dependent [13]. Expression of the *WT1* gene is detected in 75% to 100% of adults with AML, and mutations in *WT1* occur in 10% to 15% of patients with AML [14,15]. Either mutation or overexpression of *WT1* has adverse implications for survival and relapse in AML, and persistence of *WT1* expression after treatment may serve as a marker for minimal residual disease (MRD) [15-19].

Mutations in the Fms-like tyrosine kinase 3 (*FLT3*) receptor gene, most commonly internal tandem duplications (ITDs), are frequently found in AML. Overexpression of *FLT3-ITD* at diagnosis is predictive of adverse survival outcomes [6,20,21]. In contrast, *NPM1* mutation, the most common single genetic abnormality, may be associated with early blast cell clearance, better CR rates, and favorable OS [22,23]. This association appears to be stronger when *NPM1*

Financial disclosure: See Acknowledgments on page 965.

* Correspondence and reprint requests: Hee-Je Kim, MD, PhD, Department of Hematology, Catholic Blood and Marrow Transplantation Center, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-070, Korea.
E-mail address: cumckim@catholic.ac.kr (H.-J. Kim).

1083-8791/$ – see front matter © 2013 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2013.03.013

mutation is not accompanied by *FLT3-ITD* mutation [24]. However, patients in the largest subgroup of CN-AML are both *NPM1*-wild-type and *FLT3-ITD* negative [25]. In this group, no specific minimal residual marker has yet been introduced.

The objectives of this study were to assess serial *WT1* expression levels and *WT1* decrement ratios (DR) in the course of treatment in adult CN-AML. In addition, we aimed to evaluate the prognostic value of *WT1* expression as a marker of residual disease and the importance of MRD kinetics in predicting OS and DFS, specifically in the *NPM1*-wild-type/*FLT3-ITD*- negative CN-AML.

METHODS

Patients

The Catholic Medical Center Institutional Review Board approved this single-center retrospective study. Medical records were reviewed for 515 AML patients (ages 15 to 85 years; median, 45.2 years) with variable karyotypes who were diagnosed from July 2008 to December 2011. CN-AML was established mainly by chromosomal analysis yielding negative result for multiplex PCR analysis. Normal karyotype was established in bone marrow (BM) cells exclusively, after 24 and 48 hours in unsynchronized culture by GTG banding in at least 20 metaphases. The International System for Cytogenetic Nomenclature was used as a guideline for classification, and data on 206 CN-AML patients (40.0%) were available in this study.

Among the 206 patients with CN-AML, 181 had results of a *FLT3-ITD* mutation analysis (positive in 46 patients [25.4%]), 184 had results of a *NPM1* mutation analysis (positive in 65 patients [35.3%]), and 125 had results for *WT1* expression at the time of diagnosis. Because we started each marker examination at different time points, the sample size was reduced and finally consisted of 104 patients with data available for all 3 molecular markers (*FLT3-ITD* and *NPM1* mutations and *WT1* expression at the diagnostic and postinduction chemotherapy time points). Sixty-eight patients were treated by hematopoietic stem cell transplantation (HSCT) and had pre-HSCT data available; however, the post-HSCT data for 10 patients were missing. Therefore, data at 1 month post-HSCT were available for only 58 patients.

Molecular Marker Study

WT1 expression in BM samples were determined by real-time quantitative PCR (RQ-PCR) using the *WT1* ProfileQuant kit (Ipsogen, Marseille, France). Data were analyzed for assays performed at diagnosis, postinduction chemotherapy, and pre- and post-HSCT. The post-HSCT *WT1* expression results were uniformly checked at approximately 1 month after stem cell infusion. The *FLT3-ITD* mutation was evaluated by multiplex allele-specific PCR (ABSOLUTE *FLT3 TKD/ITD* PCR; Biosewoom, Korea), and the *NPM1* mutation and *NPM1* expression were measured by RQ-PCR using the *NPM1* MutaQuant kit (Ipsogen).

WT1 expression calculations were normalized as the absolute ratio of *WT1* expression to normal *ABL* expression, so a value >1.0 is suggestive of higher expression of *WT1* compared with the normal population. Assays were performed in replicates for greater accuracy in comparing results, and when the *ABL* quantification was inappropriately low, we repeated the assay 3 times. To determine the significant cut-off levels of *WT1* expression for survival outcomes at several time points, we used receiver operation characteristic (ROC) curve analysis. The most significant cut-off level was determined at *WT1* = 1.0 at diagnosis ($P = .038$), and this level was close to the median value (the median value for 104 patients was 0.951). We initially stratified patients by multiples of this level to analyze survival outcomes. After treatments, the median *WT1* expression decreased from the diagnostic level of .951 to a median value of .02 (1.5-log reduction) until before HSCT. At postinduction chemotherapy and pre-HSCT time points, the most significant *WT1* expression cut-offs for OS and DFS were at the level of 25% from the top. The significant value was .156 at postinduction chemotherapy ($P < .001$) and .050 at pre-HSCT ($P = .001$), but we could not determine a significant cut-off at the post-HSCT time point.

These values are much lower than the normal *ABL* expression level of 1.0, but we stratified patients at these levels and analyzed survival outcomes similarly. One more parameter calculated in this study was the log10 DR of the *WT1* expression level between diagnosis and each of 3 post-treatment times (postinduction chemotherapy and pre- and post-HSCT). To determine the significant level of DR, we once again used ROC curve analysis with identification of survival outcomes ($P < .01$), and the most significant level occurred at 1-log reduction. We also classified patients into 2 subgroups showing DR ≥ 1-log and <1-log, respectively.

Table 1
Serially Checked *WT1* Expression Levels from Diagnosis to Post-HSCT in CN-AML patients

	WT1 Expression RQ-PCR Level (WT1/ABL1)	*P*
At diagnosis (n = 104)		.038*
25%	.250	
50% (median)	.951†	
75%	3.833	
At postinduction chemotherapy		<.001*
25%	.005	
50% (median)	.022	
75%	.156†	
At pre-HSCT (n = 68)		.001*
25%	.006	
50% (median)	.019	
75%	.050†	
At post-HSCT (n = 58)		.144
25%	.004	
50% (median)	.008	
75%	.020	
Patient number of *WT1* DR ≥ 1-log		
At postinduction chemotherapy (n = 104)	64 (61.5%)	<.001*‡
At pre-HSCT (n = 68)	46 (67.6%)	.003*‡
At post-HSCT (n = 58)	44 (75.9%)	.144

Number of patients with *WT1* DR more than 1-log after treatments compared with diagnostic level are also displayed.

