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ORIGINAL REPORT

Wilms' Tumor 1 Gene Mutations Independently Predict Poor Outcome in Adults With Cytogenetically Normal Acute Myeloid Leukemia: A Cancer and Leukemia Group B Study

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ABSTRACT

Purpose
To analyze the prognostic impact of Wilms' tumor 1 (*WT1*) gene mutations in cytogenetically normal acute myeloid leukemia (CN-AML).

Patients and Methods
We studied 196 adults younger than 60 years with newly diagnosed primary CN-AML, who were treated similarly on Cancer and Leukemia Group B (CALGB) protocols 9621 and 19808, for *WT1* mutations in exons 7 and 9. The patients also were assessed for the presence of *FLT3* internal tandem duplications (*FLT3*-ITD), *FLT3* tyrosine kinase domain mutations (*FLT3*-TKD), *MLL* partial tandem duplications (*MLL*-PTD), *NPM1* and *CEBPA* mutations, and for the expression levels of *ERG* and *BAALC*.

Results
Twenty-one patients (10.7%) harbored *WT1* mutations. Complete remission rates were not significantly different between patients with *WT1* mutations and those with unmutated *WT1* ($P = .36$; 76% *v* 84%). Patients with *WT1* mutations had worse disease-free survival (DFS; $P < .001$; 3-year rates, 13% *v* 50%) and overall survival (OS; $P < .001$; 3-year rates, 10% *v* 56%) than patients with unmutated *WT1*. In multivariable analyses, *WT1* mutations independently predicted worse DFS ($P = .009$; hazard ratio [HR] = 2.7) when controlling for *CEBPA* mutational status, *ERG* expression level, and *FLT3*-ITD/*NPM1* molecular-risk group (ie, *FLT3*-ITD^{negative}/*NPM1*^{mutated} as low risk *v* *FLT3*-ITD^{positive} and/or *NPM1*$^{wild\text{-}type}$ as high risk). *WT1* mutations also independently predicted worse OS ($P < .001$; HR = 3.2) when controlling for *CEBPA* mutational status, *FLT3*-ITD/*NPM1* molecular-risk group, and white blood cell count.

Conclusion
We report the first evidence that *WT1* mutations independently predict extremely poor outcome in intensively treated, younger patients with CN-AML. Future trials should include testing for *WT1* mutations as part of molecularly based risk assessment and risk-adapted treatment stratification of patients with CN-AML.

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INTRODUCTION

Cytogenetically normal acute myeloid leukemia (CN-AML) is the largest cytogenetic subgroup of AML, representing approximately 45% of adult patients with AML who are younger than 60 years.[1-3] During the last decade, CN-AML has been recognized as highly heterogeneous molecularly, because several abnormalities were discovered, including mutations in *FLT3*, *NPM1*, *CEBPA*, and *MLL* genes and aberrant expression of *BAALC*, *ERG*, and *MN1* genes.[4] These genetic alterations have been associated with treatment outcome and serve as a basis for molecularly guided risk assessment in CN-AML.[4,5] However, discovery of novel genetic markers likely will improve molecular-risk stratification and will allow a more accurate prediction of response to current therapy.

The Wilms' tumor 1 (*WT1*) gene, located on chromosome 11p13,[6] encodes a transcriptional regulator that is capable of activating or repressing gene transcription, depending on the cell type, the WT1 protein isoform, and the target gene.[7] Although initially considered a tumor suppressor gene,[8] *WT1* also has been demonstrated to act as an oncogene.[7,9-11] The functional duality of *WT1* as a

tumor suppressor gene and an oncogene, however, is not well understood and appears to depend on the genomic and cellular milieu.[7] Expression of the *WT1* gene has been detected in 75% to 100% of patients with AML, but the results of studies that evaluated the impact of *WT1* expression levels at diagnosis on clinical outcome have been inconsistent.[12-17]

Intragenic *WT1* mutations are found in at least 5% of patients with sporadic Wilms' tumors,[18] which is one of the most common nonhematologic neoplasms in children.[19] In addition, *WT1* mutations have been found in rare congenital malformation syndromes with predisposition for the development of Wilms' tumors[20] and have been reported anecdotally in other cancers, including non–asbestos-related mesothelioma and juvenile granulosa cell tumor.[18]

