

Prognostic impact of *WT1* mutations in cytogenetically normal acute myeloid leukemia: a study of the German-Austrian AML Study Group

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To evaluate the incidence and clinical impact of *WT1* gene mutations in younger adult patients with cytogenetically normal acute myeloid leukemia (CN-AML), sequencing of the complete coding region was performed in diagnostic samples from 617 patients who were treated on 3 German-Austrian AML Study Group protocols. *WT1* mutations were identified in 78 (12.6%) of the 617 patients; mutations clustered in exon 7 (54 of 78) and exon 9 (13 of 78), but also occurred in exons 1, 2,

3, and 8. *WT1* mutations were significantly associated with younger age, higher serum lactate dehydrogenase levels, higher blood blast counts, and the additional presence of *FLT3*-ITD ($P < .001$) and *CEBPA* mutations ($P = .004$). There was no difference in relapse-free survival and overall survival between patients with (*WT1*^{mut}) or without *WT1* mutations. Subset analysis showed that patients with the genotype *WT1*^{mut}/*FLT3*-ITD^{pos} had a lower complete remission rate ($P = .003$) and

an inferior relapse-free survival ($P = .006$) and overall survival ($P < .001$) compared with those with the genotype *WT1*^{mut}/*FLT3*-ITD^{neg}. In conclusion, in our large cohort of younger adults with CN-AML, *WT1* mutation as a single molecular marker did not impact on outcome. However, our data suggest a negative impact of the genotype *WT1*^{mut}/*FLT3*-ITD^{pos}. (*Blood*. 2009;113:4505-4511)

Introduction

In the last decade, somatically acquired mutations have been identified in several genes in cytogenetically normal acute myeloid leukemia (CN-AML): the *nucleophosmin 1* (*NPM1*) gene, the *fms-related tyrosine kinase 3* (*FLT3*) gene, the *CCAAT/enhancer binding protein alpha* (*CEBPA*) gene, the *myeloid-lymphoid or mixed-lineage leukemia* (*MLL*) gene, the *neuroblastoma RAS viral oncogene* homolog (*NRAS*) gene, and the *runt-related transcription factor 1* (*RUNX1*) gene.^{1,2} Besides providing insights into leukemia biology, some of these markers have proved to be important for disease classification and outcome prediction.^{3,4} Thus, the discovery of novel genetic changes probably will contribute to a refined molecular classification of the disease. Recently, Summers et al⁵ revived the initial findings on *WT1* mutations in AML that were first reported by King-Underwood et al in 1996.⁶

The *Wilms' tumor 1* gene (*WT1*), cloned in 1990, is located at chromosomal band 11p13.⁷ Its corresponding protein consists of 4 C-terminal Zinc (Zn) fingers (exons 7-10) that are characteristic for transcription factors, whereas the N-terminal domain (exons 1-6), which contains proline-glutamine-rich sequences has been shown to be involved in RNA and protein interactions.^{8,9} *WT1* functions as a potent transcriptional regulator for genes involved in cellular growth and metabolism, such as extracellular matrix components, growth factors, and other transcription factors.¹⁰

Genomic deletions affecting the *WT1* gene were first identified in patients with WAGR syndrome characterized by Wilms tumor (WT), aniridia, genitourinary malformations, and mental retardation. These patients were found to have constitutional deletions of

11p13.¹¹ Twelve years later, a *WT1* mutation was described in a sporadic, unilateral WT as a heterozygous 25-bp deletion that results in loss of one of the 4 Zn finger consensus domains.¹² Subsequent studies revealed *WT1* mutations as the causative gene alteration in sporadic WT occurring with an incidence of 10% to 15%. Interestingly, in addition to the WAGR syndrome, *WT1* germline mutations have also been found in other distinct constitutional syndromes, such as Denys-Drash syndrome and Frasier syndrome, which have a predisposition for the development of WT.^{6,13,14}

The initial finding of a *WT1* mutation in a secondary AML of a WAGR patient⁶ was confirmed and extended by 2 consecutive studies in which 67 samples of different types of leukemia (AML, acute lymphoblastic leukemia, biphenotypic leukemia, undifferentiated leukemia) were analyzed.^{15,16} The overall incidence of *WT1* mutations was 12%, with 4 of 33 AML patients exhibiting *WT1* mutations.^{6,16} None of the patients with *WT1* mutation achieved complete remission (CR), and disease-free survival (DFS) and overall survival (OS) appeared to be worse. In a more recent study performed by Summers et al, *WT1* mutations were identified in 7 of 70 CN-AML patients.⁵ Of note, 6 of the 7 patients also had *FLT3* internal tandem duplications (ITDs), and all 6 patients were refractory to induction therapy.

Although its role in hematopoiesis is still not clarified, disruption of *WT1* function is currently considered to promote stem cell proliferation and to hamper cell differentiation, the hallmarks of the 2 complementation groups of gene mutations that have been

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postulated for the development of AML.^{8,10,17,18} In addition, *WT1* expression is normally restricted to the primary cells of the developing genitourinary system and to the hematopoietic progenitor cells. However, in AML, *WT1* expression can be found in 75% to 100% of AML patients.