* *P* significant at the < .05 level.

† Statistically significant cut-off levels of *WT1* expression for both OS and DFS were determined by ROC curve analysis. At diagnosis ≒ 1.0 (median, $P = .038$), at postinduction chemotherapy ≒ .15 (25% from the top, $P < .001$), at pre-HSCT ≒ .050 (25% from the top, $P = .001$).

‡ Value presenting the statistical significance of *WT1* DR more than 1-log for OS and DFS analyzed by ROC curve analysis.

Treatment

All 104 patients were treated with chemotherapy to induce remission. Seventy-four patients (71.2%) were treated according to our standard protocol, which consists of 3+7 idarubicin (IDA) plus N^4-behenoyl-1-β-D-arabinofuranosyl cytosine (BHAC) [26]. Briefly, IDA was administered daily at a dose of 12 mg/m^2 for 30 minutes intravenously on 3 consecutive days, and BHAC was administered daily at a dose of 300 mg/m^2 over a period of 4 hours on 7 consecutive days. Twelve patients (11.5%) were treated with 3+7 IDA plus cytosine arabinoside (ARA-C) at a dose of 100 mg/m^2 for 24 hours. Fifteen patients (14.4%) older than age 60 years of poor performance status were treated with low-dose ARA-C (20 mg/m^2 twice daily) plus etoposide (100 mg) for 14 days. Of the last 2 patients (1.9%), 1 was treated with daunorubicin at a dose of 60 mg/m^2 plus ARA-C and 1 was treated with fludarabine plus ARA-C.

After CR, 1 or 2 consolidation chemotherapies were administered; otherwise, patients were treated with reinduction chemotherapy. Ninety patients (86.5%) achieved CR within 2 cycles of chemotherapy, and 68 patients (65.4%) who had available an HLA-matched sibling, unrelated, or haploidentical familial donor eventually underwent allogeneic HSCT. We administered a myeloablative conditioning regimen consisting of cyclophosphamide (120 mg/kg) combined with 1320 cGy of total body irradiation or busulfex (12.8 mg/kg). In the case of advanced age or poor performance status with comorbidity, we considered a reduced-intensity conditioning regimen consisting of busulfex (6.4 mg/kg) and fludarabine (150 mg/m^2) with 400 cGy of total body irradiation. If the patient did not have an available allogeneic donor, we gave an autologous HSCT with a myeloablative conditioning regimen consisting of ARA-C (9 g/m^2), melphalan (100 mg/m^2), and 1200 cGy of total body irradiation after 2 cycles of consolidation chemotherapy that followed CR (n = 8).

Statistical Analysis

The purpose of this study was to determine the prognostic value of *WT1* expression levels and *WT1* DR at specific time points in the course of treatment. Clinical information, including molecular markers, treatment protocols, and survival outcomes, were obtained. Other clinical parameters, including age, leukocyte count, and peripheral blood (PB) and BM blast percentage at diagnosis, were treated as continuous variables. To determine the significantly sensitive and specific cut-off levels of *WT1* expression and *WT1* DR for survival outcomes, we used ROC curve analysis. All categorical

Table 2
Baseline Characteristics According to *WT1* Expression at Diagnosis and at Pre-HSCT in CN-AML

	WT1 Expression at Diagnosis (n = 104)			*WT1* Expression at Pre-HSCT (n = 68)		
	<1* (n = 52)	≥1* (n = 52)	*P*	<.05* (n = 51)	≥.05* (n = 17)	*P*
Age, yr (range)	46.2 (15-75)	48.4 (18-74)	.459	40.4 (15-66)	48.7 (30-61)	.013*
Gender (male)	31 (59.6%)	29 (55.8%)	.691	29 (56.9%)	9 (52.9%)	.778
Leukocyte count, 10^6/L (median)	34,676	45,125	.302	40,258	28,315	.343
PB blast	46.8%	50.5%	.596	49.3%	42.5%	.523
BM blast	65.4%	75.3%	.031*	69.0%	66.3%	.688
Platelet count, 10^6/L (median)	77,480	97,860	.253	91,980	73,530	.372
Induction therapy			.780			.716
Intensive chemotherapy	45 (86.5%)	44 (84.6%)		50 (98.0%)	17 (100%)	
Low-dose chemotherapy	7 (13.5%)	8 (15.4%)		1 (2.0%)	0 (0.0%)	
Disease status						
CR after 1 cycle of chemotherapy†	43 (82.7%)	35 (67.3%)	.070	46 (90.2%)	15 (88.2%)	.818
CR within 2 cycles of chemotherapy	48 (92.3%)	42 (80.8%)	.085	51 (100%)	51 (100%	1.000
Primary refractory	4 (7.7%)	10 (19.2%)	.085	0 (0.0%)	0 (0.0%)	1.000
Relapse during chemotherapy	9 (17.3%)	8 (15.4%)	.791	2 (3.9%)	2 (11.8%)	.234
Final treatment plan						
Chemotherapy alone	14 (26.4%)	22 (43.1%)	.099			
Intensive chemotherapy	9 (64.3%)	16 (72.7%)	.592			
Low-dose ARA-C	5 (35.7%)	6 (27.3%)	.592			
HSCT	38 (73.1%)	30 (57.7%)	.099			
Pre-HSCT status (non-CR)	4 (10.5%)	1 (3.3%)	.169	3 (5.9%)	2 (11.8%)	.421
Type						
Auto-HSCT	6 (15.7%)	2 (6.6%)	.246	6 (11.8%)	2 (11.8%)	1.000
Allo-MSD	19 (50.0%)	17 (56.6%)	.584	25 (49.0%)	11 (64.7%)	.262
Allo-unrelated	11 (28.9%)	8 (26.6%)	.835	15 (29.4%)	4 (23.5%)	.640
Haplo-identical	2 (5.4%)	3 (10.2%)	.457	5 (9.8%)	0 (0.0%)	.180
Source (BM)	15 (39.5%)	14 (46.6%)	.552	23 (45.1%)	6 (35.3%)	.479
Intensity (MAC)	31 (81.6%)	20 (66.6%)	.159	39 (76.5%)	12 (70.6%)	.628
Mutation status						
NPM1 mutation	14 (26.9%)	22 (42.3%)	.099	12 (23.5%)	7 (41.2%)	.160
FLT3-ITD mutation	11 (21.2%)	19 (36.5%)	.083	6 (11.8%)	5 (29.4%)	.087
NPM1(+) / FLT(−)	8 (15.4%)	12 (23.1%)	.132	9 (17.6%)	4 (23.5%)	.270
NPM1(−) / FLT(−)	33 (63.5%)	21 (40.4%)		36 (70.6%)	8 (47.1%)	
NPM1(+) / FLT(+)	6 (11.5%)	10 (19.2%)		3 (5.9%)	3 (17.6%)	
NPM1(−) / FLT(+)	5 (9.6%)	9 (17.3%)		3 (5.9%)	2 (11.8%)	