In earlier studies of acute leukemias, *WT1* mutations occurred in up to 15% of patients with AML,[21-25] in 18% of patients with biphenotypic or undifferentiated acute leukemia,[24] and rarely in those with acute lymphoblastic leukemia.[24] To our knowledge, only two reports that address the prognostic relevance of *WT1* mutations in AML have been published.[24,26] One study that included 33 adult and childhood patients with AML who had various cytogenetic findings found that none of the four patients who harbored *WT1* mutations achieved a complete remission (CR) and that their overall survival was worse than that of patients without *WT1* mutations.[24] A more recent study by Summers et al[26] focused on CN-AML and found *WT1* mutations, located primarily in exons 7 and 9, in seven (10%) of 70 patients with CN-AML.[26] Of the six patients with *WT1* mutations who received standard induction therapy, five did not achieve a CR. Interestingly, each of the five patients with *WT1* mutations who failed induction therapy harbored simultaneously a *FLT3*-internal tandem duplication (*FLT3*-ITD).[26] However, the analysis of other molecular markers with established prognostic relevance in CN-AML[4] was not reported.[26]

The objectives of our study were to assess the frequency and prognostic value of *WT1* mutations in the context of other prognostic molecular factors among younger patients with CN-AML who were treated similarly on Cancer and Leukemia Group B (CALGB) protocols that incorporated intensification therapy with autologous peripheral-blood stem-cell transplantation (APBSCT).

PATIENTS AND METHODS

Patients

We studied 196 adults who were younger than 60 years and who had untreated primary CN-AML. The diagnosis of CN-AML was based on standard cytogenetic analysis that was performed by CALGB-approved institutional cytogenetic laboratories as part of the cytogenetic companion study 8461.[1] To be considered cytogenetically normal, at least 20 metaphase cells from diagnostic bone marrow (BM) had to be evaluated, and the karyotype had to be found normal in each patient. All cytogenetic results were confirmed by central karyotype review. All patients were enrolled on two similar CALGB treatment protocols (ie, 9621 or 19808). Institutional Review Board–approved informed consent for participation in the studies was obtained from all patients.

Treatment

Details of the treatment protocols have been previously reported.[27,28] Briefly, patients on CALGB 9621 received induction chemotherapy with cytarabine, daunorubicin, and etoposide with (ADEP) or without (ADE) the multidrug resistance protein modulator PSC-833, also called valspodar.[27] Patients who had CN-AML and who achieved a CR received high-dose cytarabine (HiDAC) and etoposide for stem-cell mobilization followed by myeloablative treatment with busulfan and etoposide supported by APBSCT. Patients unable to receive APBSCT received two additional cycles of HiDAC. Patients enrolled on CALGB 19808 were treated similarly[28] to those on CALGB 9621. None of the patients received allogeneic stem-cell transplantation in first remission.

Mutational Analysis of WT1

Mononuclear cells from diagnostic BM and/or blood specimens were enriched by Ficoll density gradient centrifugation and were cryopreserved in liquid nitrogen until use. Genomic DNA was extracted from cryopreserved mononuclear cell preparations of diagnostic BM or blood by using the commercially available DNeasy Tissue Kit (Qiagen, Valencia, CA) or the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. DNA fragments that spanned the entire *WT1* exons 7 and 9 were amplified by polymerase chain reaction (PCR) by using AmpliTaq Gold (Applied Biosystems, Foster City, CA). Intronic primers previously reported for *WT1* exons 7[29] and 9[30] were used in the PCRs. PCR fragments of appropriate size were identified after agarose gel electrophoresis for all samples. Amplicons from each patient were pooled with unmutated reference amplicons, were denatured, and were cooled down slowly to 25°C. The reannealed DNA duplexes were analyzed for mutations by denaturing high-performance liquid chromatography (DHPLC)[31] by using a WAVE 3500HT DNA Fragment Analysis System (Transgenomic Inc, Omaha, NE). The individual elution peaks were compared with the unmutated reference sample. Samples with elution peaks that differed from the unmutated reference were reamplified in an independent PCR and were assessed for variations in the coding DNA by direct sequencing in both directions. The results obtained by direct sequencing were confirmed by subcloning of mutated amplicons into the pCR2.1-TOPO vector (Invitrogen) and by sequencing of 18 or fewer independent clones.

Analyses of Other Molecular Markers

Other molecular markers (ie, *FLT3*-ITD,[32] *FLT3* tyrosine kinase domain mutation [*FLT3*-TKD],[33-35] *MLL* partial tandem duplication [*MLL*-PTD],[36,37] *NPM1*[38] and *CEBPA*[39] mutations, and *ERG*[5,40] and *BAALC*[41] expression levels) were assessed as previously reported.