The aim of this study was to evaluate the incidence and clinical impact of *WT1* mutations in the context of other known gene mutations in a large cohort of younger adult patients with CN-AML who were treated within 3 consecutive treatment trials of the German-Austrian AML Study Group (AMLSG).

Methods

Patients and treatment

Diagnostic bone marrow (BM) or peripheral blood (PB) samples were analyzed from 617 younger adult patients (16-60 years) with CN-AML. Patients had been entered onto 3 consecutive multicenter AMLSG treatment trials: AML HD93 (n = 77),¹⁹ AML HD98-A (n = 268; NCT00146120),²⁰ and AMLSG 07-04 (n = 272; NCT00151242).²¹ 499 patients had de novo AML, 60 had secondary AML after myelodysplastic syndrome, 12 had treatment-related AML, and in 46 patients this information was missing. For the present study, the only criterion used to include patients was the availability of a BM or PB sample from diagnosis for gene mutation analysis. All patients gave informed consent for both treatment and genetic analysis in accordance with the Declaration of Helsinki; all studies were approved by the institutional review board of the University of Ulm. All patients received intensive double-induction and consolidation therapy. All 3 trials included a genetic randomization for allogeneic transplantation from a matched related donor. Details of the treatment plans, including dosages of cytotoxic drugs, are given in Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Cytogenetic and molecular genetic analyses

Pretreatment samples from all patients were studied centrally by chromosome banding analysis. To improve the accuracy of cytogenetic diagnosis, specimens were also analyzed by fluorescence in situ hybridization or polymerase chain reaction (PCR) for the presence of the recurring gene fusions *RUNX1-RUNX1T1*, *CBFB-MYH11*, *MLL-MLLT3*, and *PML-RARA*.⁴

Molecular diagnostics included mutation analyses of the *FLT3* (ITDs and tyrosine kinase domain [TKD] mutations at codon D835 and I836), *NPM1*, *CEBPA*, *MLL* (partial tandem duplication [PTDs]), and *NRAS* genes.⁴

Analysis of *WT1* mutations

Genomic DNA was isolated from mononuclear cells stored at -80°C using the All Prep Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. PCR of the whole coding region of the *WT1* gene (exons 1-10) was carried out using intron-exon flanking primer pairs (Table S1). Because of GC-rich sequences of exon 1, Hot Star Plus polymerase (QIAGEN) was used for DNA amplification; for the remaining exons 2 to 10, Platinum Taq polymerase (Invitrogen, Karlsruhe, Germany) was used. The total reaction volume of 25 μL contained approximately 100 ng DNA, 10 pmol of each primer, deoxynucleoside triphosphates (10 mM each), and Hot Star Plus polymerase or Platinum Taq polymerase with supplied buffers. DNA was amplified using the following PCR conditions: 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, 57°C for 30 seconds, and 72°C for 30 seconds; and finally 72°C for 7 minutes. PCR products were purified by standard methods and subsequently sequenced with M13 forward primer using the ABI Ready Reaction Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany).

In addition, PB samples from 38 healthy volunteers were analyzed for the presence of *WT1* polymorphisms. In 26 of the 78 mutated AML cases,

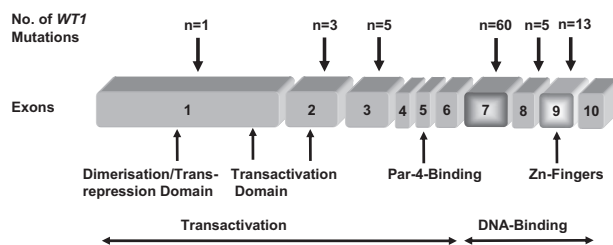


Figure 1. Structure of the *WT1* gene and localization of the 87 mutations.

DNA obtained from buccal swabs or remission material from patients in complete remission was available for *WT1* germline mutation analysis.

Statistical analyses

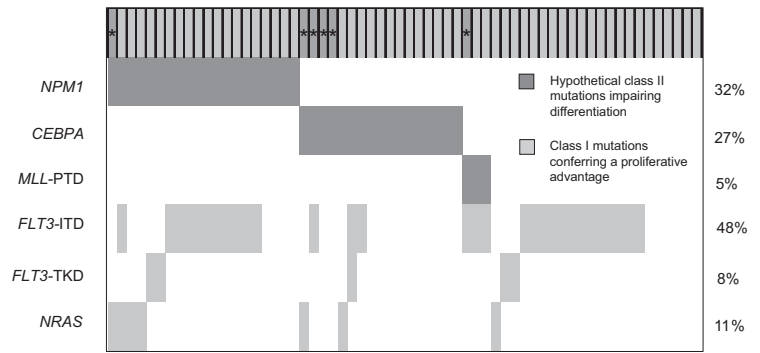
The median follow-up for survival was calculated according to the method of Korn.²² The definition of CR followed the recommended criteria.²³ OS endpoints, measured from entry into one of the prospective studies, were death (failure) and alive at last follow-up (censored).²³ Relapse-free survival (RFS) endpoints, measured from the date of documented CR, were relapse (failure), death in CR (failure), and alive in CR at last follow-up (censored).²³ Comparisons between patient characteristics (covariates) were performed by Kruskal-Wallis test for continuous variables and by Fisher exact test for categorical variables. A multivariable logistic model was used to analyze associations between presenting features and response to induction therapy. The Kaplan-Meier method was used to estimate the distribution of RFS and OS. Confidence interval estimation for the survival curves was based on the cumulative hazard function using Greenwood's formula for the standard error (SE) estimation.²⁴ A Cox model was used to identify prognostic variables.²⁵ Missing data were estimated using a multiple-imputation technique using predictive mean matching with n equals 50 for multiple imputations.²⁶ All statistical analyses were performed with the use of the R package (version 2.0-12) of the R statistical software platform (version 2.4.1).²⁷

