Clinical outcome and gene- and microRNA-expression profiling according to the Wilms tumor 1 (*WT1*) single nucleotide polymorphism rs16754 in adult *de novo* cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study

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ABSTRACT

Background

The alleles of the Wilms tumor 1 (*WT1*) polymorphism rs16754 harbor adenine (A) or guanine (G). Recently, rs16754 has been reported to affect the outcome of patients with cytogenetically normal acute myeloid leukemia. To validate this finding, we investigated pretreatment features and outcome associated with rs16754 in a large cohort of patients with cytogenetically normal acute myeloid leukemia.

Design and Methods

Four-hundred and thirty-three intensively treated and molecularly characterized cytogenetically normal patients with *de novo* acute myeloid leukemia (18-83 years old) were analyzed for rs16754. To gain biological insights, we studied the gene- and microRNA-expression profiles for associations with rs16754.

Results

Three-hundred and nine (71%) patients were homozygous for A ($WT1^{AA}$), 112 (26%) were heterozygous ($WT1^{AG}$) and 12 (3%) were homozygous for G ($WT1^{GG}$). For comparison with previous studies, we grouped $WT1^{AG}$ and $WT1^{GG}$ patients and compared them with $WT1^{AA}$ patients divided into younger (<60 years) and older (≥60 years) adults. We found no independent prognostic impact of $WT1^{AA}$. However, $WT1^{GG}$ patients, who were less often Caucasian than $WT1^{AG}$ (P=0.001) or $WT1^{AA}$ (P=0.008) patients, and had *TET2* mutations more often than $WT1^{AG}$ (P=0.02) patients, had, among patients with *FLT3*-internal tandem duplication and/or *NPM1* wild-type, better disease-free (P=0.02) and overall survival (P=0.04) than $WT1^{AA}$ and $WT1^{AG}$ patients combined. Unsupervised and supervised analyses of the gene- and microRNA-expression profiles suggested that there were no distinct expression patterns associated with any rs16754 genotype.

Conclusions

We did not observe the previously reported adverse impact of $WT1^{AA}$ but found favorable outcomes associated with the homozygous $WT1^{GG}$. Considering its low frequency, confirmatory studies are necessary. The biological significance of rs16754 remains questionable as no distinct expression profiles were associated with the genotypes.

Key words: rs16754, SNP, outcome, *WT1*, acute myeloid leukemia.

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The online version of this article has a Supplementary Appendix.

Introduction

Mutations in the Wilms tumor 1 gene (*WT1*) occur in approximately 10% of adults with cytogenetically normal acute myeloid leukemia (CN-AML), and have been reported to confer a worse outcome[1-6] or to have no prognostic impact.[7-8] The synonymous single nucleotide polymorphism (SNP) rs16754 (dbSNP Build ID: 131),[9] located in exon 7 of the *WT1* gene, which is a "hot spot" for *WT1* mutations in AML, has also recently been reported to be associated with outcome.[8,10-13] SNP rs16754 has two alleles which differ by harboring the nucleotide adenine (A) or guanine (G). They are present in a homozygous (denoted $WT1^{AA}$ or $WT1^{GG}$, respectively) or heterozygous state ($WT1^{AG}$).

Damm *et al.*[8] reported that among younger adults (17-60 years) with CN-AML, those who were homozygous for the A allele ($WT1^{AA}$) had a trend for a lower complete remission rate, and significantly shorter relapse-free survival and overall survival than patients harboring at least one G allele ($WT1^{AG}$/$WT1^{GG}$). This adverse prognostic impact of the $WT1^{AA}$ genotype on relapse-free and overall survival remained significant in multivariable analyses considering other prognostic markers, and was most pronounced in the high-risk subset of patients with *FLT3*-internal tandem duplication (*FLT3*-ITD) and/or *NPM1* wild-type.[8] In contrast, Renneville *et al.*[10] described in a preliminary report that among older CN-AML patients (aged 50 to 70 years) those with $WT1^{AA}$ had better outcomes than the group of $WT1^{AG}$/$WT1^{GG}$ patients. In pediatric AML, SNP rs16754 was not found to be of prognostic significance in two independent cohorts of CN-AML patients.[11,12] However, $WT1^{AG}$/$WT1^{GG}$ genotypes were identified as a favorable prognostic factor among children with low-risk disease, defined by Ho *et al.*[11] as patients with t(8;21)(q22;22), inv(16)(p13;q22) or t(16;16)(p13;q22), together with those carrying *CEBPA* or *NPM1* mutations. In a meeting abstract, Ma *et al.*[13] reported on cytogenetically heterogeneous adult AML patients, describing that those homozygous for the G allele ($WT1^{GG}$) had a better overall survival than the combined group of $WT1^{AA}$ and $WT1^{AG}$ patients.