Definition of Clinical End Points

CR required an absolute neutrophil count $\geq$ 1,500/μL, a platelet count $\geq$ 100,000/μL, no leukemic blasts in the blood, BM cellularity greater than 20% with maturation of all cell lines, no Auer rods, less than 5% BM blast cells, and no evidence of extramedullary leukemia, all of which had persisted for at least 1 month. Relapse was defined by $\geq$ 5% BM blasts, circulating leukemic blasts, or the development of extramedullary leukemia.[42] Disease-free survival (DFS) was measured from the date of CR until the date of relapse or death; patients alive and relapse-free at last follow-up were censored. Overall survival (OS) was measured from the date on study until the date of death, and patients alive at last follow-up were censored.

Statistical Analysis

Associations between patients with and without *WT1* mutations and baseline demographic, clinical, and molecular features were described by using Fisher's exact and Wilcoxon rank sum tests for categoric and continuous variables, respectively. Estimated probabilities of DFS and OS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. Proportional hazards models were constructed for DFS and OS to evaluate the impact of *WT1* mutations when controlling for other prognostic variables. Variables other than *WT1* mutational status that were considered for model inclusion were age, sex, race, hemoglobin, platelet count, $\log_2$(white blood cell count) [$\log_2$(WBC)], percentage of blasts in the blood and BM, *ERG* and *BAALC* expression levels (high *v* low), *CEBPA* status (mutated *v* wild-type), *FLT3*-TKD and *MLL*-PTD (present *v* absent), and molecular risk group (high *v* low) as defined by *FLT3*-ITD/*NPM1* molecular features (ie, *FLT3*-ITD$^{\text{negative}}$/*NPM1*$^{\text{mutated}}$ as low risk and *FLT3*-ITD$^{\text{positive}}$ and/or *NPM1*$^{\text{wild-type}}$ as high risk). Variables were entered by forward selection into the model by using the Wald test until no variable entered with $P < .05$. The proportional hazards assumption was checked for each variable individually. If the proportional hazards assumption was not met for a particular

variable, then an artificial time-dependent covariate was included in all models that contained that variable.[43] All analyses were performed by the CALGB Statistical Center.

RESULTS

Frequency and Types of WT1 Mutations

Twenty-one patients (10.7%) had at least one *WT1* mutation (Table 1). Mutations in exon 7 were found in 16 patients. Among these 16 patients, two patients had two mutations in exon 7 simultaneously, and one patient had a mutation in exon 7 in addition to a mutation in exon 9. One *WT1* mutation in exon 7 was a nonsense mutation; all others were frameshift mutations. The frameshift mutations were mainly caused by various small duplications; less frequent mutations were imperfect small repeats of exon 7 sequences or combined deletions/insertions. All *WT1* mutations in exon 7 resulted in a premature truncation of the WT1 protein, with loss of the zinc finger region or truncation after the second zinc finger.

Mutations in exon 9 without accompanying mutations in exon 7 were found in five patients. All *WT1* mutations in exon 9 resulted in single amino acid substitutions that affected the third zinc finger in the WT1 protein. All samples with *WT1* mutations retained the wild-type sequence in addition to the mutated sequence, which suggested heterozygosity for the mutant allele.

Clinical Characteristics, Other Molecular Markers, and Treatment

At diagnosis, patients with *WT1* mutations had greater WBCs ($P = .01$), were more often high *ERG* ($P = .01$) and *BAALC* ($P = .006$) expressers, and tended to harbor *FLT3*-ITD more often ($P = .06$) than patients with unmutated *WT1* (Table 2). The frequency of the *FLT3*-ITD^{negative}/*NPM1*^{mutated} status was not significantly different between *WT1*-mutated and *WT1*-unmutated patient groups ($P = .63$; 29% *v* 37%), nor were the proportions of patients with a high (≥ 0.7) *FLT3*-ITD/*FLT3*–wild-type allele ratio ($P = 1.00$; 19% *v* 18%). Figure 1 shows the mutation status of the *NPM1*, *FLT3* (ITD and TKD), *CEBPA*, and *MLL* (PTD) genes that were coexisting in individual patients with *WT1* mutations. There was no significant difference between the *WT1*-mutated and *WT1*-unmutated patient groups with regard to the inclusion of PSC-833 (ie, valspodar) in induction ($P = .63$) or in the proportion of patients who received APBSCT for consolidation ($P = 1.00$).

