WT1 Synonymous Single Nucleotide Polymorphism rs16754 Correlates With Higher mRNA Expression and Predicts Significantly Improved Outcome in Favorable-Risk Pediatric Acute Myeloid Leukemia: A Report From the Children's Oncology Group

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ABSTRACT

Purpose

To analyze the prevalence and clinical implications of Wilms' tumor 1 (*WT1*) single nucleotide polymorphism (SNP) rs16754 in the context of other prognostic markers in pediatric acute myeloid leukemia (AML).

Patients and Methods

Available diagnostic marrow specimens ($n = 790$) from 1,328 patients enrolled in three consecutive Children's Cancer Group/Children's Oncology Group trials were analyzed for the presence of SNP rs16754. SNP status was correlated with disease characteristics, *WT1* expression level, and clinical outcome.

Results

SNP rs16754 was present in 229 (29%) of 790 patients. The SNP was significantly more common in Asian and Hispanic patients and less common in white patients ($P < .001$). SNP rs16754 was also less common in patients with *inv*(16) ($P = .043$) and more common in patients with $-5/\text{del}(5q)$ ($P = .047$). *WT1* expression levels were significantly higher in patients with rs16754 or with *WT1* mutations compared with *WT1* wild-type patients ($P = .021$). Five-year overall survival (OS) for patients with and without the SNP was 60% and 50%, respectively ($P = .031$). Prognostic assessment by risk group demonstrated that in patients with low-risk disease, OS for those with and without SNP rs16754 was 90% versus 64% ($P < .001$) with a corresponding disease-free survival of 72% versus 53% ($P = .041$).

Conclusion

The presence of SNP rs16754 was an independent predictor of improved OS; outcome differences were most pronounced in the low-risk subgroup. The high prevalence of *WT1* SNP rs16754, and its correlation with improved outcome, identifies *WT1* SNP rs16754 as a potentially important molecular marker of prognosis in pediatric AML.

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INTRODUCTION

Single nucleotide polymorphisms (SNPs) account for much of the phenotypic diversity among individuals. Half of the known coding region SNPs in the human genome lead to a change in the resulting amino acid, whereas the other half do not (synonymous SNPs).¹ Because synonymous SNPs encode a change in the DNA sequence without altering the resultant protein sequence, such silent SNPs were long assumed to be inconsequential. However, synonymous SNPs may represent genetic markers for functional molecular alterations with which they are in linkage disequilibrium; further, recent work has

shown that synonymous SNPs may directly alter gene function and phenotype by various mechanisms, such as altering miRNA binding or protein folding, or by affecting mRNA splicing, stability, or expression.² To date, silent SNPs have been reported in association with more than 40 diseases that have a genetic basis.³ Genomic studies in both pediatric and adult AML in the past decade have identified function-altering mutations in a host of genes, including *FLT3*, *NPM1*, *NRAS*, *MLL*, Wilms' tumor 1 (*WT1*), and *CEBPA*.^{4,5} The molecular characterization of AML is being continually redefined as novel alterations are discovered. We previously identified mutations in the zinc-finger domains of the *WT1*

gene in 8% of pediatric patients with AML.⁶ Although these mutations are predicted to lead to loss of function of WT1, we found that the presence of *WT1* mutations had no independent significance in predicting outcome in pediatric AML. A recent study in adult AML also did not find *WT1* mutations to be of independent prognostic significance; however, this study reported that presence of *WT1* SNP rs16754 correlated significantly with improved survival outcomes.⁷

WT1 encodes a zinc-finger transcription factor protein that exists in multiple isoforms and is expressed primarily in tissues of the developing genitourinary and hematopoietic systems.⁸ *WT1* is overexpressed in blasts cells of the majority of acute leukemia patients.⁹ The *WT1* protein consists of a transcriptional regulatory domain (exons 1 to 6) as well as 4 C-terminal zinc-finger domains (exons 7 to 10) that facilitate DNA binding.⁸ Nearly all leukemia-associated *WT1* mutations occur within the zinc-finger domains; most of these mutations occur within a hotspot in exon 7, also the location of SNP rs16754. In a study of 249 adult patients with normal-karyotype AML, Damm et al⁷ reported that SNP rs16754 independently predicted improved overall survival (OS) and relapse-free survival (RFS) in adult AML. In our study, we determined the prevalence of SNP rs16754 in a large cohort of pediatric AML patients enrolled on three consecutive CCG/COG trials. We then analyzed biologic or clinical differences among SNP-positive and SNP-negative patients and examined the prognostic significance of harboring at least one copy of the minor SNP allele in the context of other previously validated risk factors in pediatric AML.