Thus, to further clarify the clinical significance of the rs16754 polymorphism, we investigated the prognostic impact of its genotypes in a relatively large group of 433 intensively treated *de novo* CN-AML patients aged 18 to 83 years who were comprehensively characterized for other molecular markers. Moreover, to gain insights into the potential biological diversity related to the rs16754 polymorphism, we compared the gene- and, for the first time, the microRNA-expression profiles according to the rs16754 genotypes.

Design and Methods

Patients, treatment and cytogenetic analysis

We studied pretreatment bone marrow or blood with 20% or more blasts from 433 patients enrolled on cytarabine and daunorubicin-based Cancer and Leukemia Group B (CALGB) first-line treatment protocols as summarized in the *Online Supplementary Data*. Patients with preceding hematologic disorders or treatment-related AML were excluded. In accordance with the treatment protocols, no patient received allogeneic stem-cell transplantation in first complete remission. The median follow-up for patients alive was 6.5 years (range, 2.3-11.7 years).

Cytogenetic analyses at diagnosis were performed by CALGB-approved institutional cytogenetic laboratories, and the results confirmed by central karyotype review.[14] The diagnosis of normal cytogenetics was made based on the analysis of 20 or more metaphases in bone marrow specimens cultured for 24-48 h.

All patients provided written informed consent, and all study protocols were in accordance with the Declaration of Helsinki and approved by Institutional Review Boards at each center.

Analysis of single nucleotide polymorphism rs16754 and other molecular markers

Tissue preparation is detailed in the *Online Supplementary Data*. Genomic DNA and total RNA extraction and quality control of the extracted nucleic acids were performed as reported elsewhere.[15] *WT1* exon 7 was amplified from genomic DNA in a polymerase chain reaction, and SNP rs16754 was assessed by direct sequencing. *WT1* exon 7 and 9 mutations,[2,5] *FLT3*-ITD,[16,17] *FLT3*-tyrosine kinase domain (*FLT3*-TKD),[18] *NPM1*,[19,20] *CEBPA*,[21] *MLL*-partial tandem duplication (*MLL*-PTD),[22,23] *IDH1*,[24] *IDH2*[24] and *TET2*[15] mutations, and mRNA-expression levels of *BAALC*[25-27] and *ERG*[28,29] were assessed centrally as previously reported.

Genome-wide expression analyses

Gene-expression profiling was performed using Affymetrix U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA), as previously reported.[15] The microarray data are available at *http://www.ebi.ac.uk/microarray-as/ae/* (accession n. E-TABM-1165, E-TABM-1166, and E-TABM-1167). Gene-expression profiles were compared among younger patients (<60 years) for $WT1^{AA}$ (n=64) *versus* $WT1^{AG}$ (n=30), and among older patients (≥60 years) for $WT1^{AA}$ (n=148) *versus* $WT1^{AG}$ (n=46), $WT1^{GG}$ (n=6) *versus* $WT1^{AA}$, and $WT1^{GG}$ *versus* $WT1^{AG}$. A univariable significance level of 0.001 was used to identify differentially expressed gene probe-sets. MicroRNA expression profiling was performed using custom microRNA arrays (OSU_CCC version 3.0 for younger and OSU_CCC version 4.0 for older patients), as previously reported.[15,30] MicroRNA-expression profiles were compared among younger patients for $WT1^{AA}$ (n=62) *versus* $WT1^{AG}$ (n=29), and among older patients for $WT1^{AA}$ (n=134) *versus* $WT1^{AG}$ (n=43), $WT1^{GG}$ (n=5) *versus* $WT1^{AA}$, and $WT1^{GG}$ *versus* $WT1^{AG}$. A univariable significance level of 0.005 was used to identify differentially expressed microRNA probes. A global test of significance based on a permutation procedure was performed to determine whether or not the number of differentially expressed gene probe-sets or microRNA probes was more than expected by chance; if not, no signature is reported for the comparison. Unsupervised hierarchical cluster analyses were performed using average linkage and one minus the Pearson's correlation coefficient between two gene (or microRNA) expression profiles as the distance metric. Analyses were performed using BRB-ArrayTools Version 4.1.0 Beta_2 Release developed by Dr. Richard Simon and the BRB-ArrayTools Development Team.

Statistical analysis

The clinical endpoints (complete remission, disease-free survival, overall survival) were defined according to published recommendations,[31] and are detailed in the *Online Supplementary Data*. Patients with each of the three rs16754 genotypes ($WT1^{AA}$, $WT1^{AG}$ or $WT1^{GG}$) were compared for baseline demographic, clinical and molecular features using Fisher's exact and Wilcoxon rank sum tests for categorical and continuous variables, respectively. Estimated probabilities of disease-free and overall survival were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. Analyses of clinical endpoints in the entire study cohort were

adjusted for age group (<60 years *versus* ≥60 years), when comparing $WT1^{GG}$ patients *versus* a combined group of $WT1^{AG}$ and $WT1^{AA}$ patients, and $WT1^{AA}$ patients *versus* a combined group of $WT1^{AG}$ and $WT1^{GG}$ patients.