MSD indicates matched sibling donor; MAC, myeloablative conditioning regimen.

* Statistically most significant cut-off values for both OS and DFS analyzed by ROC curve: *WT1* at diagnosis ≒ 1.000 (median = .951, *P* = .038), *WT1* at pre-HSCT ≒ .050 (25% level from the top = .050, *P* = .001).

† Remission induction chemotherapy regimens are as follows (*WT1* < 1 versus ≥1, *P* = .190): IDA/BHAC 3/7, n = 74 (36 versus 38), IDV/ARA-C 3/7, n = 12 (9 versus 3), daunorubicin +ARA-C 3/7, n = 2 (0 versus 2), fludarabine +ARA-C, n = 1 (0 versus 1). Low-dose ARA-C (20 mg/m^2 twice daily) for 14 days, n = 15 (7 versus 8).

variables were compared by chi-square analysis and Fisher's exact test, and continuous variables were compared using Student's *t*-test and Wilcoxon's rank-sum test.

OS and DFS rates were calculated using Kaplan-Meier survival curves, and log-rank analysis was used to evaluate differences between survival distributions. OS represents the proportion of patients who were alive at a specified time from the date of diagnosis and was associated with death due to any cause, both related and unrelated to the AML and treatments. DFS measured the proportion of people who remained alive or free of disease at a specified time from the date of first CR achieved and took into account death, relapse, loss to follow-up, and transfer to hopeless status as the result of disease or treatment complications. Univariate and multivariate analyses using the Cox's proportional regression model were used to calculate the survival hazard ratio (HR). All statistical analyses were performed using SAS software (version 9.2, SAS Institute, Inc., Cary, NC). Statistical significance was set at $P < .05$.

RESULTS

WT1 Expression and WT1 DR from Diagnosis to the HSCT Period: Baseline Characteristics

In Table 1, serial RQ-PCR assays of *WT1* expression from diagnosis to post-HSCT are presented by quartile. The median *WT1* level at diagnosis was .951. Significant diagnostic *WT1* RQ-PCR cut-off levels for inferior OS and DFS were determined at the level higher than 1.0 (close to the median value, $P = .038$). At postinduction chemotherapy and pre-HSCT, median *WT1* expression levels were .022 and .019. However, the most significant *WT1* RQ-PCR cut-off levels for inferior OS and DFS were determined at the levels higher than .15 and .05 (25% level from the top, $P < .001$ and $P = .001$) at postinduction chemotherapy and pre-HSCT respectively. We could not obtain a significant cut-off at the post-HSCT time point.

Table 1 also shows the greater than 1-log post-treatment *WT1* DR. The maximal decreases in *WT1* expression, up to 4-log, occurred after remission induction chemotherapy and after HSCT. A median reduction of 1.5-log occurred after induction chemotherapy and at pre-HSCT and a median reduction of 2-log occurred in the post-HSCT period. The most significant *WT1* DR for survival outcomes was determined at more than 1-log compared with the pretreatment level at both postinduction chemotherapy ($P < .001$) and pre-HSCT ($P = .003$). Sixty-four patients (61.5%) at postinduction chemotherapy, 46 patients (67.6%) at pre-HSCT, and 44 patients (75.9%) at post-HSCT achieved *WT1* DR greater than 1-log.

Baseline characteristics of patients according to *WT1* expression level at diagnosis and pre-HSCT are shown in Table 2. Among 104 patients, 52 patients showed *WT1* expression higher than 1.0, and the other 52 were lower than 1.0 at diagnosis. Between these 2 subgroups, age, leukocyte count, platelet count, and PB blast percentage did not differ significantly, but the BM blast count was higher in the group with higher *WT1* expression at diagnosis (75.3% versus 65.4%, $P = .031$). The subgroup with lower *WT1* expression at

