CLINICAL TRIALS AND OBSERVATIONS

Prevalence and prognostic implications of *WT1* mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group

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Recent studies of *WT1* mutations in acute myeloid leukemia (AML) mostly report an association with unfavorable clinical outcome. We screened 842 patients treated on 3 consecutive pediatric AML trials for *WT1* zinc-finger mutations. Eighty-five mutations were detected in 70 of 842 patients (8.3%). Mutations occurred predominantly in exon 7 (n = 74) but were also found in exons 8 (n = 5) and 9 (n = 6). Normal karyotype was observed in 35.3% of *WT1^{mut}* patients, whereas 27.5% WT1^{mut} patients harbored favorable risk cytogenetics. Patients with or without mutations had similar rates of complete remission after one course of induction chemotherapy. Overall survival (OS) for patients with *WT1* mutations was 41% versus 54% for those without mutations (*P* = .016). Corresponding event-free survival (EFS) was also significantly worse for those with *WT1* mutations (28% vs 42%; *P* = .01). However, *FLT3/ITD* was present in 36% of the *WT1^{mut}* cohort; *WT1^{mut}* patients without *FLT3/ITD* had similar OS (56% vs 56%, respectively; *P* = .8) and EFS (35% and 44%, respectively; *P* = .34) to patients who were wild type for both mutations. In current risk stratification schemes incorporating cytogenetics and *FLT3/ITD* status, the presence of *WT1* mutations has no independent prognostic significance in predicting outcome in pediatric AML. The clinical trials are registered at www.clinicaltrials.gov as #NCT00002798 and #NCT00070174. (*Blood*. 2010;116(5):702-710)

Introduction

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease that constitutes 15% to 20% of childhood leukemias. In recent years, recurring mutations in several genes with biologic and prognostic implications have been characterized in AML, particularly within the normal karyotype and/or intermediate-risk cytogenetic subset. Those genes in which mutations may affect disease classification and risk stratification schemes include *FLT3, NPM, NRAS, MLL,* and *CEBPA*.[1,2] Several series in adult AML have added mutations in the Wilms tumor 1 (*WT1*) gene to this list.[3-7]

The *WT1* gene, located on chromosome 11p13, encodes a zinc-finger protein that exists in multiple isoforms and functions as a transcription factor.[8] *WT1* is expressed primarily in tissues of the developing genitourinary and hematopoietic systems, and mutations in *WT1* occur in both syndrome-associated and sporadic cases of nephroblastoma (Wilms tumor), the most common childhood renal malignancy.[9] *WT1* is also expressed in CD34$^+$ hematopoietic progenitors and is overexpressed in a subset of acute leukemias.[10] An AML-associated somatic *WT1* mutation was first reported in the remaining allele of a patient with the WAGR contiguous gene-deletion syndrome in whom a secondary leukemia developed.[11]

The WT1 protein consists of a proline-glutamine–rich N-terminal transcriptional regulatory domain (exons 1-6), as well as 4 C-terminal zinc finger domains (exons 7-10) that facilitate DNA binding.[8] Exons 5 and 9 are subjected to alternative splicing, yielding 4 different splice isoforms. Posttranslational modifications and alternate start codons lead to additional protein products, furthering the complexity. The WT1 protein may act as a transcriptional activator or a transcriptional repressor, depending on the level of expression, the specific isoform, and the cellular context.[12] AML-associated mutations of *WT1* have been reported almost exclusively in the zinc-finger domains, resulting in a protein predicted to be incapable of binding DNA. The most commonly reported of these mutations are frameshift mutations of exon 7,[4-7] leading to a premature stop codon and a truncated protein lacking the C-terminal zinc fingers. In exon 9, missense mutations predominate; these types of mutations have been shown to interrupt DNA binding capacity by affecting amino acid residues either directly involved in DNA binding or essential to the structure of the zinc-finger motif.[13]

Recent studies from the Cancer and Leukemia Group B (CALGB),[4] Medical Research Council (MRC),[5] and AML Study Group (AMLSG)[7] have reported *WT1* zinc-finger mutations in

approximately 10% of adults with cytogenetically normal AML. Among these studies, there are subtle differences in the prognostic significance of harboring such a mutation. The MRC reported that patients with *WT1* mutations had a significantly lower complete remission (CR) rate after induction chemotherapy, whereas the CALGB and AMLSG found no statistically significant difference between the CR rates of those with or without mutations. Both the CALGB and MRC studies found the presence of a *WT1* mutation to be an independent predictor of inferior overall survival (OS) and disease-free survival (DFS); in contrast, the AMLSG study found that *WT1* mutations lacked independent prognostic significance. Notably, only the AMLSG study performed explorative subset analysis, examining outcome measures separately in subsets defined by combined *WT1* and *FLT3/ITD* status. Hollink et al[14] reported *WT1* mutations in 35 of 298 (12%) pediatric patients with de novo AML, the largest pediatric series to date. These patients were treated on AML-BFM-SG/DCOG or LAME protocols, and *WT1* mutations independently predicted poorer survival and greater risk of relapse.

In our study, we examined pediatric patients enrolled on 3 consecutive Children's Cancer Group (CCG) and Children's Oncology Group (COG) trials, providing the largest cohort of patients with AML screened for *WT1* mutations to date. Herein, we present a comprehensive evaluation of the prevalence and prognostic significance of *WT1* mutations, in the context of other validated biologic, cytogenetic, and molecular risk factors, in a large cohort of pediatric patients with de novo AML.

Methods

Patient samples

Pediatric patients with newly diagnosed de novo AML enrolled in 3 consecutive pediatric AML protocols, CCG-2941, CCG-2961, or COG-AAML03P1, were eligible for this study. Details of these studies have been previously described.[15,16] Of the 1328 patients treated on the 3 consecutive studies, 842 diagnostic specimens were available and were obtained from the COG AML Reference Laboratory. Approval by the institutional review board was obtained before mutation analysis, and this study was approved by the COG Myeloid Disease Biology Committee. Informed consent for study protocol treatment and tissue sample evaluation was obtained in accordance with the Declaration of Helsinki.