All analyses were performed by the CALGB Statistical Center.

Results

Frequencies and pretreatment features of the rs16754 genotypes

Of the 433 *de novo* CN-AML patients, 309 (71%) had the genotype $WT1^{AA}$, 112 (26%) $WT1^{AG}$, and 12 (3%) $WT1^{GG}$. The pretreatment clinical and molecular characteristics according to the rs16754 genotypes are presented in Table 1. $WT1^{GG}$ patients were significantly less often Caucasian compared with $WT1^{AA}$ (P=0.008) or $WT1^{AG}$ (P=0.001) patients. The non-Caucasian $WT1^{GG}$ patients comprised one Hispanic patient, one Native American and three Asians. In contrast, there were no Asians among the patients with the more common $WT1^{AA}$ or $WT1^{AG}$ genotype. This is consistent with the higher frequency of the $WT1^{GG}$ genotype reported in Asians.[9] $WT1^{GG}$ patients had leukemic skin infiltrates more often than $WT1^{AA}$ patients (P=0.04) and, by trend, more often than $WT1^{AG}$ patients (P=0.06). They also harbored mutations in *TET2* more frequently than $WT1^{AG}$ patients (P=0.02) and, as a trend, more frequently than $WT1^{AA}$ patients (P=0.09). In contrast, when considered as one group, mutations in the *IDH1* and *IDH2* genes tended to be less frequent in $WT1^{GG}$ patients than in $WT1^{AG}$ patients (P=0.055). Compared with $WT1^{AA}$ patients, $WT1^{AG}$ patients tended to harbor mutations more frequently in *IDH1* (P=0.06) and *WT1* (P=0.08).

Outcome according to the rs16754 genotypes

In the entire study population, we observed no significant differences in outcome among $WT1^{AA}$, $WT1^{AG}$ and $WT1^{GG}$ patients (Table 2). Considering the prognostic impact of *WT1* mutations in our cohort,[2,5] we analyzed the outcome associated with the genotypes separately in the *WT1* wild-type and *WT1*-mutated groups of patients. There were too few $WT1^{GG}$ patients with a *WT1* mutation, so only the patients with the $WT1^{AA}$ and $WT1^{AG}$ genotypes were compared in the *WT1*-mutated group. In both the *WT1* wild-type and *WT1*-mutated groups of patients, the outcomes according to the rs16754 genotypes did not differ significantly from each other (*Online Supplementary Table S1*).

The prognostic impact of rs16754 has been suggested to be more pronounced among CN-AML patients with *FLT3*-ITD and/or *NPM1* wild-type.[8] In this molecular subset of our cohort, $WT1^{GG}$ patients had a longer disease-free survival (P=0.04) and a trend to a longer overall survival (P=0.12) than $WT1^{AA}$ patients, and they tended to have longer disease-free survival (P=0.06) and overall survival (P=0.13) than $WT1^{AG}$ patients (Figure 1A,B; *Online Supplementary Table S2*). Since there was no indication that $WT1^{AA}$ and $WT1^{AG}$ patients differ in their outcomes we combined these patients into one group. Compared with this combined group of $WT1^{AA}$ and $WT1^{AG}$ patients, $WT1^{GG}$ patients had significantly better disease-free survival [P=0.02, HR=3.88 (1.23-12.22)] and overall survival [P=0.04; HR=2.3 (1.03-5.27)] in analyses which were adjusted for age group (<60 years *versus* ≥60 years) to control for differences in treatment intensity between the protocols for younger and older patients.

The rare occurrence of the $WT1^{GG}$ genotype (n=10 among patients with *FLT3*-ITD and/or *NPM1* wild-type) precluded its evaluation in multivariable models considering established prognostic markers. Of the patients disease-free at 2 years, two had an isolated *CEBPA* double mutation, which has been previously described to be associated with favorable outcomes in CN-AML.[32,33] The remaining three patients disease-free at 2 years had no clear favorable marker constellation besides low *BAALC* and/or *ERG* expression (*Online Supplementary Table S3*).

Outcome comparisons of the $WT1^{AA}$ versus the $WT1^{AG}$/$WT1^{GG}$ genotypes in different age groups

Previous studies combined the patients having at least one G allele in rs16754 into one group ($WT1^{AG}$/$WT1^{GG}$) and compared their outcome with that of $WT1^{AA}$ patients.[8,10-12] In age group-adjusted analyses of our entire cohort, we observed no significant differences between $WT1^{AA}$ and $WT1^{AG}$/$WT1^{GG}$ patients with regard to complete remission rates (P=0.21), disease-free survival (P=0.64) and overall survival (P=0.19).