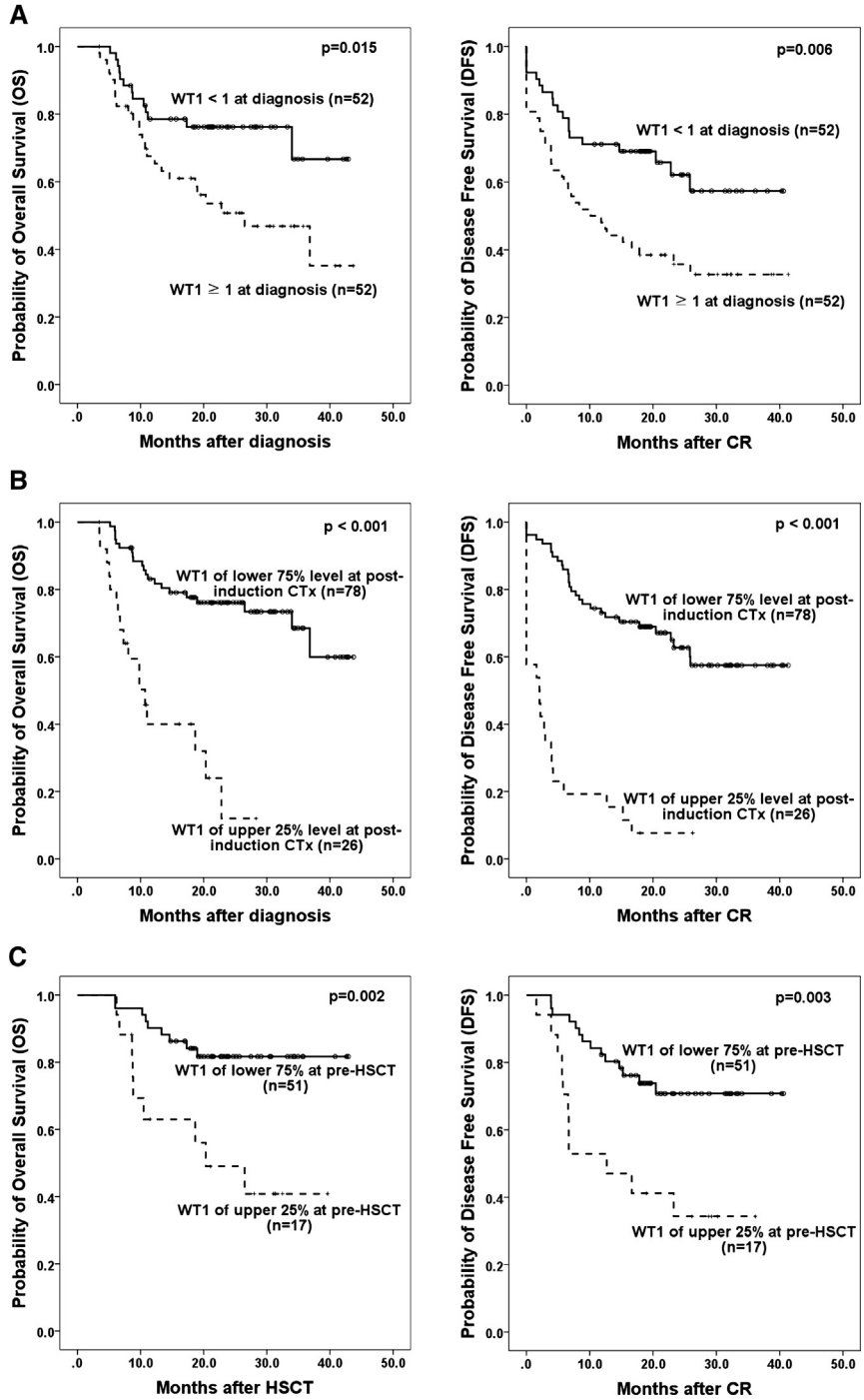

Figure 1. (A) Higher *WT1* expression ≥ 1.0 at diagnosis showed inferior OS and DFS (n = 104, 52 versus 52). (B) Higher *WT1* expression ≥0.15 (higher than upper 25% level) at postinduction chemotherapy showed inferior OS and DFS (n = 104, 52 versus 52). (C) Higher *WT1* expression ≥.05 (higher than upper 25% level) at pre-HSCT showed inferior OS and DFS after HSCT (n = 68, 17 versus 51).

diagnosis included more patients treated with HSCT (73.1% versus 57.7%, $P = .099$) and showed a relatively higher CR rate after the first cycle of induction chemotherapy (82.7% versus 67.3%, $P = .070$). The 2 subgroups did not differ significantly in postinduction therapy and status of several mutations, but *FLT3-ITD* mutations were more common in the subgroup with higher *WT1* expression at diagnosis (36.5% versus 21.2%, $P = .083$).

Sixty-eight patients treated with HSCT (5 patients were non-CR at pre-HSCT) were divided into 2 subgroups according to the pre-HSCT *WT1* expression at .05 (25% level from the top, higher than .05 [n = 17] versus lower than .05 [n = 51]). Patients were significantly older in the subgroup with higher *WT1* expression at pre-HSCT (48.7 versus 40.4 years, $P = .013$). Other parameters did not differ significantly between the 2 subgroups. As in the subgroup division based on level at diagnosis, *FLT3-ITD* mutations were more common in the subgroup with higher pre-HSCT *WT1* expression (29.4% versus 11.8%, $P = .087$), and we also identified slightly more *NPM1* mutations in the group with higher

WT1 expression (41.2 versus 23.5%, $P = .160$). Mutation frequencies did not differ significantly between 2 subgroups.

CN-AML Survival Outcomes Associated with WT1 Expression and WT1 DR from Diagnosis to the HSCT Period

The patient subgroups with higher *WT1* expression at diagnosis, at postinduction chemotherapy and preceding HSCT independently showed OS and DFS rates inferior to those of the subgroup with lower *WT1* expression (Figure 1); *WT1* levels higher than 1.0 at diagnosis (n = 52 versus 52, $P = .015$ and $P = .006$), higher than .15 at postinduction chemotherapy (n = 26 versus 78, $P < .001$ and $P < .001$), and higher than .05 preceding HSCT (n = 17 versus 51, $P = .002$ and $P = .003$) were significantly predictive. The subgroup with *WT1* DR greater than 1-log at postinduction chemotherapy (n = 64 versus 40, $P = .009$ and $P = .002$) and at pre-HSCT (n = 46 versus 22, $P = .009$ and $P = .003$) showed more favorable OS and DFS than the group with less than 1-log reduction (Figure 2). Univariate and multivariate analyses by Cox's proportional regression model are presented in Table 3. Both lower *WT1* expression at diagnosis and *WT1* DR more than 1-log at pre-HSCT predicted favorable survival outcomes in multivariate analysis.