PATIENTS AND METHODS

Patient Samples

Newly diagnosed pediatric patients with de novo AML enrolled in three consecutive pediatric AML protocols, CCG-2941, CCG-2961, and COG-AAML03P1, were eligible for this study. Details of these studies have been previously described.^{10,11} Diagnostic specimens from 790 of 1,328 patients were available for analysis. Patients with available specimens had similar clinical outcome compared with those without available specimens.⁶

Institutional review board approval was obtained before mutation analysis, and this study was approved by the COG Myeloid Disease Biology Committee. Informed consent was obtained in accordance with the Declaration of Helsinki.

Molecular Genotyping, cDNA Synthesis, and Reverse-Transcriptase Polymerase Chain Reaction

Molecular genotyping of diagnostic specimens was performed as previously described.⁶ Reverse transcription was performed on 1 μ g total RNA as per standard protocol (Invitrogen Corporation, Carlsbad, CA). Expression analysis by reverse-transcriptase polymerase chain reaction (PCR) was performed on cDNA transcripts, on a StepOne Plus real-time PCR instrument, using the TaqMan system (Applied Biosystems, Foster City, CA) per manufacturer's instructions. Patient samples were run in duplicate, with the beta glucuronidase (*GUSB*) housekeeping gene as an internal control. The TaqMan *WT1* primer/probe set was designed to hybridize within *WT1* exon 2. The $\Delta\Delta$ CT method was used to determine the relative expression levels of *WT1*. Normal bone marrow extracted from the same donor was used as a control on each run.

Statistical Methods

The Kaplan-Meier method was used to estimate OS and disease-free survival (DFS). OS was defined as time from study entry to death. DFS was defined as the time from end of course 1 for patients in complete remission

(CR) 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, where deaths from nonrelapse etiologies were considered competing events. The significance of predictor variables was tested with the log-rank statistic for OS and DFS, and with Gray's statistic for RR. Children who also received a stem-cell transplant while on study were censored at the time of transplant for all analyses. The significance of observed differences in proportions was tested by the χ^2 test and Fisher's 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 (HR) for univariate and multivariate analyses for OS and RFS. RFS was defined as the time from end of course 1 for patients in CR to relapse or death due to progressive disease, where deaths from nonrelapse etiologies were censored.

RESULTS

Prevalence of SNP rs16754

All available specimens ($n = 790$) were subjected to direct sequencing of *WT1* exon 7 to assess *WT1* SNP status. SNP rs16754 represents an A>G substitution at nucleotide position 1293, the third position of codon 352 encoding arginine (CGA>CGG). At least 1 copy of the minor allele was detected in 229 patients (29.0%), 38 (16.6%) of whom were homozygous for the SNP (191 were heterozygous).

Characteristics of the Study Population

Demographic, laboratory, and clinical characteristics of patients with and without *WT1* SNP rs16754 were compared (Table 1). There were no significant differences in sex, age, median diagnostic blast percentage, median diagnostic WBC count, or French-American-British class between SNP-positive and SNP-negative patients. There were significant differences in the racial distribution of the minor SNP allele. SNP rs16754 occurred at the highest frequency in patients of Asian (66%) and Hispanic (42%) descent and was less frequent in white (24%) and African American (22%) patients. Patients with the SNP had a lower prevalence of inversion 16 (6.3% v 12.6%; $P = .043$), and higher prevalence of $-5/\text{del}(5q)$, (3.1% v 0.8% $P = .047$) compared with their SNP-negative counterparts. No other association with cytogenetic groups were identified.