Mutation screening

Genomic DNA was extracted from the diagnostic marrow specimens with the Puregene protocol (Gentra Systems Inc). Polymerase chain reaction (PCR) amplification of the zinc-finger domains of *WT1* (exons 7-10) was performed with the use of 4 primer pairs (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). For exons 7 through 9, the PCR reaction was carried out in a mixture containing Failsafe PCR Premix (Epicentre Biotechonologies) Buffer A (exons 7 and 9) or Buffer F (exon 8), 1.25 U of Platinum Taq DNA Polymerase (Invitrogen), 5 pmol of each primer, and 10 ng of genomic DNA. For fragment-length analysis screening, forward primers were labeled with 6-FAM dye. Thermocycler conditions were as follows: 95°C for 5 minutes; 40 cycles at 95°C for 45 seconds, 59°C for 30 seconds, and 72° for 45 seconds; and a final extension step at 72°C for 7 minutes. Fluorescently labeled fragments were separated on an ABI 377*xl* automated DNA sequencer, and analysis was performed with the use of Genemapper software (Applied Biosystems). Mutations were confirmed by direct sequencing; for samples containing more than one *WT1* mutation, cloning was performed with the use of the TOPO-TA Cloning Kit (Invitrogen), and direct sequencing was performed on plasmid DNA obtained from multiple isolated clones.

For exon 10, the PCR reaction was carried out in 10× PCR Buffer (Invitrogen) containing 1.5 μL of 50mM MgCl2, 0.5 μL of 10mM deoxynucleoside triphosphates, 1.25 U of Platinum Taq DNA Polymerase, 5 pmol of each primer, and 10 ng of genomic DNA. The following conditions were used for PCR amplification: 94°C for 5 minutes; 35 cycles at 94°C for 30 seconds, 65°C for 30 seconds, and 72° for 30 seconds; and a final extension step at 72°C for 7 minutes. Direct sequencing was carried out with the use of the Big Dye Terminator v3.1 Cycle Sequencing Reaction (Applied Biosystems) and run on an ABI 3730xL DNA analyzer.

Screening for *FLT3/ITD, NPM,* and *CEBPA* mutations was performed as previously described.[17-19]

Statistical methods

The Kaplan-Meier method was used to estimate OS, event-free survival (EFS), and DFS. OS was defined as the time from study entry to death. EFS was defined as the time from study entry until failure to achieve remission by the end of 2 courses of therapy, relapse, or death. DFS was defined as the time from end of course 1 for patients in CR (bone marrow aspirate containing < 5% blasts by morphology and no evidence of extramedullary disease) until relapse or death. Estimates of relapse risk (RR) were obtained by the method of cumulative incidence that accounts for competing events. RR was defined as the time from end of course 1 for patients in CR to relapse or death due to progressive disease whereby deaths from nonprogressive disease were considered competing events. The significance of predictor variables was tested with the log-rank statistic for OS, EFS, and DFS and with Gray statistic for RR. Children who also received a stem cell transplant while on study were censored at the time of transplantation for all analyses, unless otherwise indicated. Children lost to follow-up were censored at their date of last known contact or 6 months before the cutoff date of analyses being April 14, 2005, for patients on CCG-2941, November 6, 2009, for patients on CCG-2961, and November 16, 2009, for patients on COG-AAML03P1. The significance of observed differences in proportions was tested using the χ^2 test and Fisher exact test when data were sparse. The Mann-Whitney test was used to determine the significance between differences in medians. Cox proportional hazard models were used to estimate hazard ratios (HRs) for univariate and multivariate analyses for OS, DFS, and relapse-free survival (RFS). RFS was defined as the time from end of course 1 for patients in CR to relapse or death due to progressive disease, whereby deaths from nonprogressive disease were censored.

Results

Patient population

From September 1995 to November 2005, 1328 pediatric patients with AML were treated on CCG-2941, CCG-2961, and COG-AAML03P1, 842 (63%) of whom had diagnostic bone marrow specimens available for analysis. Demographics, laboratory and clinical characteristics, and outcome for those with and without available specimens were compared. Patients without available diagnostic specimens (N = 486) had similar clinical outcomes with 5-year OS of 55% (± 5%) compared with 53% (± 4%) for those who were analyzed (P = .261). Induction CR rates and EFS from study entry were also similar. The study population differed from those not tested mainly in regard to age and diagnostic white blood cell (WBC) count, whereby those not tested were younger (median age, 6.9 vs 10.4 years; P < .001) and had lower median diagnostic WBC counts (15.1 vs 21.8×10^9/L [15 100 vs 21 800/μL]; P = .002). In addition, the population that was not tested had a higher proportion of patients with megakaryocytic leukemia (10.7% vs 4.4%; P < .001).
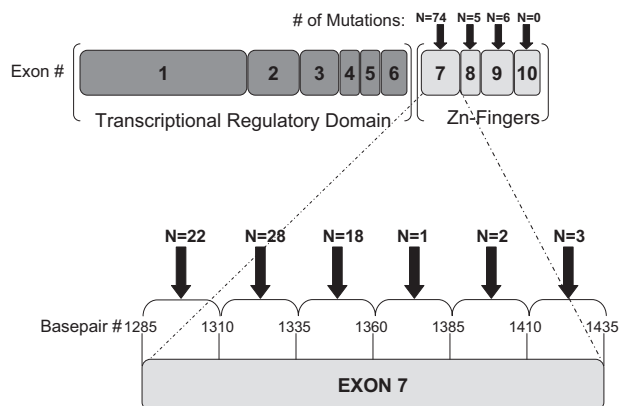
Figure 1. Location of *WT1* zinc-finger mutations. Graphical depiction of the 85 mutations detected in 70 of 842 pediatric patients with AML screened. Mutations clustered in the N-terminal portion of exon 7 but were also detected in exons 8 and 9.

WT1 mutation analysis

Initial mutation screening of the exons encoding all 4 zinc-fingers (exons 7-10) was performed by direct sequencing on 100 samples from CCG-2961. Frameshift mutations were detected in exon 7 (n = 6) and exon 8 (n = 1). Although a known single nucleotide polymorphism (SNP; c.1293A>G) was detected in exon 7 in 21 patients, no other exon 7 point mutations were detected in this initial 100-patient screen. Point mutations (n = 2) were detected in exon 9, and no mutations were detected in exon 10. Thus, in the remaining 742 available diagnostic specimens from patients enrolled on CCG-2941, CCG-2961, or COG-AAML03P1, fragment-length analysis was used to screen exons 7 and 8, whereas direct sequencing was performed on exon 9, and exon 10 was not screened further. Mutations detected by fragment-length analysis were subjected to confirmation by direct sequencing.