To allow the comparison of the outcome results in our population of patients with those previously reported for younger adults with CN-AML,[8] we performed analyses restricted to younger patients. In contrast to the reported adverse impact of $WT1^{AA}$,[8] we observed that younger patients with $WT1^{AA}$ in our study cohort had higher complete remission rates (P=0.04), similar disease-free survival (P=0.75) and a trend to a longer overall survival (P=0.09) than patients with $WT1^{AG}$/$WT1^{GG}$ (*Online Supplementary Figure S1A,B*; *Online Supplementary Table S4*). However, in multivariable analyses adjusting for other prognostic molecular markers, there were no differences in outcome between $WT1^{AA}$ and $WT1^{AG}$/$WT1^{GG}$ patients (*data not shown*). As in a previous study,[8] we also assessed the prognostic impact of rs16754 in younger patients with *FLT3*-ITD and/or *NPM1* wild-type. We found no significant outcome differences in this molecular subset (*Online Supplementary Table S4*).

Next, we compared the outcomes of the $WT1^{AA}$ and $WT1^{AG}$/$WT1^{GG}$ patients aged 60 years or older. There were no significant differences in outcome between $WT1^{AA}$ and $WT1^{AG}$/$WT1^{GG}$ patients among these older patients (*Online Supplementary Figure 1C,D*; *Online Supplementary Table S4*).

Gene- and microRNA-expression profiling of the rs16754 genotypes

To explore whether the rs16754 genotypes are associated with distinct biological features, we tested whether there was an association of the different genotypes ($WT1^{AA}$, $WT1^{AG}$ and $WT1^{GG}$) with distinct gene- and microRNA-expression patterns. Only four of the 12 $WT1^{GG}$ patients were in the younger age group and none of them had material available for gene- or microRNA-expression profiling analyses. Thus, to diminish the impact of potential age-related expression differences that might contribute to the SNP rs16754-associated signatures, particularly those involving the $WT1^{GG}$ genotype, we analyzed the expression profiles for the two age groups separately. Consequently, the analyses involving $WT1^{GG}$ were performed only in the older patients.

Pair-wise comparisons between the patients according to their rs16754 genotypes revealed no significant gene- or microRNA-expression signature associated with the

Table 1. Pretreatment clinical and molecular characteristics according to SNP rs16754 genotypes in 433 patients with cytogenetically normal *de novo* acute myeloid leukemia.

Characteristic	All Patients (n=433)	$WT1^{AA}$ (n=309)	$WT1^{AG}$ (n=112)	$WT1^{GG}$ (n=12)	P $WT1^{AA}$ versus $WT1^{AG}$	P $WT1^{AA}$ versus $WT1^{GG}$	P $WT1^{AG}$ versus $WT1^{GG}$
Age (years)					0.42	0.76	0.50
Median	62	63	60	63			
Range	18-83	19-83	18-83	24-79			
Age ≥60 years, n. (%)	242 (56)	177 (57)	57 (51)	8 (67)	0.27	0.57	0.37
Male sex, n. (%)	216 (50)	152 (49)	58 (52)	6 (50)	0.66	1.0	1.0
Race, n. (%)					0.13	0.008	0.001
Caucasian	386 (90)	275 (89)	104 (95)	7 (58)			
Non-Caucasian	44 (10)	33 (11)	6 (5)	5 (42)			
Black	24	22	2	0			
Hispanic	13	8	4	1			
Native American	2	1	0	1			
Asian	3	0	0	3			
Other	2	2	0	0			
Hemoglobin (g/dL)					0.87	0.10	0.14
Median	9.4	9.4	9.4	8.8			
Range	4.6-15.0	4.6-15.0	4.8-13.6	8.0-10.5			
Platelet count ($\times 10^9$/L)					0.24	0.96	0.67
Median	64	64	67	58			
Range	4-850	4-850	11-510	30-208			
White blood cell count ($\times 10^9$/L)					0.84	0.80	0.86
Median	26.5	26.3	25.6	37.1			
Range	0.9-450.0	0.9-450.0	1.0-261.6	1.8-273.0			
% Blood blasts					0.76	0.68	0.73
Median	60	52	58	62			
Range	0-99	0-99	0-95	0-89			
% Bone marrow blasts					0.37	0.67	0.95
Median	67	65	70	79			
Range	7-99	7-98	18-99	17-91			
French-American-British classification,* n. (%)					0.29 (M4/M5 v others)	1.0 (M4/M5 v others)	0.49 (M4/M5 v others)
M0	9 (3)	4 (2)	4 (5)	1 (11)			
M1	73 (25)	50 (24)	21 (27)	2 (22)			
M2	90 (30)	64 (30)	24 (30)	2 (22)			
M4	75 (25)	53 (25)	19 (24)	3 (33)			
M5	45 (15)	36 (17)	8 (10)	1 (11)			
M6	6 (2)	3 (1)	3 (4)	0 (0)			
Extramedullary involvement, n. (%)	111 (26)	81 (27)	25 (23)	5 (42)	0.52	0.32	0.17
Central nervous system	2 (<1)	1 (<1)	1 (1)	0 (0)	0.47	1.0	1.0
Hepatomegaly	21 (5)	15 (5)	6 (5)	0 (0)	0.80	1.0	1.0
Splenomegaly	23 (5)	17 (6)	5 (5)	1 (8)	0.81	0.51	0.47
Lymphadenopathy	34 (8)	22 (7)	10 (9)	2 (17)	0.53	0.22	0.33
Skin infiltrates	28 (7)	18 (6)	7 (6)	3 (25)	0.82	0.04	0.06
Gum hypertrophy	41 (10)	34 (11)	5 (5)	2 (17)	0.06	0.63	0.14
Mediastinal mass	2 (<1)	1 (<1)	1 (1)	0 (0)	0.46	1.0	1.0
NPM1, n. (%)					0.74	0.55	0.53
Mutated	263 (61)	187 (61)	70 (63)	6 (50)			
Wild-type	170 (39)	122 (39)	42 (38)	6 (50)			
FLT3-ITD, n. (%)					0.64	0.76	0.53
Positive	149 (34)	108 (35)	36 (32)	5 (42)			
Negative	284 (66)	201 (65)	76 (68)	7 (58)			
FLT3-ITD/*NPM1* status, n. (%)					0.64	0.35	0.21
FLT3-ITD-negative and *NPM1*-mutated	148 (34)	105 (34)	41 (37)	2 (17)			
FLT3-ITD-positive and/or *NPM1* wild-type	285 (66)	204 (66)	71 (63)	10 (83)			
FLT3-TKD, n. (%)					0.85	1.0	1.0
Positive	42 (10)	31 (10)	10 (9)	1 (8)			
Negative	388 (90)	276 (90)	101 (91)	11 (92)			
WT1, n. (%)					0.08	1.0	1.0
Mutated	39 (9)	23 (7)	15 (13)	1 (8)			
Wild-type	394 (91)	286 (93)	97 (87)	11 (92)			