The association between SNP rs16754 and other molecular alterations was investigated. *FLT3/ITD*, *NPM1*, and *CEBPA* mutations occurred at comparable frequencies in patients with and without the *WT1* SNP (Table 1). Compared with the SNP-negative patients, who had a *WT1* mutation prevalence of 10.4%, *WT1* mutations were identified in only 3.2% of those with the SNP ($P = .002$). Further evaluation demonstrated that none of the 38 patients homozygous for SNP rs16754 had a concomitant *WT1* mutation (Fig 1).

The proportion of SNP-positive and SNP-negative patients was similar when they were stratified into high-risk (-7 , $-5/\text{del}(5q)$, *FLT3/ITD* with high allelic ratio), low-risk ($t(8,21)$ $\text{inv}(16)/t(16,16)$ *NPM1*, or *CEBPA* mutations) or standard-risk groups (all other patients). Minimal residual disease data from the end of course 1, assessed by multidimensional flow cytometry, were available from 184 patients enrolled on AAML-03P1. Minimal residual disease higher than 0.01% was detected in 13 (24%) of 55 SNP-positive patients, compared with 40 (31%) of 129 SNP-negative patients ($P = .405$).

Table 1. Characteristics of Patients With and Without *WT1* SNP rs16754

Characteristic	SNP (n = 229)		No SNP (n = 561)		P
	No.	%	No.	%	
Sex					
Male	129	56.3	294	52.4	.355
Female	100	43.7	267	47.6	
Age, years					
Median		9.2		10.6	.167
Range		0.01-20.8		0.15-21.63	
0-2	65	28.4	126	22.5	.094
3-10	67	29.3	170	30.3	.837
11-21	97	42.4	265	47.2	.242
Race					
White	119	53.6	387	69.9	< .001
Black	18	8.1	65	11.7	.178
Hispanic	54	24.3	76	13.7	< .001
Asian	19	8.6	10	1.8	< .001
Other	12	5.4	16	2.9	.137
Unknown	7		7		
Median WBC, $\times 10^9/L$		20.4		22.3	.575
Range		1.0-600		0.3-860	
Bone marrow blasts, %		69		70.8	.258
Range		0-00		0-100	
Median platelets, $\times 10^3/\mu L$		46		48	.629
Range		2-610		3-800	
Median hemoglobin, μ/dL		8.1		8.2	.797
Range		2.1-30.7		0.4-38.6	
FAB classification					
M0	12	5.6	24	4.5	.656
M1	35	16.4	89	16.8	.992
M2	63	29.6	147	27.7	.679
M4	45	21.1	145	27.4	.095
M5	42	19.7	90	17.0	.437
M6	6	2.8	11	2.1	.589
M7	10	4.7	24	4.5	.924
Other/no data	16		31		
Cytogenetics					
Normal	38	23.8	87	21.9	.710
t(8;21)	29	18.1	55	13.8	.248
inv(16)	10	6.3	50	12.6	.043
11q23 abnormality	29	18.1	89	22.4	.320
t(6;9)(p23;q34)	2	1.3	9	2.3	.737
-7/7q-	8	5.0	12	3.0	.374
-5/5q-	5	3.1	3	0.8	.047
+8	13	8.1	31	7.8	.967
+21	1	0.6	3	0.8	1.000
Pseudodiploid	11	6.9	22	5.5	.681
Hyperdiploid	1	0.6	8	2.0	.458
Hypodiploid	2	1.3	3	0.8	.628
Other	11	6.9	26	6.5	.883
Unknown	69		163		
<i>FLT3</i> /ITD status					
ITD+	28	12.2	66	11.8	.951
ITD-	201	87.8	495	88.2	
Missing	0		0		
<i>CEBPA</i> status					
Mutant	7	3.3	21	4.0	.791
WT	206	96.7	500	96.0	
Missing	16		40		

(continued on following page)

Table 1. Characteristics of Patients With and Without *WT1* SNP rs16754 (continued)