Prognostic Impact of WT1 Mutations

One-hundred sixty-three (83%) of 196 patients in this study achieved a CR, and the estimated DFS and OS rates at 3 years were 46% and 51%, respectively. The median follow-up for patients who remained alive was 4.2 years (range, 1.2 to 8.9 years).

The outcome results with respect to *WT1* mutational status are summarized in Table 2. Among the 21 patients with *WT1* mutations, 16 (76%) achieved a CR. This percentage was slightly lower, but not

Table 1. *WT1* Mutations and Individual Outcome in 21 Patients With CN-AML

Patient by Mutation	Age (years)	Sex	DNA Change*†	Protein Change†‡	Individual Outcome
WT1^{mut7}					
1	44	F	1301_1304dup	R302PfsX16	Relapse/death
2	25	M	1317_1318insCTGTGCCTGGAGT	A307CfsX14	Relapse/death
3	30	F	1317_1318insCTGTGCCTGGAGT	A307CfsX14	Relapse/death
4	52	M	1325_1331dup	V311DfsX8	Relapse/death
5	33	F	1328_1329dup	V311LfsX71§	Relapse/death
6	37	F	1334delinsGG	R312GfsX5	Relapse/death
7	51	F	1327_1334dup	R312LfsX72§	Failure to achieve CR/death
8	21	F	1324_1336dup	S313DfsX8	Relapse/death
9	38	M	1326_1335dup	A314CfsX6	Relapse/death
10	50	F	1329_1338dup	A314CfsX6	Relapse/death
11	51	F	1337_1340dup	A314VfsX4	Relapse/death
12	40	M	1338dupC	A314GfsX3	Failure to achieve CR/death
13	18	M	1335_1344dup	E316VfsX4	Relapse/death
14	58	F	[1331_1337dup] + [1338C>A]	[S313CfsX6] + [S313X]	Failure to achieve CR/death
15	43	M	[1306dupT] + [1324_1333dup]	[V303CfsX14] + [R312DfsX8]	Achieved CR/alive‖
WT1^{mut9}					
16	57	M	1569_1570delinsCT	R390P	Relapse/death
17	49	F	1581G>C	R394P	Failure to achieve CR/death
18	56	F	1586G>C	D396H	Relapse/death
19	37	F	1589C>G	H397D	Relapse/death
20	41	M	1591C>A	H397Q	Failure to achieve CR/death
WT1^{mut7} and *WT1*^{mut9}					
21	22	M	[1330_1337dup(+)1581G>A]	[S313LfsX71(+)R394Q]§	Achieved CR/alive

Abbreviations: CN, cytogenetically normal; AML, acute myeloid leukemia; *WT1*^{mut7}, *WT1* mutations in exon 7; *WT1*^{mut9}, *WT1* mutations in exon 9; CR, complete response.

*Nucleotide sequence numbering is according to Genebank accession number NM_024426.

†The sequence variations are designated according to the current recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

‡The protein changes are theoretically deduced.

§The stop codons created by these *WT1*^{mut7} do not occur until the 5′ part of exon 9; DNA direct sequencing of exon 8 revealed a wild-type sequence.

‖During the preparation of the article, this patient suffered a relapse and was undergoing salvage therapy.

Table 2. Comparison of Pretreatment Clinical and Molecular Characteristics, Treatment and Outcomes of CN-AML Patients With (*WT1*^{mut}) and Without (*WT1*^{wt}) *WT1* Mutations

Characteristic	Mutation Status: *WT1*^{mut} (n = 21)	Mutation Status: *WT1*^{wt} (n = 175)	*P*
Age, years			.38
Median	41	46	
Range	18-58	19-59	
Sex, male			.65
No.	9	86	
%	43	49	
Race			.48
White			
No.	20	151	
%	95	88	
Nonwhite			
No.	1	21	
%	5	12	
Hemoglobin, g/dL			.50
Median	9.4	9.4	
Range	4.9-12.1	4.6-13.6	
Platelet count, $\times 10^9$/L			.28
Median	54	62	
Range	7-235	8-466	
WBC count, $\times 10^9$/L			.01
Median	50.9	23.8	
Range	11.3-210.0	0.9-295.0	
Blood blasts, %			.64
Median	61	59	
Range	10-93	0-97	
Bone marrow blasts, %			.78
Median	70	65	
Range	32-93	10-99	
FLT3-ITD			.06
Absent			
No.	9	113	
%	43	65	
Present			
No.	12	62	
%	57	35	
FLT3-TKD			.42
Absent			
No.	18	157	
%	86	91	
Present			
No.	3	15	
%	14	9	
NPM1			.81
Wild-type			
No.	6	60	
%	29	34	
Mutated			
No.	15	115	
%	71	66	
FLT3-ITD/*NPM1* status*			.63
Low-risk			
No.	6	64	
%	29	37	
High-risk			
No.	15	111	
%	71	63	
MLL-PTD			1.00
Absent			
No.	20	163	
%	95	93	
Present			
No.	1	12	
%	5	7	