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CEBPA, n. (%)					0.53	0.42	0.21
Mutated	65 (15)	48 (16)	14 (13)	3 (25)			
Wild-type	366 (85)	259 (84)	98 (88)	9 (75)			
MLL-PTD, n. (%)					1.0	0.48	0.49
Positive	23 (6)	16 (6)	6 (6)	1 (10)			
Negative	354 (94)	250 (94)	95 (94)	9 (90)			
IDH1, n. (%)					0.06	0.62	0.21
Mutated	52 (12)	32 (11)	20 (18)	0 (0)			
Wild-type	373 (88)	270 (89)	91 (82)	12 (100)			
IDH2, n. (%)					0.78	0.70	0.46
Mutated	78 (18)	55 (18)	22 (20)	1 (8)			
Wild-type	347 (82)	247 (82)	89 (80)	11 (92)			
IDH1/IDH2, n. (%)					0.09	0.19	0.055
IDH1 or *IDH2* mutated	130 (31)	87 (29)	42 (38)	1 (8)			
IDH1 and *IDH2* wild-type	295 (69)	215 (71)	69 (62)	11 (92)			
TET2, n. (%)					0.15	0.09	0.02
Mutated	101 (24)	75 (25)	20 (18)	6 (50)			
Wild-type	323 (76)	226 (75)	91 (82)	6 (50)			
ERG expression group**, n. (%)					0.89	0.52	0.51
High	131 (45)	96 (45)	32 (44)	3 (30)			
Low	163 (55)	115 (55)	41 (56)	7 (70)			
BAALC expression group***, n. (%)					0.43	0.53	1.0
High	153 (50)	106 (48)	41 (54)	6 (60)			
Low	152 (50)	113 (52)	35 (46)	4 (40)			

WT1^{AA}: *homozygous for nucleotide A in rs16754;* WT1^{AG}: *heterozygous A and G in rs16754;* WT1^{GG}: *homozygous for nucleotide G in rs16754;* 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. *centrally reviewed. **For patients on CALGB 9621, the cut point was the same as that used in the study by Marcucci* et al.[28] *For patients on all other protocols, the median* ERG *expression value was used as the cut point. ***Median expression was used as the cut point.*

Table 2. Outcome according to the SNP rs16754 genotypes in 433 cytogenetically normal patients with *de novo* acute myeloid leukemia.