Results

WT1 mutations

Overall, 87 *WT1* mutations were identified in 78 of the 617 (12.6%) patients (Table S2). Mutations clustered in exon 7 (n = 60) and exon 9 (n = 13) but also occurred in exons 1 (n = 1), 2 (n = 3), 3 (n = 5), and 8 (n = 5) (Figure 1). In exon 7, most of the mutations were frameshift mutations as a result of insertions (n = 39) or deletions (n = 10), ranging in length from 1 to 28 bp; 11 mutations were substitutions, in 2 of them, serine was replaced by a stop codon (S312X); 3 were synonymous mutations and 6 were missense mutations. In exon 9, most mutations (n = 11) were substitutions, with 3 of them causing a stop codon (R390X); the other 8 were missense mutations; 2 patients had an insertion leading to a frameshift. Fourteen mutations did not affect exon 7 and 9: one missense substitution in exon 1; 3 substitutions in exon 2 (2 cases with a stop codon, one missense mutation); one substitution (with a stop codon), one deletion and 3 insertions in exon 3; and 4 substitutions (one with a stop codon, 3 missense mutations) and one insertion in exon 8. Types of exon 7 and 9 mutations were similar to those recently described by King-Underwood et al,^{6,15,16} Virappane et al,²⁸ and Paschka et al.²⁹ The mutation P181S in exon 2 was previously described by Köhler et al as a germline missense mutation in a male patient with ambiguous genitalia, normal testosterone production, absence of kidney disease, and associated heart defect.³⁰ Three mutations (2 polymorphisms and 1 insertion, which resulted in a truncated protein) in exon 1 have already been

Figure 2. Frequencies and distribution of the WT1, NPM1, CEBPA, MLL, FLT3, and NRAS mutations. For WT1 mutations, each patient is represented by a gray column. Dark gray columns marked with * represent homozygous mutations. ITD denotes internal tandem duplication; PTD, partial tandem duplication; and TKD, mutation of the tyrosine kinase domain.



described by King-Underwood earlier,⁶ but these were different from mutation P13T found by us. Seventy-two patients had heterozygous and 6 patients had homozygous mutations. In all 6 patients, both alleles carried the same mutation, a finding that is consistent with acquired uniparental disomy. Data from single nucleotide polymorphism analysis were available in 2 of the 6 patients revealing acquired 11p uniparental disomy in both cases (data not shown). Three patients exhibited 2 mutations, 2 with mutations in exon 3 and exon 7, and 1 patient with mutations in exon 7 and exon 9.

None of the 38 PB samples obtained from healthy volunteers revealed WT1 sequence variations compared with the WT1 wild-type sequence (NCBI; cDNA NM_0024426; www.ncbi.org). No WT1 germline mutations were detected in the 26 cases in which DNA from buccal swabs, PB, or BM samples in CR was available.

Association with other molecular markers

WT1 mutations (WT1^{mut}) were significantly associated with the presence of FLT3-ITD ($P < .001$; marker constellation analyzed in 612 patients) and CEBPA mutations ($P = .004$; marker constellation analyzed in 552 patients); 41 of 78 (53%) WT1^{mut} patients also harbored a FLT3-ITD compared with 171 of 534 (32%) WT1^{wt} patients, and 17 of 72 (24%) WT1^{mut} patients had additional CEBPA mutations compared with 51 of 480 (11%) WT1^{wt} patients. The median of the FLT3-ITD mutant/wild-type ratio in WT1^{mut} patients was 0.53 (range, 0.02-4.10) compared with 0.51 in WT1^{wt} patients (range, 0.006-14.2, Wilcoxon test, $P = .65$). In contrast, there was an inverse relationship between the presence of WT1 mutations and NPM1 mutations: 24 of 75 (32%) WT1^{mut} patients had NPM1 mutation, whereas 286 of 523 (55%) WT1^{wt} patients had an additional NPM1 mutation ($P < .001$). There was no significant association with regard to FLT3-TKD mutations ($P = .66$), MLL-PTD ($P = .64$), or NRAS mutations ($P = .30$; Figure 2).

Patient characteristics by WT1 mutation status

WT1 mutations were significantly associated with younger age ($P = .007$), higher lactate dehydrogenase (LDH) serum levels ($P = .007$), and higher PB blast counts ($P = .01$). Of note, LDH and PB blast counts were significantly associated with the combined mutation status WT1^{mut}/FLT3-ITD^{pos}, in that highest values were present in double-positive cases (median LDH level, 755.5 U/L; median blast count, 71%) followed by cases harboring FLT3-ITD (559.5 U/L; 55%) or WT1^{mut} (422 U/L; 35%); lowest values were found in patients with the WT1^{wt}/FLT3-ITD^{neg} genotype (362 U/L; 27%; $P = .007$ and $P = .02$ for overall comparison, respectively). Patients' characteristics according to WT1 and FLT3-ITD mutation status are given in Table 1.