Subgroups were further selected according to the *WT1* expression at diagnosis ($\geq$1.0 or $<$1.0) and *WT1* DR ($>$1-log or $<$1-log) preceding HSCT and analyzed for survival outcomes. We identified four subgroups: Subgroup1 (n = 20), *WT1* at diagnosis $<$1.0 and *WT1* DR $\geq$ 1-log; Subgroup 2 (n = 18), diagnostic *WT1* $<$ 1.0 and *WT1* DR $<$ 1-log; Subgroup 3 (n = 26), diagnostic *WT1* $\geq$ 1.0 and *WT1* DR $\geq$ 1-log; and Subgroup 4 (n = 4), diagnostic *WT1* $\geq$ 1.0 and *WT1* DR $<$ 1-log. Patients with higher diagnostic *WT1* expression showed more than 1-log reduction in 86.6% of patients (Subgroup 3), and this was significantly higher than in the group with lower diagnostic *WT1* expression ($P = .003$): more than 1-log reduction in 52.6% (Subgroup 1). Patients of lower *WT1* expression at diagnosis with more than 1-log reduction in *WT1* expression from diagnosis to pre-HSCT (Subgroup 1) comprised the most favorable-risk group, with respect to OS ($P = .002$, HR = .029 [95% confidence interval {CI}, .002-.263]) and DFS ($P = .002$, HR = .029 [95% CI, .003-.259]) after HSCT (Figure 3). Subgroup 3, with higher *WT1* expression at diagnosis and more than 1-log reduction at the time of HSCT, showed more favorable OS ($P = .010$, HR = .203 [95% CI, .060-.681]) and DFS ($P = .031$, HR = .281 [95% CI, .089-.887]) than Subgroup 2 with less than 1-log reduction in *WT1* expression from diagnosis to pre-HSCT. All HRs of Subgroups 1, 2, and 3 were compared with Subgroup 4, which presented the worst survival outcomes.

Analysis in the Subgroup of NPM1-wild-type/FLT3-ITD–Negative CN-AML

The *NPM1*-wild-type/*FLT3-ITD*–negative CN-AML subgroup included 54 patients, and 44 of these were treated with HSCT. Within this subgroup, patients with higher *WT1* expression at diagnosis and at postinduction chemotherapy showed inferior OS and DFS as compared with

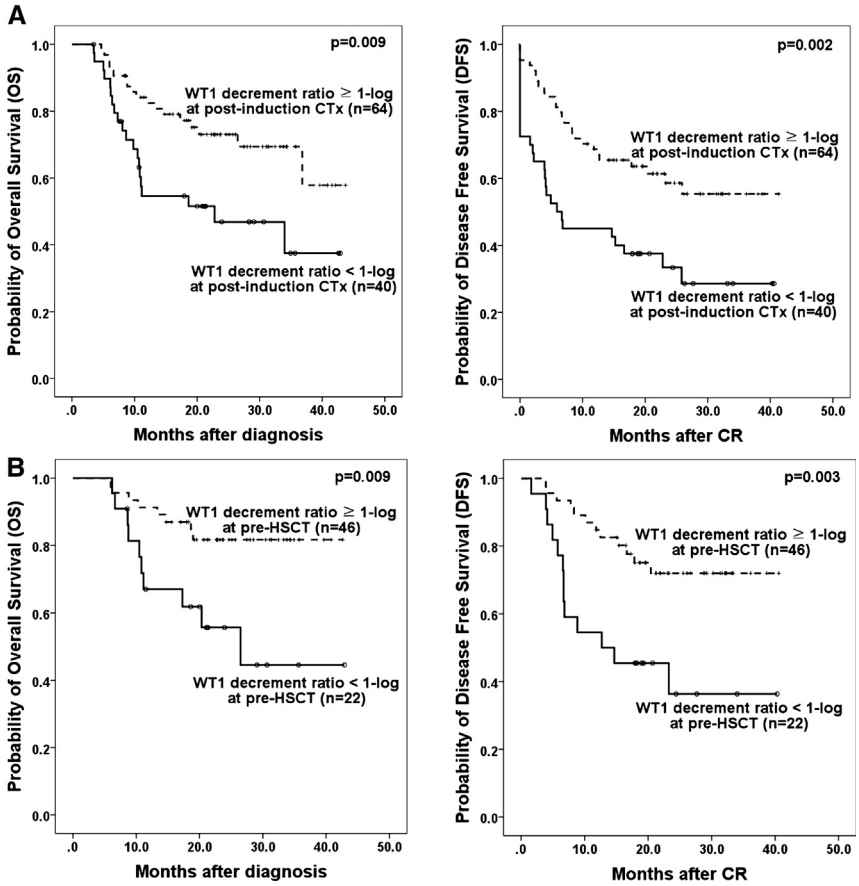

Figure 2. (A) *WT1* DR more than 1-log at postinduction chemotherapy showed superior OS and DFS (n = 104, 64 versus 40). (B) *WT1* DR more than 1-log at pre-HSCT also showed superior OS and DFS after HSCT (n = 68, 46 versus 22).

Table 3
Univariate and Multivariate Analyses of OS and DFS Calculated Using Cox's Proportional Regression Model in CN-AML Patients Treated with HSCT (n = 68)

	OS				DFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR	*P* (95% CI)	HR	*P* (95% CI)	HR	*P* (95% CI)	HR	*P* (95% CI)
Age, yr	1.010	.322 (.990-1.031)			1.007	.439 (.989-1.025)		
Leukocyte (≥50,000)	.971	.935 (.485-1.947)			1.009	.976 (.555-1.836)		
BM blast (≥70%)	1.289	.439 (.678-2.451)			1.422	.227 (.803-2.519)		
FLT3-ITD positivity	4.064	<.001* (2.128-7.761)			4.129	<.001* (2.358-7.229)		
Higher *WT1* ≥ 1.0 at diagnosis (median)	2.180	.019* (1.138-4.175)	4.257	.010* (1.421-12.752)	2.138	.008* (1.218-3.753)	3.519	.010* (1.343-9.220)
Higher *WT1* ≥ .15 at postinduction chemotherapy (upper 25%)	4.857	<.001* (2.498-9.442)			6.478	<.001* (3.651-11.492)		
Higher *WT1* ≥ .05 at pre-HSCT (upper 25%)	3.841	.003* (1.593-9.264)			3.182	.004* (1.439-7.036)		
WT1 DR ≥ 1-log at pre-HSCT	.310	.009* (.128-.751)	.154	.001* (.053-.445)	.325	.005* (.148-.715)	.174	<.001* (.068-.441)

* $P < 0.05$.

the subgroup with lower *WT1* expression (Figure 4A, B); patients with higher than 1.0 at diagnosis (n = 21 versus 33, $P = .080$ and $P = .010$) and higher than .15 at postinduction chemotherapy (n = 7 versus 47, $P = .027$ and $P = .001$). Patients with *WT1* DR greater than 1-log at pre-HSCT also showed more favorable OS ($P = .048$) and DFS ($P = .060$) after HSCT than did those with *WT1* DR less than 1-log (Figure 4C).