End Point	*WT1*^{AA} (n=309)	*WT1*^{AG} (n=112)	*WT1*^{GG} (n=12)	*P* *WT1*^{AA} *versus WT1*^{AG}	*P* *WT1*^{AA} *versus WT1*^{GG}	*P* *WT1*^{AG} *versus WT1*^{GG}
Complete remission, n. (%)	237 (77)	81 (72)	8 (67)	0.37	0.49	0.74
Disease-free survival				0.44	0.22	0.18
Median (years)	1.2	1.0	Not reached			
% Disease-free at 3 years, % (95% CI)	32 (26-38)	26 (17-36)	50 (15-77)			
% Disease-free at 5 years, % (95% CI)	27 (22-33)	25 (16-34)	50 (15-77)			
Overall survival				0.22	0.80	0.59
Median (years)	1.5	1.3	1.3			
% Alive at 3 years, % (95% CI)	36 (30-41)	27 (19-35)	42 (15-67)			
% Alive at 5 years, % (95% CI)	30 (25-35)	22 (15-30)	31 (18-58)			

WT1^{AA}: *homozygous for nucleotide A in rs16754;* WT1^{AG}: *heterozygous A and G in rs16754;* WT1^{GG}: *homozygous for nucleotide G in rs16754; CI: confidence interval.*

rs16754 genotypes in the younger or older patients. Moreover, in unsupervised cluster analyses of the gene- and microRNA-expression profiles, there were no evident patterns of clustering of the patients according to the rs16754 genotypes (Figure 2).

Discussion

The conflicting reports concerning the prognostic significance of SNP rs16754[8,10-13] prompted us to evaluate the clinical impact of this polymorphism in a relatively large cohort of intensively treated *de novo* CN-AML patients who we had previously comprehensively characterized at the molecular level.[15] To gain insights into the biological features of the polymorphism, we also examined the gene- and microRNA-expression profiles according to the rs16754 genotypes.

The frequencies and the race distribution of the rs16754 genotypes in our cohort of CN-AML patients were in accordance with those expected in a normal population (dbSNP Build ID: 131).[9] This suggests that none of the rs16754 genotypes is associated with a predisposition to AML. Our results also suggest that the *WT1*^{GG} genotype, in addition to being more frequent in non-Caucasians, might be associated with other distinct pretreatment characteristics, such as a higher frequency of leukemic skin infiltrates and *TET2* mutations.

While we found no significant differences in outcome between *WT1*^{AA}, *WT1*^{AG} and *WT1*^{GG} patients in the entire study cohort, we observed that *WT1*^{GG} patients had a more favorable outcome than *WT1*^{AA} or *WT1*^{AG} patients within

the subset of patients with *FLT3*-ITD and/or *NPM1* wild-type. Unfortunately, too few patients had $WT1^{GG}$ so we could not evaluate whether its prognostic impact was independent of other molecular markers in multivariable models. Thus, it is at present uncertain whether the better outcome of these patients can be attributed to the presence of the $WT1^{GG}$ genotype. However, while no differences in outcome between $WT1^{GG}$ and $WT1^{AG}$ patients were found in a pediatric AML cohort,[11] a favorable prognostic impact of the $WT1^{GG}$ genotype compared with $WT1^{AG}$ and $WT1^{AA}$ was described in a meeting abstract on a cohort of adult AML patients.[13] Unfortunately, neither study included outcome analyses of the $WT1^{GG}$ genotype restricted to CN-AML or to the molecular high-risk subset of patients with *FLT3*-ITD and/or *NPM1* wild-type. We observed a potentially favorable impact of $WT1^{GG}$ in this subset of patients, and Damm *et al.*[8] also found that the outcome differences according to rs16754 in their CN-AML cohort were more pronounced in this molecular subgroup. The report by Damm *et al.*[8] of $WT1^{AA}$ patients having worse outcomes than patients harboring at least one G allele in rs16754 ($WT1^{AG}$/$WT1^{GG}$) also suggests that it is the presence of the G allele that con-