Response to induction therapy

In univariable analysis, there was no difference in the rates of CR (77% vs 79%), refractory disease (RD; 19% vs 14%), and early death (4% vs 7%) between WT1^{mut} and WT1^{wt} patients, respectively.

Based on the previous observation that the concurrent presence of WT1 mutation and FLT3-ITD may be associated with induction failure⁵ and based on our findings showing that patients with the genotype WT1^{mut}/FLT3-ITD^{pos} appeared to have a high proliferative activity indicated by high blood blast counts and high LDH serum levels, a subset analysis was performed exploring the impact of an additional FLT3-ITD in patients with WT1 mutations. Patients with the genotype WT1^{mut}/FLT3-ITD^{pos} (n = 40) had a significantly lower CR rate (63%) and a significantly higher RD rate (33%) compared with patients (n = 37) with WT1 mutations in the absence of FLT3-ITD (92% and 5%; $P = .003$ and $P = .003$, respectively). Patients with the WT1^{wt}/FLT3-ITD^{pos} (n = 169) and WT1^{wt}/FLT3-ITD^{neg} (n = 357) genotypes had comparable CR rates

Table 1. Patient characteristics according to the WT1 and FLT3-ITD mutation status

Characteristic	WT1 ^{wt} /FLT3-ITD ^{neg}	WT1 ^{mut} /FLT3-ITD ^{neg}	WT1 ^{wt} /FLT3-ITD ^{pos}	WT1 ^{mut} /FLT3-ITD ^{pos}	P
Sex, no. female/no. male	178/185	23/14	104/67	22/19	.05
Type of AML, n*					.35
De novo	288	29	146	33	
s-AML	43	3	10	2	
t-AML	9	0	3	0	
Missing	23	5	12	6	
Median age, y (range)	49.4 (16-60.9)	41.9 (23-59.8)	47.2 (18.3-60.9)	46.4 (19.4-58.8)	.007
Median WBC count, ×10 ⁹ /L (range)	13.5 (0.2-369)	13.9 (0.2-369)	39.2 (0.2-345)	30.1 (1.1-220)	.22
Median PB blasts, % (range)	27 (0-99)	35 (0-98)	55 (0-100)	71 (1-96)	.01
Median BM blasts, % (range)	70 (0-100)	72 (7-96)	87 (2-100)	88 (20-100)	.14
Median LDH, U/L (range)	362 (89-2371)	422 (131-4566)	559.5 (122-4091)	755.5 (184-2992)	.007

WBC denotes white blood cell; PB, peripheral blood; BM, bone marrow; LDH, lactate dehydrogenase (normal value < 250 U/L); s-AML, secondary AML; and t-AML, therapy-associated AML.

*Status on FLT3-ITD missing in 3 de novo AML and in 2 secondary AML after myelodysplastic syndrome.

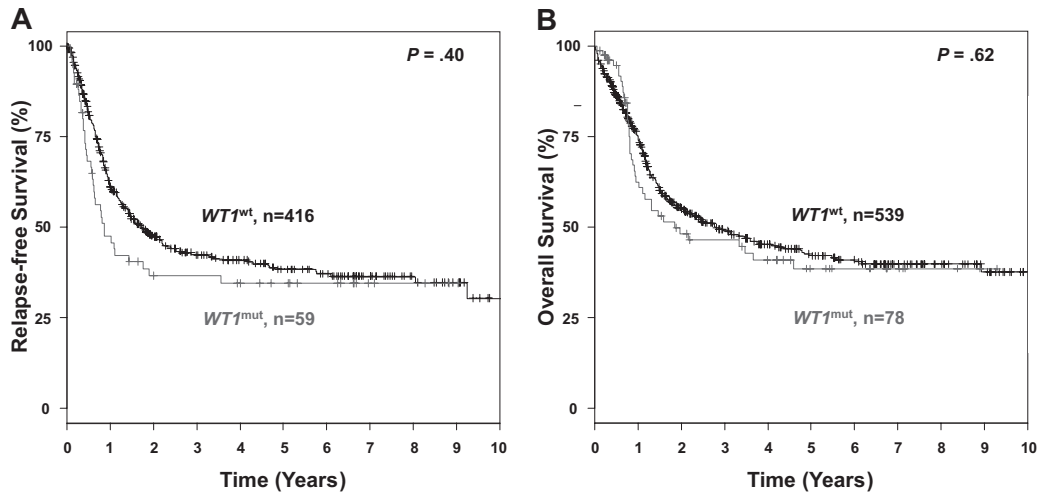


Figure 3. Kaplan-Meier survival estimates, according to *WT1* mutation status. Data are shown for relapse-free survival (A) and overall survival (B).

(78% and 79%, respectively) and RD rates (6.5% and 8%, respectively; missing values for response evaluation $n = 9$).