(continued in next column)

Table 2. Comparison of Pretreatment Clinical and Molecular Characteristics, Treatment and Outcomes of CN-AML Patients With (*WT1*^{mut}) and Without (*WT1*^{wt}) *WT1* Mutations (continued)

Characteristic	Mutation Status: *WT1*^{mut} (n = 21)	Mutation Status: *WT1*^{wt} (n = 175)	*P*
CEBPA			.24
Wild-type			
No.	15	121	
%	71	82	
Mutated			
No.	6	26	
%	29	18	
ERG expression†			.01
Low			
No.	7	88	
%	37	68	
High			
No.	12	41	
%	63	32	
BAALC expression‡			.006
Low			
No.	4	74	
%	21	57	
High			
No.	15	55	
%	79	43	
Induction treatment			.63
ADE			
No.	15	111	
%	71	63	
ADEP			
No.	6	64	
%	29	37	
Complete remission rate			.36
No.	16	147	
%	76	84	
Receiving APBSCT for consolidation			1.00
No.	11	101	
%	69	69	
Disease-free survival, years			< .001
Median	0.6	3.4	
Disease-free at 3 years			
%	13	50	
95% CI	2 to 33	42 to 58	
Overall survival, years			< .001
Median	0.8	4.5	
Alive at 3 years			
%	10	56	
95% CI	2 to 26	49 to 64	

Abbreviations: CN, cytogenetically normal; AML, acute myeloid leukemia; *WT1*^{mut}, *WT1* mutations; *WT1*^{wt}, unmutated (wild-type) *WT1* alleles; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation of the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene; ADE, cytarabine, daunorubicin, and etoposide; ADEP, cytarabine, daunorubicin, etoposide, and valspodar; APBSCT, autologous peripheral-blood stem-cell transplantation; CI, confidence interval.

**FLT3*-ITD^{negative}/*NPM1*^{mutated} molecular status defines the low-risk group. *FLT3*-ITD^{positive} and/or *NPM1*$^{wild\text{-}type}$ molecular status defines the high-risk group.

†In patients on protocol 9621, cut point was the same as in Marcucci et al.[40] In patients on protocol 19808, cut point was the same as in Marcucci et al.[5]

‡In patients on protocol 9621, cut point was the same as in Baldus et al.[41] In patients on protocol 19808, the median *BAALC* expression value was used for cut point.