Overall, 85 *WT1* zinc-finger mutations were detected in 70 of the 842 patients (8.3%) screened (supplemental Table 2). Mutations clustered overwhelmingly in exon 7 (74 mutations in 59 patients; Figure 1), but they were also detected in exon 8 (n = 5) and exon 9 (n = 6). Nearly all exon 7 mutations were frameshift mutations leading to a stop codon, including insertions (n = 62) ranging between 1 and 31 base pairs and deletions (n = 9) ranging between 1 and 38 base pairs. Also detected were 3 single base pair substitution, missense mutations. Most exon 7 mutations occurred within 2 "mutational hotspot" regions: between base pairs 1295 and 1309 (n = 22) or between base pairs 1323 and 1340 (n = 33). For patients in whom 2 mutated peaks were detected on fragment-length analysis, or in whom 2 mutations were detected on confirmatory sequencing, TOPO-TA cloning (Invitrogen) was performed. Plasmid DNA from multiple isolated clones was then directly sequenced. In this manner 15 of the 70 patients (21.4%) with mutations were shown to harbor biallelic *WT1* mutations in exon 7: 4 patients had homozygous frameshift mutations, 8 patients had 2 different frameshift mutations, and 3 patients had both a frameshift and a missense mutation.

Eleven mutations were detected outside of exon 7, including 5 frameshift mutations in exon 8 and 6 missense mutations in exon 9. None of these 11 patients carried concomitant exon 7 mutations. Exon 8 frameshift mutations included insertions (n = 4) ranging from 2 to 23 base pairs, and one deletion of 22 base pairs, all of which were predicted to lead to a premature stop codon. Exon 9 mutations included substitutions at 3 amino acid residues known to be affected by constitutional *WT1* mutations in Denys-Drash syndrome (DDS), a congenital anomaly syndrome consisting of the

triad of ambiguous genitalia, congenital nephrotic syndrome, and predisposition to Wilms tumor. These amino acid changes included H397A (n = 1), D396N (n = 2), R394P (n = 2), and R394W (n = 1). Such DDS-associated exon 9 point mutations have been shown to decrease the DNA-binding affinity of the WT1 protein.[20]

Characteristics of the study population

Demographic, laboratory, and clinical characteristics of patients with or without *WT1* mutations were compared (Table 1). There were no significant differences in sex, race, median diagnostic blast percentage, or median diagnostic WBC count between patients with and patients without *WT1* mutations. However, *WT1* mutations were less common in the youngest patients with AML aged from birth to 2 years; this age group accounted for 7.1% of patients with mutations, as opposed to 25% of those without (P = .001). Such mutations were also less common in patients with French-American-British class M5 (these accounted for 6% of patients with *WT1* mutations and 18.7% of patients without; P = .014).

We also evaluated associations between *WT1* mutations and cytogenetic and molecular alterations (Table 1). In terms of cytogenetics, *WT1* mutations were found most frequently in the normal karyotype subset (35.3% of *WT1*[mut] patients had normal karyotype compared with 20.0% of those without *WT1* mutations; P = .017). A substantial number (27.5%) of *WT1*[mut] patients with known cytogenetics were also found in the "favorable-risk," core-binding factor (CBF-AML) cytogenetic subgroup. This was primarily because of a higher incidence of inv(16), which was present in 17.6% of *WT1*[mut] patients, but only to 11.5% of patients without *WT1* mutations (P = .283); in addition, 9.8% of patients with *WT1* mutations had t(8;21). An overlap between CBF translocations and *WT1* mutations has not been previously reported. To verify our results, we confirmed the presence of the *WT1* mutation, by fragment-length analysis, and the translocation, by reverse transcription–PCR, in diagnostic RNA from all patients with both the mutation and the cytogenetic abnormality; RNA testing confirmed the coexistence of both abnormalities in each patient. Two patients (3.9%) with *WT1* mutations were classified as cytogenetically high risk because of the presence of monosomy 7.

Regarding other molecular alterations, there was also a substantial overlap between *WT1* mutations and *FLT3/ITD*, ie, 35.7% of those carrying a *WT1* mutation were *FLT3/ITD* positive as opposed to 9.8% of patients without *WT1* mutations (P < .001). In addition, 11q23 alterations were rare in patients with *WT1* mutations (7.8% vs 23.1%; P = .019) compared with patients with wild-type *WT1*. *CEBPA* mutations were found in 4.5% of those with *WT1* mutations; *NPM* mutations occurred in 4.3%, and *FLT3* point mutations occurred in 7.1%. These percentages did not differ significantly from the prevalence of these mutations in patients without *WT1* mutations.

Clinical outcome and prognostic effect of *WT1* mutations

The CR rate was determined for all patients after the first course of induction therapy. Patients with *WT1* mutations had a lower rate of CR (72.1%) compared with those without mutations (80.6%), but this difference was not statistically significant (P = .127). Clinical outcome data were examined for the 842 patients with known *WT1* mutation status (Figure 2). Actuarial OS from study entry for patients with *WT1* mutations was 41% plus or minus 13% versus 54% plus or minus 4% for those without *WT1* mutations; (HR = 1.52; P = .017). Corresponding EFS was also significantly worse for those with *WT1* mutations (28% ± 12% vs 42% ± 4%;