As a consequence of these findings, we performed an explorative multivariable logistic regression analysis, including the variables *FLT3*-ITD and *WT1*^{mut} as single markers as well as with an interaction term. This model revealed *CEBPA*^{mut} (odds ratio [OR], 2.67; $P = .02$) and *NPM1*^{mut} (OR, 2.13; $P = .001$) to be significantly associated with response to induction therapy, whereas logarithm of white blood cell (WBC; OR, 0.63; $P = .009$) and the genotype *WT1*^{mut} with concurrent *FLT3*-ITD^{pos} (OR 0.19; $P = .03$) were significantly associated with induction failure.

Survival analysis

The median follow-up time for survival was 44 months. In univariable analysis, there was no difference in RFS ($P = .40$) and OS ($P = .62$) between the *WT1*^{mut} and the *WT1*^{wt} groups (Figure 3). Results for survival were very similar when only exon 7 and 9 mutations were considered, or when only de novo AML were included in statistical analyses. When considering only those patients who received either repetitive cycles of high-dose cytarabine or autologous stem cell transplantation for consolidation and excluding those who received allogeneic transplantation, again, no difference in RFS ($P = .23$) or OS was found ($P = .46$). Subset

analysis according to the *FLT3*-ITD status (excluding patients who received an allogeneic transplantation) revealed a significantly inferior RFS ($P = .006$) and OS ($P < .001$) for patients with the genotype *WT1*^{mut}/*FLT3*-ITD^{pos} compared with patients with the genotype *WT1*^{mut}/*FLT3*-ITD^{neg} (Figure 4).

Multivariable analysis on the endpoints RFS and OS revealed a significant impact of the genotypes *NPM1*^{mut}/*FLT3*-ITD^{neg} (hazard ratio [HR], 0.45 and 0.52, respectively), *CEBPA*^{mut} (HR, 0.51 and 0.39, respectively), *MLL*-PTD (HR, 1.57 only for RFS), age (10 years difference, HR 1.2 and 1.3), and logarithm of WBC (HR 1.55 and 1.58, respectively). *WT1* mutations had no significant impact.

Discussion

WT1 mutations in acute leukemia were indeed reported more than 10 years ago,⁶ but it is only recently that these mutations received attention again.⁵ In this study, we assessed the incidence and prognostic significance of *WT1* mutations in a very large cohort of younger adult patients with CN-AML who were prospectively enrolled into 3 AMLSG protocols.

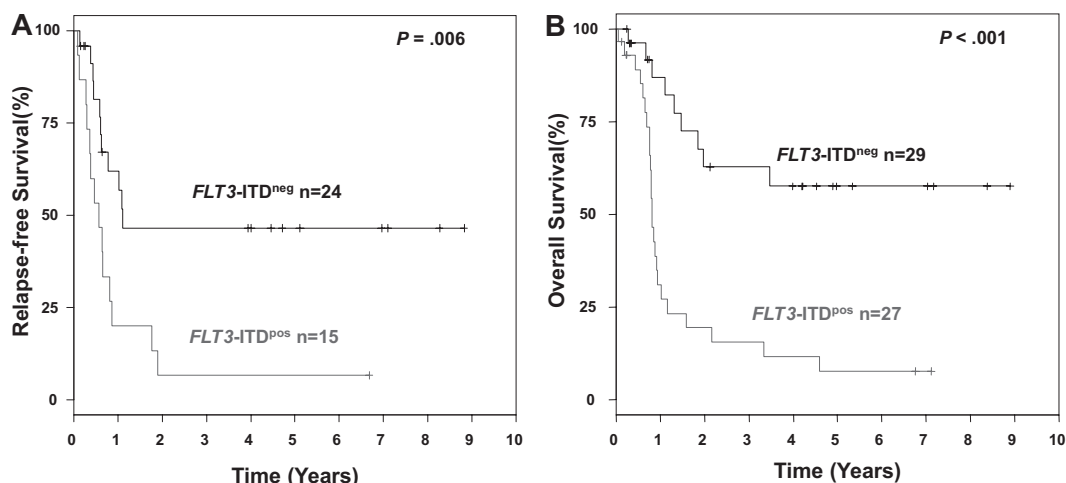


Figure 4. Kaplan-Meier survival estimates for patients with *WT1* mutation, according to *FLT3*-ITD mutation status. Data are shown for relapse-free survival (A) and overall survival (B). Patients after allogeneic stem cell transplantation have been excluded from this analysis.

Table 2. Comparison of the WT1 mutation studies performed by AMLSG, CALGB, and MRC