DISCUSSION

As reported in previous studies, BM and PB samples obtained from healthy volunteers express *WT1* at very low levels [27,28]. We also found that *WT1* is overexpressed in most AML patients at diagnosis, and various decremental responses were observed along the timeline of the treatments, including HSCT. A number of studies have pointed to this consistently overexpressed gene as a potential target for new immunological therapies for CN-AML, using *WT1* gene expression as a marker for MRD assessment [17,29]. Evaluation of MRD after the first cycle of induction chemotherapy is helpful in predicting outcome and may inform the selection of postremission treatment type and intensity. At times before and after HSCT, MRD evaluation is important for early detection of relapse, and based on this evaluation, the conditioning regimen intensity or immunosuppressive agents for post-HSCT management may be adjusted. Our present findings support the use of *WT1* expression as an MRD marker in CN-AML and as a predictive indicator of survival outcomes. These findings are especially relevant because adult patients with *NPM1*-wild-type/*FLT3-ITD*–negative CN-AML comprise the largest subgroup in adult CN-AML.

Evidence increasingly supports using *WT1* expression levels from BM or PB as an MRD marker at diagnosis and after treatments. Cilloni et al. reported that increased *WT1* expression from PB samples probably represents a circulating blast cell population proportional to the leukemia burden and may therefore serve as an early predictor of relapse after treatments [17,30]. Using BM and PB samples from 96 adult AML patients with variable cytogenetic risk, Gray et al. found significant association of *WT1* expression from PB with BM blast counts but not with peripheral leukocyte counts. These authors also showed the utility of using PB samples for *WT1* analysis in detecting MRD [16]. We used only BM for assays of *WT1* expression in samples from 104 adult CN-AML patients and found a BM blast count that was statistically higher in the group with higher *WT1* expression at diagnosis.

The significance of either *WT1* mutation or expression level at diagnosis for OS or leukemia-free survival in CN-AML remains uncertain [16,19,30,31]. In contrast, changes in *WT1* expression in the course of treatment may prove to be more informative. In the present study, increases in *WT1*

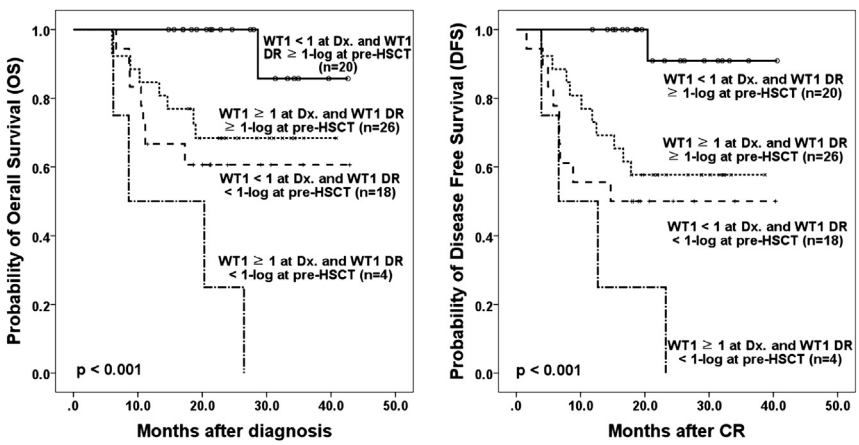

Figure 3. Subgroup analyses associated with relations between diagnostic *WT1* expression and *WT1* DR at pre-HSCT. Four subgroups: Subroup1 (n = 20), diagnostic *WT1* < 1.0 and *WT1* DR ≥ 1-log; Subgroup 2 (n = 18), diagnostic *WT1* < 1.0 and *WT1* DR < 1-log; Subgroup 3 (n = 26), diagnostic *WT1* ≥ 1.0 and *WT1* DR ≥ 1-log; Subgroup 4 (n = 4), diagnostic *WT1* ≥ 1.0 and *WT1* DR < 1-log. Subgroup 1 showed the most favorable outcomes. Subgroup 3 showed more favorable survival curves than Subgroup 2.