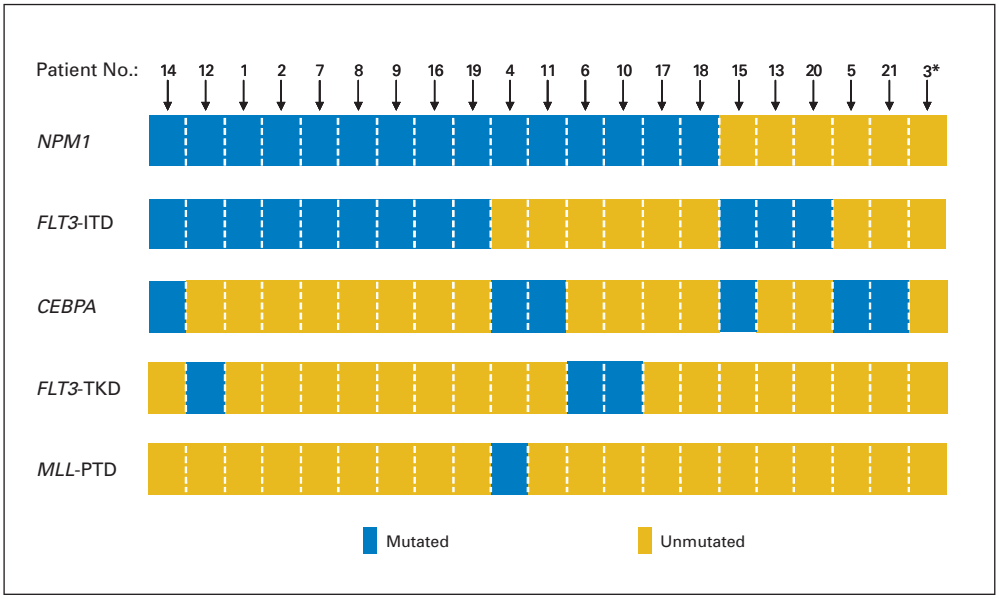

Fig 1. Coexistence of *WT1* mutations with mutations in other genes, such as *NPM1*, *FLT3* (ITD), *CEBPA*, *FLT3* (TKD), and *MLL* (PTD) among 21 patients who harbored *WT1* mutations. (*) Indicates the presence of the *CEBPA* polymorphism c.1175_1180dup in patient 3.

significantly different, than the 84% of patients with unmutated *WT1* that achieved a CR ($P = .36$). All five patients with *WT1* mutations who did not achieve a CR had resistant disease. Interestingly, four of them also harbored an *NPM1* mutation, which in three of these patients coexisted with *FLT3*-ITD; the fifth patient had *FLT3*-ITD but no *NPM1* mutation.

Of the patients who achieved a CR, those with *WT1* mutations relapsed more frequently than those with unmutated *WT1* (88% *v* 51%; $P = .007$). The estimated 3-year DFS rate was only 13% for patients with *WT1* mutations compared with 50% for patients with unmutated *WT1* (Fig 2A).

In a multivariable analysis (Table 3), *WT1* mutational status independently predicted worse DFS ($P = .009$) when controlling for *CEBPA* mutational status ($P = .004$), the *FLT3*-ITD/*NPM1* molecular-risk group ($P = .006$), and *ERG* expression level ($P = .04$); the estimated risk of relapse or death was almost three times higher for patients who had *WT1* mutations compared with patients who had unmutated *WT1* (hazard ratio [HR] = 2.7; Table 3). At the time of the analysis, all relapses in the *WT1*-mutated patients occurred within 9 months of CR achievement; there were only two of the 16 patients with *WT1* mutations who achieved a CR and had not relapsed—one at 4 years, and one at 7 years. However, during preparation of this manuscript, one of these patients experienced a relapse at 5 years after CR achievement. This patient currently is undergoing salvage therapy.

Likewise, patients with *WT1* mutations had shorter OS than patients with unmutated *WT1* ($P < .001$). The estimated OS rates at 3 years were 10% and 56% for patients with and without *WT1* mutations, respectively (Fig 2B). *WT1* mutations independently predicted a higher risk of death ($P < .001$; Table 3) when controlling for the *FLT3*-ITD/*NPM1* molecular-risk group ($P = .004$), *CEBPA* mutational status ($P = .02$), and WBC ($P = .04$); the estimated risk of death was more than three times higher for patients who had *WT1* mutations compared with patients who had unmutated *WT1* (HR = 3.2; Table 3). Notably, none of the five patients with *WT1* mutations who failed to achieve a CR and none of the 14 who experienced an early relapse had successful salvage treatment. All of these patients died within 17 months of study enrollment.

DISCUSSION

We present here a relatively large study that assessed the prognostic value of *WT1* mutations in younger adults who had primary CN-AML and who received similar intensive treatment that did not include allogeneic stem-cell transplantation in first CR. We show that the presence of *WT1* mutations at diagnosis is associated with an extremely poor outcome and that it independently predicts a higher risk of relapse and death when other molecular markers with established prognostic significance and clinical variables are taken into consideration.

Previous, relatively small studies on patients with AML who had diverse cytogenetic findings and/or secondary AML found *WT1* mutations in ≤ 15% of the patients.[21-25] To date, however, only Summers et al[26] assessed the incidence and prognostic impact of *WT1* mutations exclusively in CN-AML. This study included 70 patients with CN-AML, ranging in age between 19 and 78 years; seven (10%) of these patients had *WT1* mutations, including five patients with heterozygous mutations in exon 7, one patient with concurrent mutations in exon 7 and exon 9, and one patient with a homozygous mutation in exon 9. Consistent with these results,[26] we found *WT1* mutations in 10.7% of patients with CN-AML, and mutations in exon 7 also were more frequent than those in exon 9. Although we found the CR rate of patients with *WT1* mutations to be lower than that of patients with unmutated *WT1*, we did not observe a statistically significant difference. However, 14 (88%) of 16 patients who had *WT1* mutations and who attained a CR relapsed within the first 9 months of CR achievement.