Table 1. Characteristics of patients with or without *WT1* mutations

	WT1 mutant	*WT1* wild-type	*P*
Study			
CCG-2941	1 (1.4)	38 (4.9)	.243
CCG-2961	46 (65.7)	495 (64.1)	.891
AAML03P1	23 (32.9)	239 (31.0)	.846
Sex			
Male	37 (52.9)	411 (53.2)	.949
Female	33 (47.1)	361 (46.8)	
Age, y			
Median (range)	11.5 (0.85-18.3)	10.1 (0.01-21.63)	.031*
0-2	5 (7.1)	193 (25.0)	.001*
3-10	25 (35.7)	225 (29.1)	.310
11-21	40 (57.1)	354 (5.9)	.092
Race			
White	47 (67.1)	492 (65.1)	.829
Black	12 (17.1)	85 (11.2)	.203
Hispanic	8 (1.4)	125 (16.5)	.346
Asian	2 (2.9)	26 (3.4)	.999
Other	1 (1.4)	28 (3.7)	.503
Unknown	0	16	
WBC count, $\times 10^3/\mu$L, median (range)	35 (1.2-3260)	20.5 (0.3-860)	.145
BM blasts, %	75.5 (5-100)	70 (0-100)	.122
Platelet count, $\times 10^3/\mu$L, median (range)	54 (4-800)	47.5 (2-46 000)	.252
Hemoglobin level, g/dL, median (range)	8.2 (3.1-13.7)	8.3 (0.4-38.6)	.935
FAB classification			
M0	3 (4.5)	32 (4.4)	.999
M1	11 (16.4)	119 (16.3)	.875
M2	22 (32.8)	202 (27.7)	.457
M4	23 (34.3)	191 (26.2)	.199
M5	4 (6.0)	136 (18.7)	.014*
M6	2 (3.0)	15 (2.1)	.648
M7	2 (3.0)	33 (4.5)	.760
Other/no data, n	3	44	
Cytogenetics			
Normal	18 (35.3)	108 (20.0)	.017*
t(8;21)	5 (9.8)	87 (16.1)	.327
inv(16)	9 (17.6)	62 (11.5)	.283
Abnormal 11	4 (7.8)	125 (23.1)	.019*
t(6;9)(p23;q34)	3 (5.9)	9 (1.7)	.076
−7/7q−	2 (3.9)	18 (3.3)	.687
−5/5q−	0 (0.0)	7 (1.3)	.999
+8	3 (5.9)	41 (7.6)	.999
+21	0 (0.0)	4 (0.7)	.999
Pseudodiploid	4 (7.8)	31 (5.7)	.531
Hyperdiploid	2 (3.9)	8 (1.5)	.210
Hypodiploid	0 (0.0)	5 (0.9)	.999
Other	1 (2.0)	36 (6.7)	.356
Unknown, n	19	231	
***FLT3/ITD* status**			
ITD+	25 (35.7)	76 (9.8)	< .001*
ITD−	45 (64.3)	696 (90.2)	
Missing, n	0	0	
***FLT3* PM status**			
FLT3 PM+	5 (7.1)	47 (6.1)	.611
FLT3 PM−	65 (92.9)	725 (93.9)	
Missing, n	0	0	
***CEBPA* status**			
CEBPA mutant	3 (4.5)	28 (3.9)	.743
CEBPA WT	64 (95.5)	688 (96.1)	
Missing, n	3	56	
***NPM* status**			
NPM mutant	3 (4.3)	43 (6.4)	.792
NPM WT	67 (95.7)	632 (93.6)	
Missing, n	0	97	

Table 1. Characteristics of patients with or without *WT1* mutations (continued)

	WT1 mutant	*WT1* wild-type	*P*
Course 1 response			
CR	49 (72.1)	603 (80.6)	.127
Not in CR	19 (27.9)	145 (19.4)	
Unevaluable, n	27	24	
Course 2 response			
CR	44 (69.8)	547 (77.6)	.355
Not in CR	19 (30.2)	158 (22.4)	
Unevaluable, n	7	67	

Data are reported as n (%) except where otherwise noted. BM indicates bone marrow; FAB, French-American-British; PM, point mutation; and WT, wild-type.
*Statistically significant (P = .05).

HR = 1.50; *P* = .011). Of the 652 patients who achieved an initial CR, RR at 5 years from remission was higher for patients with *WT1* mutations (51% ± 16%) than for patients without *WT1* mutations (40% ± 4%; *P* = .136) but not significantly different. Corresponding DFS from CR in patients with and without *WT1* mutations was 38% plus or minus 15% and 50% plus or minus 5%, respectively (HR = 1.39; *P* = .119).

Differences in outcome measures between patients with and without *WT1* mutations were similar when analyses were restricted to patients having normal cytogenetics: (OS: 20% ± 22% vs 44% ± 11%, *P* = .024; EFS: 32% ± 24% vs 57% ± 11%, *P* = .012; RR: 40% ± 31% vs 44% ± 12%, *P* = .905; DFS: 31% ± 30% vs 46% ± 12%, *P* = .181). However, when outcome analyses were restricted to standard-risk patients (excluding those patients risk-stratified by virtue of favorable cytogenetics, unfavorable cytogenetics, *CEBPA* mutations, or *FLT3/ITD*), no significant differences were found between patients with and without *WT1* mutations: (OS: 53% ± 24% vs 47% ± 6%, *P* = .935; EFS: 30% ± 21% vs 37% ± 6%, *P* = .334; RR: 58% ± 29% vs 45% ± 7%, *P* = .313; DFS: 33% ± 27% vs 46% ± 7%, *P* = .349).

Of the 842 patients in this study, 128 total patients (15%) underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) from a matched sibling donor after the third course of chemotherapy (intensification 1) for patients on AAML03P1 and after the second course of induction for patients on CCG-2941 and CCG-2961. For patients in CR by the end of induction 2 or intensification 1, we directly compared the outcome of *WT1* mutation–positive patients who received an allo-HSC transplant (n = 9) with that of patients who were treated with chemotherapy only (n = 33). In patients with *WT1* mutations, RR (cumulative incidence) at 5 years from the end of course 2 was 22% plus or minus 28% for the HSC transplant recipients versus 53% plus or minus 18% for patients who were treated with chemotherapy only (*P* = .159). Corresponding OS at 5 years from the end of course 2 was 78% plus or minus 28% versus 64% plus or minus 17%

(*P* = .321) for HSC transplant recipients and chemotherapy-only recipients, respectively. Patients with *WT1* mutations did appear to benefit from allo-HSCT, but differences were not statistically significant. Of note, however, of the 9 patients with *WT1* mutations who underwent HSCT, only 1 patient was "high-risk" by virtue of *FLT3/ITD* with high allelic ratio. Within the subset of 128 patients who received a HSC transplant, we also compared the outcome of patients with *WT1* mutations (n = 9) with patients without *WT1* mutations (n = 119) and found that *WT1* status did not have any significant effect on outcome after HSCT in our study (RR from transplantation, 22% ± 28% for *WT1*-mutated patients vs 14% ± 7% for *WT1* wild-type patients [*P* = .546]; corresponding 5-year OS, 78% ± 28% for patients with *WT1* mutations vs 72% ± 9% for patients without [*P* = .642]).