Characteristic	SNP (n = 229)		No SNP (n = 561)		P
	No.	%	No.	%	
<i>NPM1</i> status					
Mutant	14	6.8	43	8.4	.592
WT	191	93.2	471	91.6	
Missing	24		47		
<i>WT1</i> status					
Mutant	7	3.2	56	10.4	.002
Negative	210	96.8	485	89.6	
Missing	12		20		
Risk groups					
Standard	79	46.5	215	49.0	.642
Low	58	34.1	163	37.1	.549
High	33	19.4	61	13.9	.118
Unknown	59		122		
Course 1 response					
CR	177	81.2	438	80.2	.837
Not in CR	41	18.8	108	19.8	
Unevaluable	11		15		
Course 2 response					
CR	161	79.3	398	77.1	.594
Not in CR	42	20.7	118	22.9	
Unevaluable	26		45		

NOTE. Bold font indicates statistical significance.

Abbreviations: WT1, Wilms' tumor 1; SNP, single nucleotide polymorphism; FAB, French-American-British; ITD, internal tandem duplication; WT, wild-type; CR, complete response.

Clinical Outcome and Prognostic Impact of WT1 SNP

Clinical outcome data were examined for the patients with known *WT1* SNP rs16754 status (Fig 2). Patients with or without SNP rs16754 had similar CR rates after one course of induction (81.2% v 80.2%, $P = .837$). Overall survival at 5 years from study entry for SNP-positive patients was 60% (SE [$\pm$] 7%) versus 50% $\pm$ 5% for those without the SNP (HR, 0.76; $P = .031$). The corresponding DFS from CR was 51% $\pm$ 8% and 47% $\pm$ 5% for those with and without SNP rs16754 (HR, 0.86; $P = .415$). For patients who achieved an initial remission ($n = 615$), those with and without the SNP had similar relapse rates (40% $\pm$ 8% v 43% $\pm$ 5%; HR, 0.92; $P = .763$) and similar treatment-related mortality (8% $\pm$ 4% v 11% $\pm$ 3%; HR, 0.79; $P = .442$).

Prognostic Factors

We performed univariate and multivariate Cox regression analysis to evaluate whether presence of the *WT1* SNP as well as other known prognostic factors (ie, cytogenetics, mutations, diagnostic WBC, and race) were predictors of OS and RFS in the entire study cohort (Table 2). Patients with SNP rs16754 had an improved survival with an HR of 0.76 for death from enrollment ($P = .031$), and an HR of 0.92 for relapse from achieving an initial remission ($P = .6$). Patients with high-risk features had an HR of 1.76 for death from diagnosis ($P < .001$) and an HR of 1.91 for increased risk of relapse ($P < .001$), whereas low-risk group assignment was associated with improved survival (HR, 0.48; $P < .001$) and lower risk of relapse (HR, 0.49; $P < .001$). Diagnostic WBC higher than $50 \times 10^9/L$ was a predictor of decreased OS (HR, 1.30; $P = .005$) and higher risk of relapse (HR, 1.26; $P = .047$). Non-white patients had decreased OS (HR, 1.34; $P = .001$) but not significantly reduced RFS (HR, 1.17; $P = .152$).

In the multivariate analysis, *WT1* SNP rs16754 was an independent prognostic marker for improved OS with an HR for death of 0.64 compared with SNP-negative patients ($P = .004$). In this model, HR for relapse from remission for patients with *WT1* SNP was 0.79 compared with their SNP negative counterparts ($P = .197$).

Prognostic Significance of SNP rs16754 in Risk Groups

The prognostic impact of SNP rs16754 was evaluated in specific clinical risk groups (Fig 3). In patients with standard-risk disease (no high or low risk features, $n = 294$), *WT1* SNP was identified in 79 patients (26%). Those with and without SNP rs16754 had identical OS at 5 years from diagnosis of 47% and a similar RR (48% $\pm$ 14% v 45% $\pm$ 9%; $P = .77$) and DFS (45% $\pm$ 14% v 47% $\pm$ 9%; $P = .88$) from remission. *WT1* SNP was identified in 58 patients (26%) with low-risk AML (CBF translocations, *CEBPA* or *NPM1* mutations, $n = 221$). In contrast to standard-risk patients, low-risk patients with SNP rs16754 had an actuarial OS of 90% $\pm$ 8%, versus 64% $\pm$ 9% for low-risk SNP-negative patients (HR, 0.27; $P = .001$). In low-risk patients who achieved an initial remission, DFS at 5 years from remission for SNP-positive patients was 72% $\pm$ 13% versus 53% $\pm$ 10% for SNP-negative patients ($P = .041$), with a corresponding RR of 21% $\pm$ 12% versus 41% $\pm$ 9% (HR, 0.49; $P = .179$) compared with their SNP-negative low-risk counterparts.