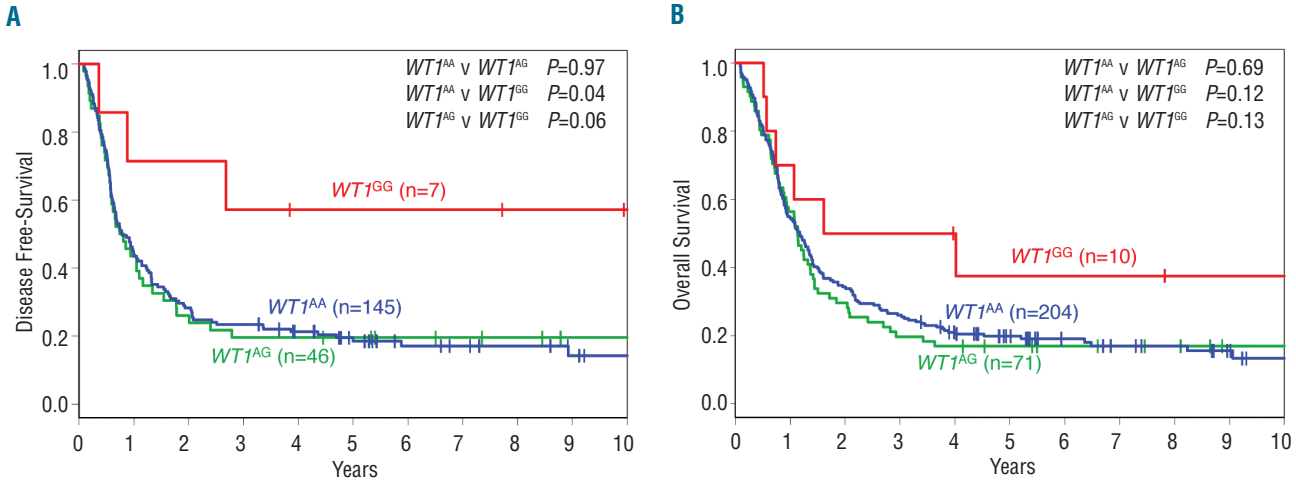

Figure 1. Disease-free survival (A) and overall survival (B) of cytogenetically normal *de novo* acute myeloid leukemia patients with *FLT3*-ITD and/or *NPM1* wild-type according to the genotypes of SNP rs16754. $WT1^{AA}$: patients homozygous for nucleotide A in rs16754; $WT1^{AG}$: heterozygous A and G; $WT1^{GG}$: homozygous for nucleotide G.

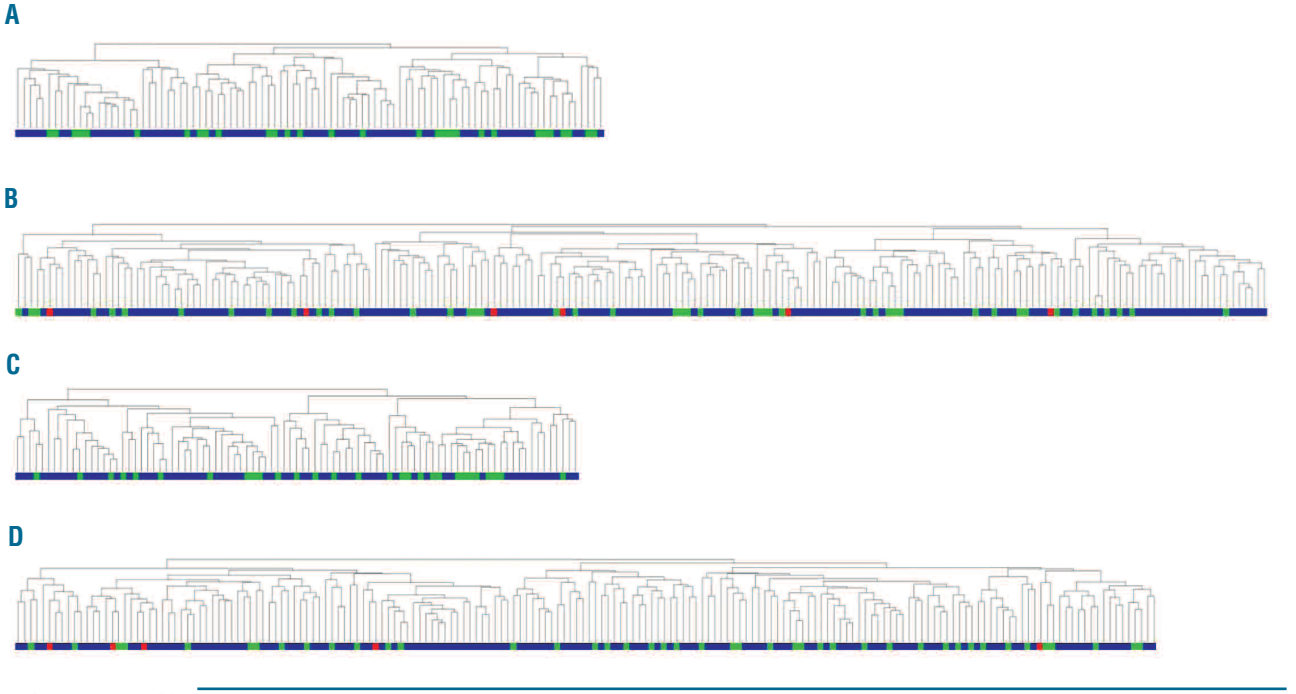

Figure 2. Unsupervised cluster analyses of the gene-expression profiles of younger (A) and older patients (B), and of the microRNA-expression profiles of younger (C) and older (D) patients with cytogenetically normal *de novo* acute myeloid leukemia. The tree diagram displays the clusters of patients generated by hierarchical clustering of gene- and microRNA-expression profiles, respectively. The SNP rs16754 genotype of each patient is indicated as follows: blue - $WT1^{AA}$ patients, green - $WT1^{AG}$ patients and red - $WT1^{GG}$ patients.

tributes to a prognostically favorable phenotype.