Given the substantial overlap between *WT1* and *FLT3/ITD* mutations, we examined outcome measures separately for *WT1^mut^/ITD^+^* patients compared with *WT1^mu+^/ITD^−^* patients (Table 2). Only 12 of 23 patients (48%) with *WT1^mut^/ITD^+^* achieved CR after induction 1, as opposed to 37 of 45 patients (82.2%) with *WT1^mut^/ITD^−^* (*P* = .020). Patients with both *WT1* and *FLT3/ITD* mutations had an extremely dismal prognosis (OS, 15% ± 15%; EFS, 15% ± 16%), yet patients with *WT1* mutations who were *ITD* negative had similar outcomes to patients who were *WT1* wild-type and *ITD* negative (Table 2; Figure 3). OS from study entry was 56% plus or minus 16% for the *WT1^mut^/ITD^−^* patients compared with 56% plus or minus 4% for patients with neither mutation (*P* = .80). Corresponding EFS was 35% plus or minus 16% compared with 44% plus or minus 4%, respectively (*P* = .336), and 5-year RR from CR was similar between the 2 groups (44% ± 18% vs 38% ± 5%; *P* = .456). Thus, *WT1^mut^/ITD^+^* patients had shorter survival due to both a higher rate of induction failure and a higher rate of disease recurrence, whereas *WT1^mut^/ITD^−^* patients had a similar outcome to patients who were wild type for both mutations. The prognostic significance of having a *WT1* mutation was also determined in patients with a normal karyotype
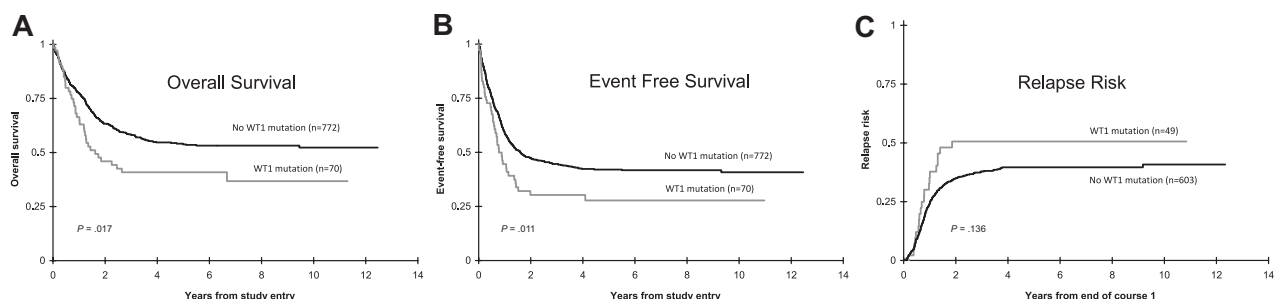

Figure 2. Clinical significance of *WT1* mutations in pediatric AML. Kaplan-Meier estimates show that patients with *WT1* mutations have shorter (A) OS and (B) EFS from study entry than patients without these mutations. (C) Cumulative incidence of relapse was also worse for patients with *WT1* mutations.

Table 2. Clinical outcomes of patients stratified by *WT1* and *FLT3/ITD* status

	WT1 wild-type	*WT1* mutant	*P*
CR rate at end of course 1			
FLT3/ITD neg, %	81.5	82.2	.932
FLT3/ITD pos, %	72.4	52.2	.119
P	.078	.020	
OS			
FLT3/ITD neg, %, mean ± SD (n)	56 ± 4 (696)	56 ± 16 (45)	.8
FLT3/ITD pos, %, mean ± SD (n)	35 ± 13 (76)	15 ± 15 (25)	.004
P	.026	.001	
EFS			
FLT3/ITD neg, %, mean ± SD (n)	44 ± 4 (696)	35 ± 16 (45)	.336
FLT3/ITD pos, %, mean ± SD (n)	24 ± 12 (76)	15 ± 16 (25)	.036
P	.017	.019	
RR			
FLT3/ITD neg, %, mean ± SD (n)	38 ± 5 (548)	44 ± 18 (37)	.456
FLT3/ITD pos, %, mean ± SD (n)	61 ± 15 (55)	70 ± 30 (12)	.403
P	.005	.115	
***FLT3/ITD,* allelic ratio**			.131
Low, n (%)	25 (33)	4 (16)	
High, n (%)	51 (67)	21 (84)	

who were *FLT3/ITD* negative. No significant differences were found in the OS (51% ± 32% and 58% ± 12%, respectively; *P* = .31) or in the corresponding EFS values (31% ± 29% and 44% ± 12%, respectively; *P* = .408).

Conversely, the presence of *FLT3/ITD* remained a significant marker of unfavorable prognosis, even in the patients without *WT1* mutations (Table 2; Figure 3). *WT1⁻/ITD⁺* patients had significantly lower OS (35% ± 13% vs 56% ± 4%; *P* = .026) and EFS (24% ± 12% vs 44% ± 4%; *P* = .017) than did patients who were wild type for both mutations. Corresponding RR was 61% plus or minus 15% for *WT1⁻/ITD⁺* patients. As expected, patients with *FLT3/ITD* with a high allelic ratio (AR > 0.4)[21] had an even worse outcome. This remained true in the cohort of patients negative for *WT1* mutations, whereby patients with *FLT3/ITD* with high AR had an OS of 29% plus or minus 16%, EFS of 17% plus or minus 12%, and an RR of 77% plus or minus 15%.

Subset analysis was then performed for patients with CBF-AML. Patients with both *WT1ᵐᵘᵗ* and CBF-AML had similar OS and EFS compared with patients with CBF-AML and wild-type *WT1* (OS: 77% ± 23% vs 74% ± 8%, *P* = .972; EFS: 60% ± 28% vs 58% ± 9%, *P* = .885). DFS (65% ± 29% vs 60% ± 9%) and RR (28% ± 27% vs 27% ± 9%) were also not significantly different, suggesting that the favorable prognostic effect of the CBF translocation "trumps" the prognostic effect of the *WT1* mutation.