In patients with high-risk disease (-7 , $-5/del5q$ or high risk FLT3/ITD, $n = 94$), the *WT1* SNP was identified in 33 patients (35%). Actuarial OS at 5 years from diagnosis was 41% $\pm$ 22% for SNP-positive patients versus 21% $\pm$ 12% for SNP-negative patients ($P = .083$). In high-risk patients who achieved an initial remission, DFS at 5 years from remission for SNP-positive patients was 30% $\pm$

Table 2. Univariate and Multivariate Cox Regression Analysis of *WT1* SNP rs16754 and Other Validated Risk Factors

Analysis	No.	OS From Study Entry			RFS From End of Course 1		
		HR	95% CI	<i>P</i>	HR	95% CI	<i>P</i>
Univariate							
<i>WT1</i> SNP rs16754							
SNP negative	561	1			1		
SNP positive	229	0.76	0.59 to 0.98	.031	0.92	0.68 to 1.25	.605
Risk group							
Standard	522	1			1		
Low	305	0.48	0.37 to 0.63	< .001	0.49	0.37 to 0.66	< .001
High	123	1.76	1.34 to 2.29	< .001	1.91	1.36 to 2.68	< .001
WBC, ×10 ⁹ /L							
≤ 50	939	1			1		
> 50	388	1.30	1.08 to 1.56	.005	1.26	1.00 to 1.58	.047
Race							
White	842	1			1		
Non-white	452	1.34	1.13 to 1.60	.001	1.17	0.94 to 1.46	.152
Multivariate							
<i>WT1</i> SNP rs16754							
SNP negative	439	1			1		
SNP positive	170	0.64	0.47 to 0.86	.004	0.79	0.56 to 1.13	.197
Risk group							
Standard	294	1			1		
Low	221	0.44	0.32 to 0.61	< .001	0.52	0.36 to 0.75	< .001
High	94	1.47	1.06 to 2.02	.020	2.22	1.48 to 3.33	< .001
WBC							
≤ 50	404	1			1		
> 50	205	1.31	1.01 to 1.71	.044	1.15	0.83 to 1.60	.406
Race							
White	386	1			1		
Non-white	210	1.49	1.14 to 1.94	.003	1.35	0.98 to 1.87	.071

NOTE. Bold font indicates statistical significance.

Abbreviations: WT1, Wilms' tumor 1; SNP, single nucleotide polymorphism; OS, overall survival; RFS, relapse-free survival; HR, hazard ratio.

22% versus $10\% \pm 11\%$ for SNP-negative patients ($P = .273$), with a corresponding RR of $60\% \pm 23\%$ versus $78\% \pm 15\%$ ($P = .179$) compared with their SNP-negative counterparts.

We further evaluated the prognostic significance of SNP rs16754 in patients with normal karyotype. Of the 125 patients without cyto-

genetic abnormalities, 38 had SNP rs16754 (30%). Overall survival at 5 years from study entry for patients with and without WT1 SNP was $45\% \pm 18\%$ versus $39\% \pm 12\%$ ($P = .522$). In patients who achieved an initial remission, DFS at 5 years from remission for SNP-positive patients was $51\% \pm 20\%$ versus $41\% \pm 14\%$ for SNP-negative patients ($P = .273$), with a corresponding RR of $46\% \pm 20\%$ versus $47\% \pm 14\%$ ($P = .882$) compared with their SNP-negative counterparts.