	AMLSG	CALGB	MRC
Patients, n	617	196	470
Type of AML	De novo, s-AML, t-AML	De novo	94% de novo AML
Age, y	16-60	18-60	15-60; 13 patients > 60
Median follow-up time, mo	33	50.4	122.4 (range, 27-213)
Trials	AML HD93, AML HD98-A, AMLSG 07-04	9621, 19808	MRC10, MRC12
Exons	1-10	7 and 9	7 and 9
No. of mutated patients	78/617 (13%)	21/196 (11%)	47/470 (10%)
Associated mutations (<i>WT1</i> ^{mut})	<i>FLT3</i> -ITD (<i>P</i> = .001) <i>CEBPA</i> ^{mut} (<i>P</i> = .004) <i>NPM1</i> ^{mut} (inverse, <i>P</i> = .001)	<i>FLT3</i> -ITD (<i>P</i> = .06)	<i>FLT3</i> -ITD (<i>P</i> = .08) <i>NPM1</i> ^{mut} (inverse, <i>P</i> = .05)
Clinical characteristics (<i>WT1</i> ^{mut})	↓ age (<i>P</i> = .007) ↑ LDH serum levels (<i>P</i> = .007) ↑ PB blast counts (<i>P</i> = .01)	↑ WBC counts (<i>P</i> = .01)	No difference in age, sex, type of AML, WBC count
Response to induction (<i>WT1</i> ^{mut})	No difference in rate of CR, RD, and ED	No difference in rate of CR	Inferior response to induction therapy (<i>P</i> = .02)
Survival analysis (<i>WT1</i>^{mut})			
Univariable analysis	No difference in RFS and OS	↓ DFS (<i>P</i> < .001), ↓ OS (<i>P</i> < .001)	↓ RFS (<i>P</i> = .005), ↓ OS (<i>P</i> = .007)
Multivariable analysis	No difference in RFS and OS	↓ DFS (<i>P</i> = .009), ↓ OS (<i>P</i> < .001)	↓ RFS (<i>P</i> < .02), ↓ OS (<i>P</i> < .04)
Explorative subset analysis	<i>WT1</i> ^{mut} / <i>FLT3</i> -ITD ^{neg} : ↑ CR (92%), ↓ RD (5%) <i>WT1</i> ^{mut} / <i>FLT3</i> -ITD ^{pos} : ↓ CR (63%), ↑ RD (33%) ↓ RFS (<i>P</i> = .006), ↓ OS (<i>P</i> < .001)	Not performed	Not performed
Cumulative dosages of high-dose cytarabine	18-54 g/m ²	13-25 g/m ²	1-8 g/m ²

AMLSG denotes German-Austrian AML Study Group; CALGB, Cancer and Leukemia Group B; MRC, Medical Research Council; s-AML, secondary AML; t-AML, therapy-associated AML; WBC, white blood cell; LDH, lactate dehydrogenase; PB, peripheral blood; CR, complete remission; RD, refractory disease; ED, early death; RFS, relapse-free survival; OS, overall survival; and DFS, disease-free survival.

WT1 mutations were identified in 78 of the 617 (12.6%) patients. This percentage of cases is slightly higher than the 10% and 10.7% frequencies that were recently reported in the studies by Virappane et al²⁸ and Paschka et al,²⁹ respectively (Table 2). The difference may be explained by the fact that in our study the entire *WT1* coding sequence was analyzed, and mutations other than those in exons 7 or 9 were identified.

WT1 mutations in our study were significantly associated with younger age, higher LDH serum levels, and higher PB blast counts, with the latter 2 factors probably reflecting a higher proliferative activity of these leukemias. In the study by Cancer and Leukemia Group B (CALGB),²⁹ there was an association of *WT1* mutations with higher WBC counts; however, no association with surrogate markers for proliferation was found in the Medical Research Council (MRC) study.²⁸ In accordance with the findings by Summers et al,⁵ in our study the presence of *WT1* mutations was significantly associated with *FLT3*-ITD; in addition, we found that, among hypothetical class II mutations, *CEBPA* mutations, but not *NPM1* mutations, were significantly associated with *WT1* mutations. In both the MRC and CALGB studies, there also was a higher frequency of *FLT3*-ITD among *WT1* mutated cases; however, this did not reach statistical significance (Table 2).

Of note, in our study, we did not observe any prognostic impact of *WT1* mutations on achievement of CR, RFS, and OS in either univariable or multivariable analysis. This finding is in marked contrast to the data by CALGB and by MRC that evaluated the prognostic significance of *WT1* mutations in 196 and 470 younger adults with CN-AML, respectively.^{28,29} With regard to response to induction therapy, in the study by CALGB, there was also no difference in CR rates between patients with or without *WT1* mutations; whereas in the study by MRC, patients with *WT1* mutations had a significantly inferior CR rate because of a higher rate of resistant disease. In the study by CALGB, patients with *WT1* mutations had inferior DFS and OS; the 3-year OS rate was 10%

and 56% for patients with and without *WT1* mutations, respectively, and in multivariable analysis, *WT1* mutation independently predicted for DFS and OS. Similarly, in the study by MRC, patients with *WT1* mutations had inferior RFS and OS; the 5-year OS rate was 26% and 47% for patients with and without *WT1* mutations, respectively; in multivariable analysis, *WT1* mutation was an independent adverse prognostic factor.

The observation by Summers et al⁵ that the presence of a *FLT3*-ITD in addition to *WT1* mutation may be associated with induction failure and inferior outcome prompted us to perform an exploratory subset analysis taking into account the *FLT3*-ITD status. Of note, comparing the laboratory characteristics among the various genotypes, the genotype *WT1*^{mut}/*FLT3*-ITD^{pos} was associated with highest serum LDH levels and highest blood blast counts, suggesting that the 2 mutations cooperate and confer a high proliferative capacity to these leukemias. In accordance with the findings by Summers et al,⁵ the *WT1*^{mut}/*FLT3*-ITD^{pos} genotype was significantly associated with induction failure and inferior RFS and OS (Figure 4). This effect could only be shown for response to induction in multivariable analysis and for RFS and OS in univariable analysis.