Outcomes for those with "double," biallelic *WT1* mutations were compared with outcomes for patients with single *WT1* mutations. Although numbers were too small to detect significant differences, patients with biallelic mutations appeared to have worse outcome than patients with single mutations (OS: 33% ± 24% vs 43% ± 15%, HR = 1.22, *P* = .587; EFS: 20% ± 21% vs 31% ± 14%, HR = 1.48, *P* = .249).

Prognostic factors

We performed Cox regression analyses to evaluate the status of the following mutations as predictors of OS and RFS in separate univariate models: *WT1, NPM, CEBPA,* and *FLT3/ITD*. In univariate analysis, the presence of *FLT3/ITD* was the strongest predictor of decreased survival (HR = 1.77; *P* < .001) and increased risk of relapse (HR = 1.87; *P* < .001). *WT1* mutations also predicted poor outcome in the univariate model (lower OS: HR = 1.52, *P* = .017; lower RFS: HR = 1.49, *P* = .092). *CEBPA* mutations predicted improved OS (HR = 0.43; *P* = .027) and RFS (HR = 0.42; *P* = .052), whereas *NPM* mutations were not prognostic in these analyses.

Cox regression analyses were then performed to evaluate *WT1* mutation status as a predictor of EFS and RR alongside the following known prognostic groups: high diagnostic WBC count, high-risk cytogenetic/molecular features (defined as −5/del(5q), −7, or presence of *FLT3*/ITD with AR > 0.4), as well as favorable-risk cytogenetic/molecular features (CBF-AML or presence of *CEBPA* mutation). These factors were analyzed as predictors of EFS and RR in univariate and multivariate models (Table 3). In the univariate model, the presence of a *WT1* mutation was a significant
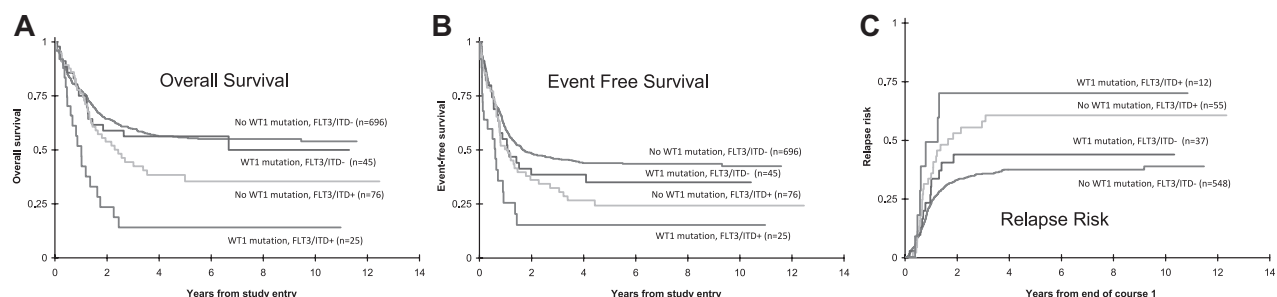

Figure 3. Outcomes stratified by combined *WT1* and *FLT3/ITD* mutation status. When stratifying patients by combined *FLT3/ITD* and *WT1* status, patients with both mutations had the worst (A) OS, (B) EFS, and (C) RR. Of the 2 mutations, *FLT3/ITD* imparts a stronger negative effective on survival outcome.

Table 3. Cox regression analysis of *WT1* mutations and other specific prognostic factors

	EFS from study entry				RFS from end of course 1			
	N	HR	95% CI	*P*	N	HR	95% CI	*P*
Univariate Cox analyses								
WT1 mutation present (vs absent)	70	1.50	1.10-2.05	.011	49	1.49	0.94-2.36	.092
High-risk cytogenetic/molecular (vs standard)	123	1.79	1.4-2.28	< .001	79	1.94	1.39-2.72	< .001
Favorable-risk cytogenetic/molecular (vs standard)	249	0.51	0.40-0.66	< .001	227	0.47	0.34-0.65	< .001
WBC count greater than 50×10^9/L (vs less than 50×10^9/L	388	1.32	1.13-1.56	< .001	283	1.26	1.00-1.58	.047
Multivariate Cox analyses								
WT1 mutation present (vs absent)	59	1.33	0.94-1.88	.105	41	1.33	0.81-2.20	.261
High-risk cytogenetic/molecular (vs standard)	98	1.52	1.14-2.04	.004	63	1.93	1.29-2.89	.002
Favorable-risk cytogenetic/molecular (vs standard)	189	0.50	0.38-0.66	< .001	171	0.48	0.32-0.70	< .001
WBC count greater than 50×10^9/L (vs less than 50×10^9/L)	212	1.35	1.08-1.69	.010	151	1.26	0.91-1.74	.158

prognostic factor for decreased EFS with an HR of 1.50 ($P = .011$). In separate univariate models, high-risk cytogenetic/molecular features (HR = 1.79; $P < .001$), and diagnostic WBC count greater than 50×10^9/L (HR = 1.32; $P < .001$) were associated with worse EFS. Patients with favorable-risk cytogenetic/molecular features had superior OS (HR = 0.51; $P < .001$) and RFS (HR = 0.47; $P < .001$). In a multivariate model that included the above-mentioned prognostic factors, the presence of *WT1* mutations loses independent prognostic significance for EFS ($P = .105$) and RFS ($P = .261$), whereas WBC count greater than 50×10^9/L, the presence of high-risk cytogenetic or molecular features, or the presence of favorable-risk cytogenetic or molecular features all remained as independent prognostic factors for EFS. Only WBC count greater than 50×10^9/L did not remain significant in a multivariate model for RFS.

Discussion

In this retrospective study, we report that mutations in the zinc-finger domains of the *WT1* gene were detected in 70 of 842 patients with pediatric AML, corresponding to 8.3% of our study population, and that these mutations were significantly associated with unfavorable outcome in univariate, but not multivariate, analysis. This is the largest study of *WT1* mutations in AML to date. We demonstrate that *WT1* mutations are associated with shorter OS and EFS as well as higher risk of relapse. However, we found a substantial overlap between *WT1* mutations and other established prognostic markers already in clinical use, notably *FLT3/ITD* and CBF translocations. The presence of *WT1* mutations as well as CBF translocations was confirmed at the RNA level in these patients, as this is an association that has not been previously reported.