All but one patient with CN-AML who had *WT1* mutations in our study had at least one additional mutation in the other genes analyzed (ie, *NPM1*, *FLT3*, *CEBPA*, and *MLL;* Fig 1). The most common among these were mutations in the *NPM1* gene (71%), followed by *FLT3*-ITD (57%); other mutations were much less frequent. It is striking that four of five patients with *WT1* mutations who did not achieve a CR also harbored an *NPM1* mutation, which has been reported previously to impact favorably on the probability of CR achievement.[44-46] Consistent with the study of

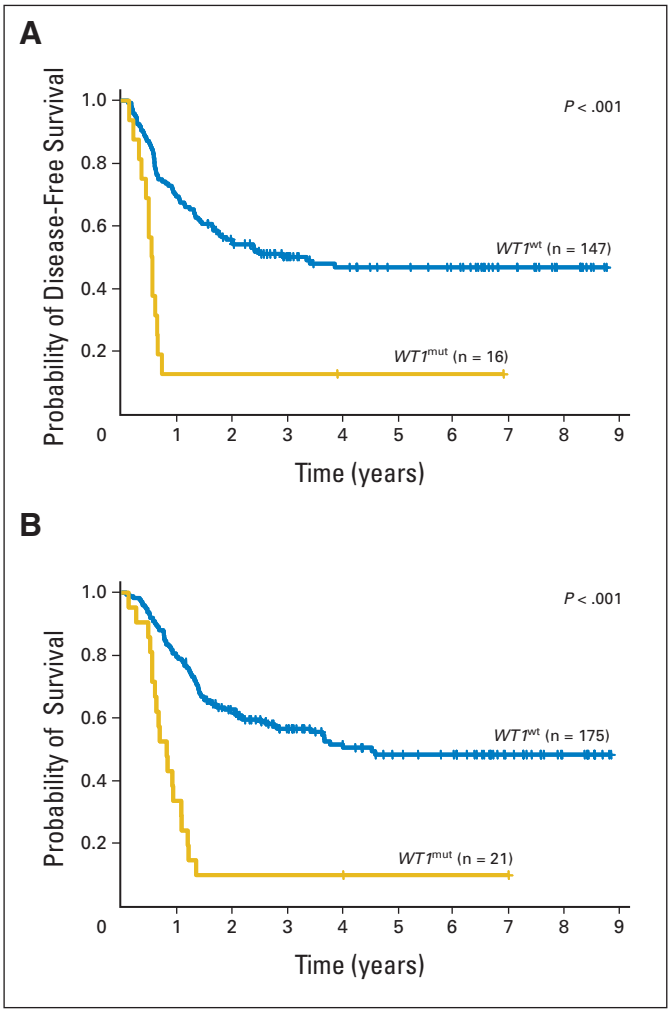

Fig 2. (A) Disease-free and (B) overall survival of patients with CN-AML according to mutational *WT1* status (ie, patients with *WT1* mutations [$WT1^{mut}$] *v* patients without *WT1* mutations [$WT1^{wt}$]).

Table 3. Multivariable Analyses for DFS and OS

Endpoint	Variables in the Final Models	HR	95% CI	*P*
DFS	*WT1*: $WT1^{mut}$ *v* $WT1^{wt}$	2.7	1.3 to 5.5	.009
	FLT3-ITD/*NPM1* status: high *v* low risk*	2.5	1.3 to 4.7	.006
	CEBPA: $CEBPA^{mut}$ *v* $CEBPA^{wt}$	0.3	0.2 to 0.7	.004
	ERG expression: high *v* low†	1.9	1.0 to 3.5	.04
OS	*WT1*: $WT1^{mut}$ *v* $WT1^{wt}$	3.2	1.7 to 6.2	< .001
	FLT3-ITD/*NPM1* status: high *v* low risk*	2.7	1.4 to 5.2	.004
	CEBPA: $CEBPA^{mut}$ *v* $CEBPA^{wt}$	0.4	0.2 to 0.8	.02
	Log_2(WBC) two-fold increase‡	1.9	1.2 to 3.2	.04

NOTE. Hazard ratios greater than or less than 1 indicate an increased or decreased risk, respectively, of an event for greater values of continuous variables and for the first category listed for dichotomous variables.