The marked differences between our data and those by CALGB and MRC remain elusive. One possible explanation is that the discrepancies may be a consequence of differences in treatment. Indeed, comparing the treatment protocols, the most striking difference was in the cumulative dosages of high-dose cytarabine given for consolidation treatment. Both MRC 10 and MRC 12 protocols used relatively low cumulative doses of high-dose cytarabine (~ 6 g/m² and ~ 1-8 g/m², respectively). Similarly, in CALGB protocol 9621 (dose escalation study of daunorubicin and etoposide) and protocol 19808, the cumulative dosages of high-dose cytarabine ranged between 13 g/m² and 25 g/m². In contrast, in our treatment protocols, the dosages of high-dose cytarabine ranged between 18 g/m² and 54 g/m², with the majority of patients

receiving more than 36 g/m² (Table 2). These data would suggest that the negative impact of *WT1* mutations reported by others may be overcome by the use of repetitive cycles of high-dose cytarabine, especially in the subgroup of patients with an *FLT3-ITD*^{neg} genotype. Of note, similar differential treatment effects of various dosages of cytarabine have previously been reported for other genotypic subsets of AML, ie, for AML with *RUNX1-RUNX1T1* and *CBFB-MYH11* gene fusions³¹ and, more recently, for AML with *RAS* mutations.³² Thus, it will be important to prospectively evaluate the impact of *WT1* mutations with respect to different treatments, in particular to the use of repetitive cycles of high-dose cytarabine for consolidation treatment.

In accordance with previous studies, the majority of *WT1* mutations in our study were frameshift mutations occurring in exon 7, followed by single amino acid substitutions in exon 9; whereas frameshift mutations in exon 9 were rare and have been identified in only 2 of our patients. Because exon 7 encodes the first Zn finger of *WT1*, mutations would result in truncated proteins lacking the 4 Zn fingers that contain functional domains, such as the DNA-binding portion, the nuclear localization signal and the binding domains for interacting proteins. Exon 9 frameshift mutations are predicted to lack zinc fingers 3 and 4; exon 9 substitutions, in particular mutations at codon D396 and H397, would also be expected to impair DNA binding and have been shown to destabilize the helix of Zn finger 3.³³ The missense mutation R390X in exon 9 has previously been reported as somatic as well as germline *WT1* mutation in WT.^{14,34,35} Beyond the mutation hotspot regions in exons 7 and 9, we found mutations in exons 1, 2, 3, and 8. Exon 8 frameshift mutations are predicted to result in truncated proteins lacking the second Zn finger; the frameshift mutations in exon 2 and 3 would result in truncated proteins lacking the trans-repression and the trans-activation domain localized at exon 1 to 4 as well as the 4 zinc fingers. Mutations in exon 1 have been reported previously and would lead to an almost complete lack of the protein from the affected allele.⁶ Nevertheless, it is unknown whether the various *WT1* mutation types that so far have been described in AML also have different functional consequences and therefore differ in their impact on clinical outcome. Of note, there are marked differences in *WT1* mutation types between the distinct disease entities. In AML, the mutations predominantly cluster in exon 7 (mostly frameshift mutations resulting from insertions or deletions) and 9 (mostly substitutions); whereas in patients with WAGR, Denys-Drash syndrome, or Frasier syndrome, the majority of *WT1* mutations are point mutations located either in Zn finger 2 and 3 (exon 8 and 9) or in cases with Frasier syndrome in intron 9.

In WT, large deletions or intragenic mutations involving especially exons 7 and 9 have been found. These distinct mutation types suggest different pathogenic mechanism of mutant *WT1* in differentiating hematopoietic cells and cells of the genitourinary system.

In conclusion, in our large cohort of patients with CN-AML, the presence of *WT1* mutation per se did not have an impact on treatment outcome; however, the *WT1*^{mut}/*FLT3-ITD*^{pos} genotype appeared to be associated with worse clinical course. Testing for *WT1* mutations should become part of the molecular risk assessment in future clinical trials to resolve the discrepancies observed among the different studies and to further evaluate whether the use of repetitive cycles of high-dose cytarabine may indeed overcome the strong negative prognostic impact reported in the studies by MRC and CALGB.

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Authorship

Contribution: V.I.G., H.D., and K.D. designed research, analyzed data, and wrote the paper; R.F.S. analyzed data and critically reviewed the paper; S.M. and A.B. performed experiments; and L.B., A.C., B.S., J.K., and A.G. contributed intellectually and critically reviewed the paper.

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A complete list of the members of the German-Austrian AML Study Group (AMLSG) appears in the Appendix, available online on the *Blood* website.