We found that *WT1* mutation status lacks independent prognostic significance in multivariate analysis, including other established prognostic markers. This is in contrast to adult studies reported by the CALGB[4] and the MRC.[5] We also recently reported that *KIT* mutations lack independent prognostic significance in pediatric CBF-AML,[22] as opposed to their widely reported association with unfavorable outcome in adult CBF-AML. These differences in the prognostic value of molecular markers in pediatric versus adult AML may reflect underlying differences in disease biology or differences in the interaction of this biology with pediatric versus adult AML treatment schemas. Routine mutation screening of the *WT1* gene at diagnosis, for the purpose of risk stratification in

future pediatric trials, is not warranted on the basis of the results of our study.

The overlap between *WT1* mutations and *FLT3/ITD* has both biologic as well as prognostic implications. Subset analysis identifies the *WT1mut/FLT3/ITD+* group as particularly high risk, suggesting the chemoresistance and resultant effect on outcome conferred by these mutations is potentiated when they occur together. These patients have dismal survival outcomes due to both failure of induction chemotherapy as well as higher rates of recurrence, features known to be associated with *FLT3/ITD+* disease. These patients are already stratified into the maximally intensive therapy arm on the basis of *FLT3* status alone on current COG protocols. Notably, when excluding the *FLT3/ITD+* subgroup in our study, patients with *WT1* mutations in the absence of *FLT3/ITD* had similar outcomes to patients who had neither mutation. Although the prior pediatric AML study reported independent prognostic significance for *WT1* mutations, Hollink et al[14] also failed to detect a statistically significant difference in OS between *WT1mut* and *WT1* wild-type patients when restricting analysis to *FLT3/ITD−* patients. In contrast with the prior pediatric study, our study finds that *FLT3/ITD* remains a significant predictor of poor outcome even in patients without *WT1* mutations. The relative sizes of the studies may account for this difference, because our study may have been large enough to detect a difference that was not statistically significant in the Dutch study. In addition, had allelic ratio been used to stratify patients into a high-risk *FLT3/ITD* group, Hollink et al[14] may have been able to demonstrate a more pronounced difference in outcome that was based on *ITD* status.

When considering only the exons comprising the zinc-finger domains, *WT1* mutations were found in approximately 10% of patients in 3 preceding adult AML studies.[4,5,7] Note that these 3 studies restricted their cohorts to normal-karyotype AML. The mutation incidence of 8.7% in our study is higher than the 5% reported in unselected adult patients with AML by the Acute Leukemia French Association[6] but less than the 12% incidence reported in the prior pediatric study.[14] This discrepancy may be explained in part by the fact that Hollink et al[14] reported rare missense mutations in exon 7; although we discovered 3 such mutations on confirmatory sequencing of patients with length mutations in the opposite allele, our method of screening by fragment-length analysis in exon 7 would not detect patients who harbored only missense mutations without concomitant insertions or deletions.

Most of the *WT1* mutations in our study were found in exon 7, as has been previously described. We also detected 5 novel

frameshift mutations in exon 8. The frameshift mutations in exons 7 and 8 are predicted to result in a truncated protein lacking a significant portion of the zinc-finger domains. In exon 9, nearly all of the previously described mutations are single base pair substitutions; we detected 4 such missense mutations. Five of the exon 9 mutations in our study replaced either the arginine at residue 394 or the aspartic acid at residue 396 with a different amino acid. Because R394 and D396 are the residues most commonly affected by germline *WT1* mutations in DDS, functional studies have been performed which show mutations at these codons abrogate the WT1 protein's DNA-binding capacity.[23] The remaining exon 9 mutation in our cohort was H397A, an amino acid residue also implicated in some cases of DDS. Replacing histidine with a different amino acid would disrupt the precise cysteine-histidine spacing that is crucial to the structure of the zinc-finger. The zinc-finger domains in *WT1* are responsible for DNA binding, nuclear localization, and protein interaction. Mutations leading to loss, or alteration, of the zinc-fingers may lead to the loss of these integral functions.

All of the mutations we detected in the zinc-finger domains of *WT1* are predicted to either abolish or diminish the function of the *WT1* protein. Inactivation of both alleles would be predicted to completely eliminate normal *WT1* function. We detected mutations affecting both alleles in 15 of the 70 patients with *WT1* mutations; in each case of "double" mutation, both mutations were found in exon 7. The true incidence of biallelic double mutations may be higher than reported in our study, because mutations in C-terminal exons, which we did not screen, have been reported rarely in conjunction with exon 7 mutations.[14] Four of the biallelic mutations we detected appear to be homozygous by direct sequencing; previous work has shown that segmental uniparental disomy may be a mechanism of acquiring loss of heterozygosity of a mutated *WT1* gene in AML.[24] We did not detect any double mutations involving exon 9. There are data to suggest that exon 9 missense mutations may behave in a dominant-negative manner,[23] in which case biallelic inactivation would not be required for complete loss of function.

The exact effect of the loss of function of *WT1* on either the development or progression of leukemia is unknown. The traditional model of molecular-genetic cooperativity in myeloid leukemogenesis posits that "class II" events, which impair differentiation, must be coupled with "class I" events, which confer a proliferative advantage.[25] In our study, *WT1* mutations show significant overlap with CBF translocations, which are classic class II events, as well as with the class I mutation *FLT3/ITD,* so the role of *WT1* mutations in the stepwise evolution of leukemia is uncertain. In addition, although *WT1* mutations in AML appear to result in loss of *WT1* function, marked overexpression of wild-type *WT1* is a common finding in AML.[26-28] Further, in one study, patients with pediatric AML with *WT1* exon 7 mutations had significantly higher levels of *WT1* expression than patients with wild-type *WT1*.[28] This apparent contradiction, in which a single gene might function as both an oncogene as well as a tumor suppressor, may stem from the ability of the WT1 protein to function either as a transcriptional activator or repressor, depending on a multitude of factors.[12] There is much still to be learned about the biology of *WT1* in AML; further insight into the roles that this fascinating gene plays in leukemogenesis may eventually pave the way for targeted therapies.