Abbreviations: DFS, disease-free survival; OS, overall survival; HR, hazard ratio; $WT1^{mut}$, presence of *WT1* mutations; $WT1^{wt}$, absence of *WT1* mutations; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; $CEBPA^{mut}$, presence of *CEBPA* mutations; $CEBPA^{wt}$, absence of *CEBPA* mutations; Log_2(WBC), log-transformed white blood cell count.

*The high-risk molecular group is defined by the presence of *FLT3*-ITD and/or the absence of *NPM1* mutations. The low-risk molecular group is defined by the absence of *FLT3*-ITD and by the simultaneous presence of an *NPM1* mutation.

†*ERG* expression did not meet the proportional hazards assumption. The *P* value corresponds to the Wald statistic of a two degree of freedom test that evaluated whether the coefficients for *ERG* expression and an artificial time-dependent covariate were equal to zero. The risk of an event was similar for high and low *ERG* expressers shortly after achieving complete remission, but the risk for high *ERG* expressers increased over time. The hazard ratio evaluated at 1 year is presented.

‡Log_2(WBC) did not meet the proportional hazards assumption. The *P* value corresponds to the Wald statistic of a two degree of freedom test that evaluated whether the coefficients for log_2(WBC) and an artificial time-dependent covariate were equal to zero. The risk of an event was larger for those with greater WBC counts soon after going on study, but this risk decreased over time. The hazard ratio presented is for a two-fold increase in WBC when evaluated at 3 months.

Summers et al,[26] patients who had *WT1* mutations in our study tended to be *FLT3*-ITD–positive more often than patients with unmutated *WT1*. Thus, we performed multivariable analyses to assess whether the impact of *WT1* mutations on outcome was independent from other established prognostic molecular markers and clinical characteristics. We show that mutations in the *WT1* gene are indeed independent predictors for worse DFS and OS in younger patients who have primary CN-AML. Interestingly, all six patients with *WT1* mutations who belonged to the low-risk molecular category by virtue of having *FLT3*-$ITD^{negative}$/$NPM1^{mutated}$ status died after they experienced a failure to achieve CR or relapse (Appendix Fig A1, online only), which suggests that the presence of *WT1* mutations may be capable of overcoming the reported favorable prognostic impact of the coexistence of the *NPM1* mutation with the lack of *FLT3*-ITD in this CN-AML subset.[37,46,47]

Patients with mutated *WT1* were also high expressers of *ERG* and *BAALC* more frequently than patients with unmutated *WT1*. Overexpression of both the *ERG*[5,40] and the *BAALC*[41,48] genes has been associated with an adverse prognosis. In our study, *WT1* mutations appeared to impact adversely on DFS and OS, regardless of the expression status of these genes.

The *WT1* gene consists of 10 exons and encodes a transcriptional regulator that is characterized by two major functional domains—an N-terminal transcriptional regulatory domain and a C-terminal DNA and/or RNA binding domain that is composed of four zinc fingers.[9,49] Mutational analyses in our study focused on *WT1* exons 7 and 9, because these regions have been recognized previously as mutational hot spots in CN-AML.[26] *WT1* exons 7 and 9 encode the first and third zinc finger, respectively, in the WT1 protein.[9,49] All mutations in exon 7 of the *WT1* gene found in the present study led to a premature truncation of the protein and eliminated all or, less frequently, the last two zinc fingers. *WT1* mutations in exon 9 led to single amino acid substitutions within the third zinc finger that affects residues expected to be crucial for the DNA binding ability.[50] Thus, *WT1* mutations would be expected to abolish, impair, or change the DNA binding ability of the WT1 protein to its target genes, including to those that encode proteins involved in the regulation of normal hematopoiesis (*RARA, CSF1*), apoptosis (*BCL2, BCL2A1, BAK1*), cell cycle (*CCNE1, CDKN1A*), gene transcription (*MYC, PAX2, MYB, EGR1*), and cell proliferation (*TGFB1, PDGFA*).[9] Although preliminary in vitro[10,51] and in vivo[11] studies have implicated involvement of the WT1 protein in leukemogenesis, its role still is not understood fully,[9] and the mechanisms by which *WT1* mutations confer leukemic cell resistance to therapy remain to be elucidated.

In conclusion, we show for the first time that mutations in the *WT1* gene represent a strong, independent predictor of poor outcome in intensively treated patients with CN-AML. On the basis of these

results, we propose that upcoming clinical trials incorporate molecular testing for *WT1* mutations in patients with CN-AML at diagnosis to prospectively confirm their prognostic significance, with the ultimate goals of improving current molecularly based risk stratification of CN-AML and of developing targeted therapies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