In accordance with previous studies,[8,10-12] we also conducted outcome analyses with patients harboring at least one G allele in rs16754 combined into one *WT1*^{AG}/*WT1*^{GG} group. Considering the entire study cohort, no significant differences in outcome were observed between *WT1*^{AA} and *WT1*^{AG}/*WT1*^{GG} patients. In the younger age group, we observed higher complete remission rates and longer overall survival of *WT1*^{AA} patients compared with the *WT1*^{AG}/*WT1*^{GG} group, but this favorable impact of *WT1*^{AA} was not significant in multivariable analyses. The lack of prognostic impact of *WT1*^{AA} in our study contrasts with the previously reported adverse outcome associated with this genotype in younger adults with CN-AML.[8] In preliminary results from a cohort of older (age 50 to 70 years) CN-AML patients, Renneville *et al.*[10] reported that *WT1*^{AA} patients had a favorable outcome. However, when we restricted our analyses to patients aged 60 years or older, we observed no significant differences in outcome between *WT1*^{AA} and *WT1*^{AG}/*WT1*^{GG} patients.

Similar to what has been suggested for mutations in *WT1*,[7] treatment differences could account for the discrepancies among the studies investigating the impact of *WT1* SNP rs16754. While no patient in our cohort received allogeneic stem cell transplantation in first complete remission, approximately 20% of the patients investigated by Damm *et al.*[8] received such consolidation. The significance of rs16754 may also vary among cytogenetic groups, as Ho *et al.*[11] observed in pediatric AML that *WT1*^{AG}/*WT1*^{GG} had no prognostic impact in CN-AML, but was associated with a favorable outcome in a subset of patients denoted as having low-risk disease, which comprised children with t(8;21), inv(16) or t(16;16), or those with *CEBPA* or *NPM1* mutations. Moreover, Damm *et al.*[34] reported that adult patients with core-binding factor AML and *WT1*^{AG}/*WT1*^{GG} genotype had a trend to a longer overall survival than patients with *WT1*^{AA}.

Another potential factor influencing response to treatment is race. *WT1*^{GG} is more frequent among Asians. Although we are not aware of studies demonstrating that adult Asian patients with CN-AML respond to treatment differently from Caucasians and Blacks, there are studies suggesting the existence of differences in treatment outcomes between Caucasians and Blacks with AML.[35,36] Thus, the racial composition of study cohorts might affect the impact of rs16754 in different studies.

A few molecular markers, such as *FLT3*-ITD and mutations in the *NPM1* and *CEBPA* genes,[37] are well established as prognostic factors in CN-AML, and further promising candidates, *e.g.*, mutations in the *IDH1/IDH2*[24] or *TET2*[15] genes, are under investigation. Based on the current data, the role of SNP rs16754 in predicting outcome of CN-AML patients appears to be minor when compared to that of the aforementioned gene mutations. Although in some studies specific SNP rs16754 genotypes have been shown to be associated with outcome, the results are inconsistent.[8,10-13] Thus, further molecular studies should clarify the prognostic role of the rs16754 genotypes before testing for SNP rs16754 should be considered for inclusion in the work-up of patients with CN-AML.

SNP rs16754 attracted particular attention because of its localization in a "hot spot" for *WT1* mutations in AML, but its biological effects have not been well characterized. A gene set enrichment analysis on nine younger CN-AML patients with the *WT1*^{AA} and eight with the *WT1*^{AG} genotype suggested the existence of biological differences according to the polymorphism.[8] However, our comparison of gene-expression profiles among relatively large CN-AML cohorts of younger *WT1*^{AA} (n=64) and *WT1*^{AG} (n=30) patients and older *WT1*^{AA} (n=148) and *WT1*^{AG} (n=46) patients did not identify any signatures of genes differentially expressed between these genotypes. In addition, we compared the gene-expression profiles of the patients with the prognostically favorable *WT1*^{GG} with those of *WT1*^{AA} and *WT1*^{AG} patients, but we did not find a significant gene-expression signature. Likewise, we could not identify any microRNA-expression signature associated with the SNP rs16754 genotypes. Moreover, in hierarchical cluster analyses, which group patients with similar gene- or microRNA-expression profiles, we observed no clustering of patients according to their rs16754 genotype. Our data, therefore, suggest that the rs16754 polymorphism does not lead to robust biological differences among malignant blasts with different genotypes.

In summary, unlike previous observations in younger adults with CN-AML,[8] *WT1*^{AA} patients did not have worse outcomes than patients of the *WT1*^{AG}/*WT1*^{GG} group in our CN-AML series. The inconsistent outcome results according to rs16754 among different studies may reflect differences in patients' characteristics or treatments administered. In our cohort, the *WT1*^{GG} genotype appeared to be associated with distinct clinical and molecular characteristics and potentially better outcomes compared with the *WT1*^{AG} or *WT1*^{AA} genotypes. Because of the relatively low frequency of the *WT1*^{GG} genotype in our study population, large collaborative studies should be performed to further evaluate whether the rs16754 polymorphism adds prognostic information to established molecular markers in CN-AML. These studies should include both large Asian populations, since the *WT1*^{GG} genotype is more frequent among Asians, and large populations of Caucasians and Blacks, which would allow evaluation of the clinical significance of *WT1* SNP rs16754 within ethnically homogeneous cohorts.

Authorship and Disclosures

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