Authorship

Contribution: P.A.H. and R.Z. designed and performed research, analyzed data, and wrote the manuscript; T.A.A. served as senior statistician, performed statistical analyses, and edited the manuscript; R.B.G. performed statistical analyses and edited the manuscript; K.L.M. performed research and edited the manuscript; J.A.P., D.L.S., N.A.H., S.C.R., B.H., J.L.F., and B.L. analyzed data and edited the manuscript; and S.M. designed the research, analyzed data, and wrote the manuscript.

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A complete list of the members of the Children's Oncology Group appears in the supplemental Appendix.

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References

1. Mrozek K, Dohner H, Bloomfield CD. Influence of new molecular prognostic markers in patients with karyotypically normal acute myeloid leukemia: recent advances. *Curr Opin Hematol.* 2007; 14(2):106-114.

2. Gaidzik V, Dohner K. Prognostic implications of gene mutations in acute myeloid leukemia with normal cytogenetics. *Semin Oncol.* 2008;35(4): 346-355.

3. Summers K, Stevens J, Kakkas I, et al. Wilms' tumour 1 mutations are associated with FLT3-ITD and failure of standard induction chemotherapy in patients with normal karyotype AML. *Leukemia.* 2007;21(3):550-551.

4. 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 leuke-

mia group B study. *J Clin Oncol.* 2008;26(28): 4595-4602.

5. Virappane P, Gale R, Hills R, et al. Mutation of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: the United Kingdom Medical Research Council Adult Leukaemia working party. *J Clin Oncol.* 2008; 26(33):5429-5435.

6. Renneville A, Boissel N, Zurawski V, et al. Wilms tumor 1 gene mutations are associated with a higher risk of recurrence in young adults with acute myeloid leukemia: a study from the Acute Leukemia French Association. *Cancer.* 2009; 115(16):3719-3727.

7. 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(19):4505-4511.

8. Scharnhorst V, van der Eb AJ, Jochemsen AG. WT1 proteins: functions in growth and differentiation. *Gene.* 2001;273(2):141-161.

9. Little SE, Hanks SP, King-Underwood L, et al. Frequency and heritability of WT1 mutations in nonsyndromic Wilms' tumor patients: a UK Children's Cancer Study Group study. *J Clin Oncol.* 2004;22(20):4140-4146.

10. Miwa H, Beran M, Saunders GF. Expression of the Wilms' tumor gene (WT1) in human leukemias. *Leukemia.* 1992;6(5):405-409.

11. Pritchard-Jones K, Renshaw J, King-Underwood L. The Wilms tumour (WT1) gene is mutated in a secondary leukaemia in a WAGR patient. *Hum Mol Genet.* 1994;3(9):1633-1637.

12. Yang L, Han Y, Suarez Saiz F, et al. A tumor suppressor and oncogene: the WT1 story. *Leukemia.* 2007;21(5):868-876.

13. Little M, Wells C. A clinical overview of WT1 gene mutations. *Hum Mutat.* 1997;9(3):209-225.

14. Hollink IH, van den Heuvel-Eibrink MM, Zimmermann M, et al. Clinical relevance of Wilms tumor 1 gene mutations in childhood acute myeloid leukemia. *Blood.* 2009;113(23):5951-5960.

15. Lange BJ, Smith FO, Feusner J, et al. Outcomes in CCG-2961, a children's oncology group phase 3 trial for untreated pediatric acute myeloid leukemia: a report from the children's oncology group. *Blood.* 2008;111(3):1044-1053.

16. Franklin J, Alonzo TA, Hurwitz CA, et al. COG AAML03P1: efficacy and safety in a pilot study of intensive chemotherapy including gemtuzumab in children newly diagnosed with acute myeloid leukemia (AML). *Blood.* [abstracts]. 2008;112(11): Abstract 136.

17. Meshinchi S, Woods WG, Stirewalt DL, et al. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. *Blood.* 2001;97(1):89-94.

18. Brown P, McIntyre E, Rau R, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. *Blood.* 2007;110(3):979-985.

19. Ho PA, Alonzo TA, Gerbing RB, et al. Prevalence and prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. *Blood.* 2009;113(26):6558-6566.

20. Borel F, Barilla KC, Hamilton TB, et al. Effects of Denys-Drash syndrome point mutations on the DNA binding activity of the Wilms' tumor suppressor protein WT1. *Biochemistry.* 1996;35(37): 12070-12076.

21. Meshinchi S, Alonzo TA, Stirewalt DL, et al. Clinical implications of FLT3 mutations in pediatric AML. *Blood.* 2006;108:3654-3661.

22. Pollard JA, Alonzo TA, Gerbing RB, et al. Prevalence and prognostic significance of KIT mutations in pediatric patients with core binding factor AML enrolled on serial pediatric cooperative trials for de novo AML. *Blood.* 2010;115(12):2372-2379.

23. Little M, Holmes G, Bickmore W, et al. DNA binding capacity of the WT1 protein is abolished by Denys-Drash syndrome WT1 point mutations. *Hum Mol Genet.* 1995;4(3):351-358.

24. Fitzgibbon J, Smith LL, Raghavan M, et al. Association between acquired uniparental disomy and homozygous gene mutation in acute myeloid leukemias. *Cancer Res.* 2005;65(20):9152-9154.

25. Renneville A, Roumier C, Biggio V, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia.* 2008;22(5): 915-931.

26. Østergaard, Olesen LH, Hasle H, Kjeldsen E, Hokland P. WT1 gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukemia patients–results from a single-centre study. *Br J Haematol.* 2004; 125:590-600.

27. Noronha SA, Farrar JE, Alonzo TA, et al. WT1 expression at diagnosis does not predict survival in pediatric AML: a report from the Children's Oncology Group. *Pediatr Blood Cancer.* 2009;53(6): 1136-1139.

28. Willasch AM, Gruhn B, Coliva T, et al. Standardization of WT1 mRNA quantitation for minimal residual disease monitoring in childhood AML and implications of WT1 gene mutations: a European multicenter study. *Leukemia.* 2009;23(8):1472-1479.