Within the SNP-positive group, homozygous ($n = 38$) and heterozygous ($n = 191$) patients had similar 5-year OS ($61\% \pm 8\%$ v $56\% \pm 18\%$, $P = .960$), DFS ($46\% \pm 8\%$ v $41\% \pm 17\%$, $P = .720$), and RR ($38\% \pm 9\%$ v $50\% \pm 20\%$, $P = .312$).

SNP rs16754 Is Present in the mRNA Transcript

Because the presence of this synonymous SNP imparts prognostic significance, we sought to determine a potential mechanism by which a silent polymorphism may be translated into a functional alteration. Differential RNA editing is one mechanism by which a silent genomic mutation may create a functional alteration in the pre-mRNA transcript, leading to a change in the protein product. An example of RNA editing has been previously described in codon 280 of *WT1*.¹² To determine whether differential RNA editing might occur as a result of the synonymous SNP rs16754, we sequenced *WT1* exon 7 from cDNA transcripts obtained from 50 patients at random from

Fig 1. Prevalence of Wilms' tumor 1 (*WT1*) mutation in patients with homozygous and heterozygous single nucleotide polymorphism (SNP) rs16754 compared with *WT1* wild-type patients.

Fig 2. Prognostic significance of Wilms' tumor 1 single nucleotide polymorphism (SNP) rs16754 mutations in pediatric acute myeloid leukemia. Clinical outcome for patients with and without SNP rs16754. Kaplan-Meier estimates of (A) overall survival and (B) disease-free survival are shown. (C) Cumulative incidence of relapse is also shown stratified by SNP status.

COG AAML-03P1. Of the 50 patients, 13 (26%) had at least 1 minor allele of the *WT1* SNP; 4/13 SNP-positive patients were homozygous for the SNP. Correlation of genotyping data performed on genomic DNA with these cDNA data showed identical sequences, demonstrating that RNA editing is not involved in the pathogenesis of AML in regard to SNP rs16754.

SNP rs16754 Is Associated With Elevated mRNA Expression

WT1 is highly expressed in most patients with AML.¹³ We questioned whether patients with SNP rs16754 may have an altered *WT1*

expression level. *WT1* expression levels were evaluated by quantitative reverse-transcriptase PCR in 114 unselected patient samples from COG-AAML03P1 with known *WT1* SNP rs16754 (SNP positive, n = 30) and *WT1* mutation (mutation positive, n = 13) status (Fig 4). *WT1* expression levels for each sample were normalized to the expression in normal marrow control. *WT1* expression was highly variable, ranging from 0.00- to 2949.42-fold normal marrow expression (median 70.4-fold normal bone marrow). Eleven patients did not have detectable *WT1* expression (10%) and 97 patients (85%) had *WT1* expression higher than observed in normal marrow controls.

Fig 3. Prognostic impact of Wilms' tumor 1 single nucleotide polymorphism (SNP) rs16754 in specific clinical risk groups. Estimates of the probability of (A, D, G) overall survival, (B, E, H) disease-free survival, and relapse risk (C, F, I) for (A, B, C) low-risk, (D, E, F) standard-risk, and (G, H, I) high-risk acute myeloid leukemia.

Fig 4. Levels of Wilms' tumor 1 (*WT1*) expression by single nucleotide polymorphism (SNP) and mutation status (Mut). Median expression within each cohort is indicated by the dashed line. NBM, normal bone marrow.

Median *WT1* expression in *WT1* wild-type patients (without SNP rs16754 or a *WT1* mutation, $n = 71$) was 40.61 times that of normal marrow controls (range, 0.00 to 2949.42), whereas the median expression in patients with the *WT1* SNP ($n = 30$) was 215.76 times that of normal marrow control (range, 0.00 to 1,399.23; $P = .0214$). We further compared *WT1* expression levels in those with and without *WT1* mutations. Of the 114 patient samples tested, 13 (11.4%) were positive for a *WT1* mutations (12 exon 7 mutations, one exon 8 mutation) and only one patient harbored both SNP rs16754 and a *WT1* mutation. Median *WT1* expression level for this cohort was 327.14-fold normal marrow control (range, 54.17- to 902.3-fold; $P = .0005$) compared with the *WT1* wild-type cohort.