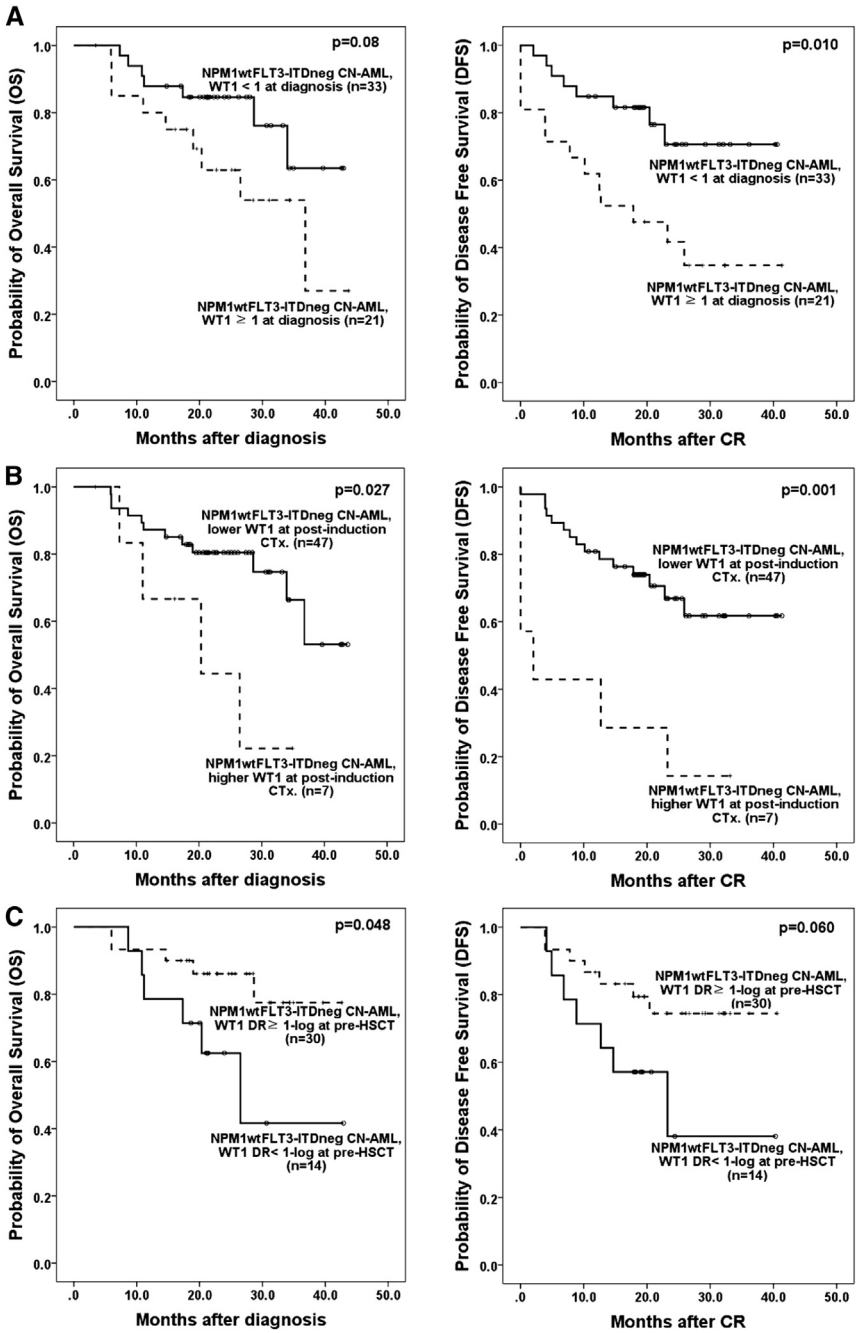

Figure 4. *WT1* expression analysis in the *NPM1*-wild-type/*FLT3-ITD*−negative CN-AML. (A) Higher *WT1* expression at diagnosis showed inferior OS and DFS. (B) Higher *WT1* expression at postinduction chemotherapy also showed inferior OS and DFS. (C) *WT1* DR more than 1-log also showed superior OS and DFS after HSCT in the *NPM1*-wild-type/*FLT3-ITD*−negative CN-AML subgroup.

expression at several time points from diagnosis to the HSCT period were independently associated with inferior OS and DFS. The *WT1* DR at pre-HSCT showed power to predict survival outcomes after HSCT. A *WT1* DR $>$ 1-log (median $=$ 1.5-log) after the first cycle of induction chemotherapy and at pre-HSCT predicted favorable outcomes. Previous studies reported that failure to reduction of at least 2-log units compared with pretreatment level independently predicted more relapse [16,17]. In a study of 91 patients with subtype or karyotype not specified, Cilloni et al. found that a reduction in *WT1* expression of at least 2-log compared with the pretreatment level after the first cycle of chemotherapy corresponded to a decreased risk of subsequent relapse. At baseline, *WT1* expression levels in these patients exceeded 2×10^4 copies per 10^4 copies of *ABL* (corresponding to the value of 2.0 in our study) [17], and this allowed better discrimination of log reductions in expression. However, our study targeted only CN-AML and included patients with *WT1* expression levels both lower ($<$1.0) and higher ($\geq$1.0) than the level at diagnosis. The data showed that a reduction in expression greater than 1-log from pretreatment up to HSCT was significantly and positively associated with favorable OS and DFS after HSCT.

Important for the purpose of predicting survival after HSCT, the analysis by subgroup showed that the *WT1* DR from diagnosis up to HSCT predicted survival outcomes after HSCT

more accurately than did the level of *WT1* expression at diagnosis. Even for patients with lower *WT1* expression at diagnosis, a decrease of less than 1-log by the time of HSCT predicted less favorable outcomes than decreases greater than 1-log. Similarly, among patients with higher *WT1* expression at diagnosis, a reduction of more than 1-log up to the time of HSCT predicted more favorable survival outcomes. This result justifies the quantitative monitoring of changes in *WT1* expression in parallel with chemotherapy to evaluate the feasibility of HSCT for patients with CN-AML. For this purpose, however, the factors that influence treatment-related reduction of *WT1* expression must be investigated.

To simplify the search for a reliable marker of residual disease in adult with CN-AML, we sought a subgroup with the lowest possible prevalence of specific mutations. Thus, for this study we selected adult patients with *NPM1*-wild-type/*FLT3-ITD*–negative CN-AML. Patients with this combination comprise the largest subgroup of adult CN-AML and are classified as having intermediate-1 risk based on the European Leukemia Net guidelines. Patients with *NPM1*-wild-type/*FLT3-ITD*–positive and *NPM1*-mutated/*FLT3-ITD*–positive CN-AML share this classification, whereas the *NPM1*-mutated/*FLT3-ITD*–negative CN-AML is classified as having more favorable risk [25]. For these intermediate-1 risk group patients, the relevant prognostic factors remain to be fully elucidated. However, in those in the *NPM1*-wild-type/*FLT3-ITD*–negative CN-AML category, we found that low *WT1* expression at diagnosis and *WT1* DR more than 1-log at pre-HSCT may have favorable implications for OS and DFS after HSCT.

Our study is limited by the retrospective design, particularly by the progressive loss of some data for the post-HSCT period. The study is noteworthy, however, in that consistently significant results were observed at several stages in the treatment of CN-AML. Overall, the findings support use of *WT1* expression as a marker of residual disease and underscore the importance of *WT1*-based MRD kinetics in monitoring and treatment of CN-AML.

In conclusion, serial monitoring of *WT1* expression of BM in the time from diagnosis to the HSCT period may predict survival outcomes in adult CN-AML. Even at the very low levels detected during follow-up, *WT1* expression and kinetic properties of expression analyzed as the *WT1* DR have important implications. Specifically in *NPM1*-wild-type/*FLT3-ITD*–negative adult CN-AML, *WT1* expression at diagnosis and the *WT1* DR determined before HSCT may predict survival outcomes after HSCT.