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References

- Döhner K, Döhner H. Molecular characterization of acute myeloid leukemia. *Haematologica*. 2008; 93:976-982.
- Mrózek K, Heerema NA, Bloomfield CD. Cytogenetics in acute leukemia. *Blood Rev*. 2004;18: 115-136.
- Mrózek K, Döhner H, Bloomfield C. Influence of new molecular prognostic markers in patients with karyotypically normal acute myeloid leukemia: recent advances. *Curr Opin Hematol*. 2007; 14:106-114.
- Schlenk RF, Döhner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med*. 2008;358: 1909-1918.
- 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;21:550-551.
- King-Underwood L, Renshaw J, Pritchard-Jones K. Mutations in the Wilms' tumor gene *WT1* in leukemias. *Blood*. 1996;87:2171-2179.
- Call KM, Glaser T, Ito CY, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell*. 1990;60:509-520.
- Hohenstein P, Hastie ND. The many facets of the Wilms' tumour gene, *WT1*. *Hum Mol Genet*. 2006;15:R196-R201.
- Menke AL, van der Eb AJ, Jochemsen AG. The Wilms' tumor 1 gene: oncogene or tumor suppressor gene? *Int Rev Cytol*. 1998;181:151-212.
- Yang L, Han Y, Suarez Saiz F, Minden MD. A tumor suppressor and oncogene: the *WT1* story. *Leukemia*. 2007;21:868-876.
- Riccardi VM, Sujansky E, Smith AC, Francke U. Chromosomal imbalance in the Aniridia-Wilms' tumor association: 11p interstitial deletion. *Pediatrics*. 1978;61:604-610.
- Haber DA, Buckler AJ, Glaser T, et al. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilm's tumor. *Cell*. 1990;61:1257-1269.
- Little M, Wells C. A clinical overview of *WT1* gene mutations. *Hum Mutat*. 1997;9:209-225.
- 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;22:4140-4146.
- Pritchard-Jones K, Renshaw J, King-Underwood L. The Wilms tumor (*WT1*) gene is mutated in a secondary leukemia in a WAGR patient. *Hum Mol Genet*. 1994;3:1633-1637.
- King-Underwood L, Pritchard-Jones K. Wilms'

- tumor (WT1) gene mutations occur mainly in acute myeloid leukemia and may confer drug resistance. *Blood*. 1998;91:2961-2968.
17. Ariyaratana S, Loeb DM. The role of the Wilms tumour gene (WT1) in normal and malignant haematopoiesis. *Expert Rev Mol Med*. 2007;9:1-17.
 18. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet*. 2002;3:179-198.
 19. Schlenk RF, Benner A, Hartmann F, et al. Risk-adapted postremission therapy in acute myeloid leukemia: results of the German multicenter AMLHD93 treatment trial. *Leukemia*. 2003;17:1521-1528.
 20. Schlenk RF, Dohner K, Kerz M, et al. Risk-adapted therapy in younger adults with acute myeloid leukemia: results of the AMLHD98A Trial of the AMLSG [abstract]. *Blood*. 2006;108:14a.
 21. Schlenk RF, Bommer M, Schmid M, et al. Valproic acid as adjunct to induction therapy in patients with acute myeloid leukemia: first results of two randomized studies of the AMLSG [abstract]. *Blood*. 2005;106:2800a.
 22. Korn EL. Censoring distributions as a measure of follow-up in survival analysis. *Stat Med*. 1986;5:255-260.
 23. Cheson BD, Bennett JM, Kopecky KJ, et al. International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia: Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. *J Clin Oncol*. 2003;21:4642-4649.
 24. Therneau TM, Grambsch PM. *Modeling Survival Data: Extending the Cox Model*. New York, NY: Springer Verlag; 2000.
 25. Cox DR. Regression models and life tables (with discussion). *J R Stat Soc*. 1972;34:187-220.
 26. Harrell FE. *Regression Modeling Strategies: With Applications to Linear Models, Logistic Regression, and Survival Analysis*. New York, NY: Springer Verlag; 2001.
 27. R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2007.
 28. Virappane P, Gale RA, Hills R, et al. Mutations of the Wilms' tumor 1 gene is a poor prognostic factor associated with chemoresistance in normal karyotype acute myeloid leukemia. *J Clin Oncol*. In press.
 29. Paschka P, Marcucci G, Ruppert AS, et al. Wilms Tumor 1 gene mutations independently predict poor outcome in adults with cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2008;26:4595-4602.
 30. Köhler B, Pienkowski C, Audrian F, et al. An N-terminal WT1 mutation (P181S) in an XY patient with ambiguous genitalia, normal testosterone production, absence of kidney disease and associated heart defect: enlarging the phenotype spectrum of WT1 defects. *Eur J Endocrinol*. 2004;150:825-830.
 31. Bloomfield C, Lawrence D, Byrd JC, et al. Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. *Cancer Res*. 1998;58:4173-4179.
 32. Neubauer A, Maharry K, Mrozek K, et al. Patients with acute myeloid leukemia and RAS mutations benefit most from postremission high-dose cytarabine: a Cancer and Leukemia Group B study. *J Clin Oncol*. 2008;26:4603-4609.
 33. Stoll R, Lee BM, Debler EW, et al. Structure of the Wilms tumor suppressor protein zinc finger domain bound to DNA. *J Mol Biol*. 2007;372:1227-1245.
 34. Schumacher V, Schneider S, Figge A, et al. Correlation of germ-line mutations and two hit inactivation of the WT1 gene with Wilms tumors of stromal-predominant histology. *Proc Natl Acad Sci U S A*. 1997;94:3972-3977.
 35. Quek HH, Chow VT, Tock EP. The third zinc finger of the WT1 gene is mutated in Wilms' tumour but not in a broad range of other urogenital tumours. *Anticancer Res*. 1993;13:1575-1580.