DISCUSSION

In this study, we identified *WT1* SNP rs16754 in 29% of childhood patients with AML, the presence of which was either directly ($-5/\text{del}5q$) or inversely ($\text{inv } 16$, *WT1* mutation) associated with specific disease characteristics. Further, rs16754 was disproportionately distributed across ethnic groups, with higher prevalence in Asian and Hispanic patients. More importantly, despite being a silent polymorphism, the presence of this SNP highly correlated with improved disease outcome. The protective impact of this polymorphism appears to be most pronounced in patients with favorable risk features.

The elucidation of possible mechanisms by which a synonymous SNP may be biologically significant is an important pursuit. Such a SNP may be commonly inherited as part of a haplotype and exist in linkage disequilibrium with yet-unidentified, disease-associated molecular markers. Studies are ongoing to identify possible disease-associated alterations that may exist in linkage disequilibrium with SNP rs16754. Alternatively, a synonymous SNP may lead to alterations in a microRNA binding site, alternative splicing, protein folding, or mRNA expression. In the case of the *MDR1* gene, Kimchi-Sarfaty et al¹⁴ showed that a synonymous SNP substituting a rare codon for a common codon encoding the same amino acid results in decreased function of the encoded P-glycoprotein. As the tRNA pool for each codon is proportional to how frequently that codon is uti-

lized, replacing a commonly used codon with a rare codon (or vice versa) may directly impact translation kinetics of the protein in question. Altering translation kinetics is known to affect protein folding, resulting in altered protein function. In the case of *WT1* SNP rs16754, the minor allele results in a CGA>CGG transversion, which represents a change from a rare (CGA, 6.2 per thousand) codon encoding arginine to a more frequently used codon (CGG, 11.4 per thousand; frequencies obtained from the Codon Usage Database¹⁵). Substitution of a rare codon for a more frequently used codon leads to increased translation kinetics, which can also have functional protein consequences, as has been demonstrated in vitro in *E coli*.¹⁶

It is notable that the incidence of *WT1* zinc-finger mutations was lower in SNP-positive patients than in their SNP-negative counterparts. Further, such mutations occurred only in heterozygous patients; none of the homozygous SNP-positive patients also had a concomitant *WT1* mutation. Although the mechanism is unclear, if further validated, it is intriguing to speculate that harboring the *WT1* SNP rs16754 may render unnecessary the acquisition of a *WT1* mutation during myeloid leukemogenesis.

In multivariate analysis, SNP rs16754 is an independent predictor of improved OS. This *WT1* SNP may be in linkage disequilibrium with a haplotype of another gene which affects response to chemotherapy. Survival differences were most pronounced in patients assigned to the low-risk group, with SNP-positive patients having an excellent OS of 90%. However, the prognostic utility of SNP status in this risk group is uncertain, as SNP-negative low-risk patients have better survival outcomes than standard-risk patients. Likewise, although SNP-positive high-risk patients trended toward improved outcomes compared with SNP-negative high-risk patients, their outcomes were still inferior to those of standard-risk patients. Thus, on the basis of SNP status alone, we can make no recommendation for change in risk stratification of patients with AML. Of note, the presence of SNP rs16754 had no impact on survival outcomes in pediatric AML patients currently assigned to the standard-risk group.

We also found that median expression levels of *WT1* in patients with SNP rs16754 were significantly higher than those in patients with wild type *WT1*. SNP rs16754 is a putative cis-acting regulatory SNP; such SNPs may regulate expression levels of their respective genes, contributing to allelic imbalance.¹⁷ Although its role in leukemogenesis is unclear, the frequency and degree of *WT1* overexpression in AML suggests that this expression can be used both as target of immunotherapy, as well as a potential marker of minimal residual disease.¹³ Identifying a population with more favorable outcome within the subset of patients with high *WT1* expression may help in refining the utility of this parameter in response evaluation. Thus, *WT1* mutation and *WT1* SNP status should be prospectively evaluated alongside *WT1* expression in future pediatric AML trials.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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