ACKNOWLEDGMENTS

Financial disclosure: Supported by a grant from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea (1020370).

REFERENCES

1. Slovak ML, Kopecky KJ, Cassileth PA, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: A Southwest Oncology Group/Eastern Cooperative Oncology Group Study. *Blood*. 2000;96:4075-4083.
2. Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: Results from Cancer and Leukemia Group B (CALGB 8461). *Blood*. 2002;100:4325-4336.
3. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood*. 1998;92:2322-2333.
4. Baldus CD, Thiede C, Soucek S, et al. BAALC expression and FLT3 internal tandem duplication mutations in acute myeloid leukemia patients with normal cytogenetics: Prognostic implications. *J Clin Oncol*. 2006;24:790-797.
5. Frohling S, Schlenk RF, Breitruck J, et al. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: A study of the AML Study Group Ulm. *Blood*. 2002;100:4372-4380.
6. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: Association with FAB subtypes and identification of subgroups with poor prognosis. *Blood*. 2002;99:4326-4335.
7. Schnittger S, Schoch C, Kern W, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood*. 2005;106:3733-3739.
8. Green CL, Koo KK, Hills RK, et al. Prognostic significance of CEBPA mutations in a large cohort of younger adult patients with acute myeloid leukemia: Impact of double CEBPA mutations and the interaction with FLT3 and NPM1 mutations. *J Clin Oncol*. 2010;28:2739-2747.
9. Baldus CD, Tanner SM, Ruppert AS, et al. BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients with normal cytogenetics: A Cancer and Leukemia Group B Study. *Blood*. 2003;102:1613-1618.
10. Haferlach T, Kern W, Schoch C, et al. A new prognostic score for patients with acute myeloid leukemia based on cytogenetics and early blast clearance in trials of the German AML Cooperative Group. *Haematologica*. 2004;89:408-418.
11. Mrozek K, Marcucci G, Paschka P, et al. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: Are we ready for a prognostically prioritized molecular classification? *Blood*. 2007;109:431-448.
12. Haber DA, Buckler AJ, Glaser T, et al. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. *Cell*. 1990;61:1257-1269.
13. Yang L, Han Y, Suarez Saiz F, Minden MD. A tumor suppressor and oncogene: The WT1 story. *Leukemia*. 2007;21:868-876.
14. King-Underwood L, Pritchard-Jones K. Wilms' tumor (WT1) gene mutations occur mainly in acute myeloid leukemia and may confer drug resistance. *Blood*. 1998;91:2961-2968.
15. Paschka P, Marcucci G, Ruppert AS, et al. Wilms' tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: A cancer and leukemia group B study. *J Clin Oncol*. 2008;26:4595-4602.
16. Gray JX, McMillen L, Mollee P, et al. WT1 expression as a marker of minimal residual disease predicts outcome in acute myeloid leukemia when measured post-consolidation. *Leuk Res*. 2012;36:453-458.
17. Cilloni D, Renneville A, Hermitte F, et al. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: A European Leukemia Net study. *J Clin Oncol*. 2009;27:5195-5201.
18. Weisser M, Kern W, Rauhut S, et al. Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia. *Leukemia*. 2005;19:1416-1423.
19. Lapillonne H, Renneville A, Auvrignon A, et al. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. *J Clin Oncol*. 2006;24:1507-1515.
20. Kim YK, Kim HN, Lee SR, et al. Prognostic significance of nucleophosmin mutations and FLT3 internal tandem duplication in adult patients with cytogenetically normal acute myeloid leukemia. *Korean J Hematol*. 2010;45:36-45.
21. Park SH, Chi HS, Min SK, et al. Prognostic significance of the FLT3 ITD mutation in patients with normal-karyotype acute myeloid leukemia in relapse. *Korean J Hematol*. 2011;46:88-95.
22. Schneider F, Hoster E, Unterhalt M, et al. NPM1 but not FLT3-ITD mutations predict early blast cell clearance and CR rate in patients with normal karyotype AML (NK-AML) or high-risk myelodysplastic syndrome (MDS). *Blood*. 2009;113:5250-5253.
23. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med*. 2005;352:254-266.
24. Thiede C, Koch S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood*. 2006;107:4011-4020.
25. Rollig C, Bornhauser M, Thiede C, et al. Long-term prognosis of acute myeloid leukemia according to the new genetic risk classification of the European Leukemia Net recommendations: Evaluation of the proposed reporting system. *J Clin Oncol*. 2011;29:2758-2765.
26. Park HS, Kim DW, Kim CC, et al. Induction chemotherapy with idarubicin plus N4-behenoyl-1-beta-D-arabinofuranosylcytosine in acute

myelogenous leukemia: A newly designed induction regimen—a prospective, cooperative multicenter study. *Semin Hematol.* 1996;33: 24-29.
27. Cilloni D, Gottardi E, Messa F, et al. Significant correlation between the degree of WT1 expression and the International Prognostic Scoring System Score in patients with myelodysplastic syndromes. *J Clin Oncol.* 2003;21:1988-1995.
28. Cilloni D, Gottardi E, De Micheli D, et al. Quantitative assessment of WT1 expression by real time quantitative PCR may be a useful tool for monitoring minimal residual disease in acute leukemia patients. *Leukemia*. 2002;16:2115-2121.
29. Najima Y, Ohashi K, Kawamura M, et al. Molecular monitoring of BAALC expression in patients with CD34-positive acute leukemia. *Int J Hematol.* 2010;91:636-645.
30. Cilloni D, Messa F, Arruga F, et al. Early prediction of treatment outcome in acute myeloid leukemia by measurement of WT1 transcript levels in peripheral blood samples collected after chemotherapy. *Haematologica*. 2008;93:921-924.
31. Gaidzik VI, Schlenk RF, Moschny S, et al. Prognostic impact of WT1 mutations in cytogenetically normal acute myeloid leukemia: a study of the German-Austrian AML Study Group. *Blood*. 2009;113: 4505-4511.