Prognostic implications of mutations and expression of the Wilms tumor 1 (*WT1*) gene in adult acute T-lymphoblastic leukemia

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ABSTRACT

Background
The role of the *Wilms tumor 1* gene (*WT1*) in acute leukemias has been underscored by mutations found in acute myeloid leukemia identifying patients with inferior survival. Furthermore, aberrant expression of *WT1* in acute myeloid leukemia was associated with an increased risk of relapse. No larger studies have performed a combined approach including *WT1* mutation and expression analyses in acute T-lymphoblastic leukemia.

Design and Methods
We analyzed the *WT1* mutations and the expression status in a total of 252 consecutive adult patients with newly diagnosed T-lymphoblastic leukemia, who were registered on the GMALL 06/99 and 07/03 protocols and had sufficient material available. The GMALL protocols included intensive chemotherapy as well as stem cell transplantation according to a risk-based model with indication for stem cell transplantation in first complete remission for early and mature T-lymphoblastic leukemia patients; patients with thymic T-lymphoblastic leukemia were allocated to a standard risk group and treated with intensive chemotherapy.

Results
Twenty of the 238 patients analyzed had *WT1* mutations (*WT1mut*) in exon 7. *WT1mut* cases were characterized by immature features such as an early immunophenotype and higher *WT1* expression. In thymic T-lymphoblastic leukemia, *WT1mut* patients had an inferior relapse-free survival compared to *WT1* wild-type patients. T-lymphoblastic leukemia patients with aberrant *WT1* expression (high or negative) showed a higher relapse rate and an inferior outcome compared to patients with intermediate *WT1* expression. In the standard risk group of thymic T-lymphoblastic leukemia, aberrant *WT1* expression was predictive for an inferior relapse-free survival as compared to patients with intermediate expression. In multivariate analysis, *WT1* expression was of independent prognostic significance for relapse-free survival.

Conclusions
WT1 mutations were associated with an inferior relapse-free survival in standard risk thymic T-lymphoblastic leukemia patients. Moreover, altered expression associated with inferior outcome also suggests a role of *WT1* in T-lymphoblastic leukemia and the potential use of molecularly-based treatment stratification to improve outcome.

Key words: adult acute T-lymphoblastic leukemia, *WT1*, gene expression, mutations.

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The online version of this article has a Supplementary Appendix.

Introduction

The Wilms tumor 1 gene, *WT1*, encodes a transcription factor involved in normal and malignant hematopoiesis.[1] *WT1* was first recognized as a tumor suppressor when congenital malformation syndromes with predisposition to childhood kidney cancer were linked to *WT1* germline mutations.[2] In acute leukemia, *WT1* mutations have been reported in 10% of patients with acute myeloid leukemia (AML). However, mutations were also observed in selected cases of acute T-lymphoblastic leukemia (T-ALL), as well as undifferentiated/biphenotypic leukemia.[3] The mutations reported included frame shift mutations in exon 7, resulting in a truncated protein lacking the major DNA binding portion of the WT1 protein.[4] Most importantly, in intermediate risk AML with normal cytogenetics, *WT1* mutations identified patients with an inferior outcome.[5,6]

Apart from *WT1* mutations, overexpression of *WT1* was found in AML patients and to a lower extent in acute lymphoblastic leukemia (ALL).[7] There are conflicting studies regarding the prognostic impact of *WT1* expression levels in newly diagnosed AML. Some studies have associated high *WT1* expression levels in pre-treatment leukemic samples with an inferior outcome, whereas other studies could not demonstrate the prognostic significance of *WT1* expression levels in AML patients.[8-10] Only a few studies have so far evaluated the prognostic relevance of *WT1* expression in ALL and have suggested an association between high expression and an inferior outcome.[11,12]

In normal hematopoiesis, expression of *WT1* is mainly restricted to the progenitor compartment, whereas mature hematopoietic cells lack *WT1* expression. Enforced expression of *WT1* in CD34 positive hematopoietic precursors has been shown to induce growth arrest.[13,14] The ability of *WT1* to repress or activate gene transcription is dependent on *WT1* levels, isoforms, cell type, and interaction of WT1 with other proteins.[15] Moreover, the complexity of its role in hematopoiesis may be explained by its differentiation dependent function, as it was shown that *WT1* maintains primitive stem cells in a quiescence state, while it promotes differentiation of more mature progenitors.[16]

So far, studies have mainly focused on AML and underscored the role of *WT1* by exploring its aberrant expression and mutational status. Interestingly *WT1* mutations were predominantly observed in immature AML subtypes, in biphenotypic acute leukemias, and overexpression of *WT1* was correlated with a more undifferentiated phenotype. In a recent study by Tosello *et al.* mutations in the *WT1* gene were found in a subset of adult as well as pediatric T-ALL patients.[17] Together these findings suggest that altered *WT1* function might also be implicated in other leukemic subgroups and reflect transformation of a primitive hematopoietic cell of origin with retained multilineage potential.[3]

We had previously shown that AML and T-ALL share molecular markers with prognostic significance that are indicative of an immature leukemic subtype.[18] Thus, we have examined the prognostic implications of *WT1* mutations and expression levels in a large cohort of adult T-ALL within the German Multicenter ALL (GMALL) study group. As multiple genetic hits cooperate in different cellular pathways and are required for leukemic transformation,[19,20] *WT1* alterations were analyzed in the context of other genetic alterations.

Design and Methods

Patients and treatment

We studied a total of 252 consecutive adult patients with newly diagnosed T-ALL who were registered on the GMALL 06/99 and 07/03 protocols and had sufficient material available.[21,22] The GMALL protocols included intensive chemotherapy as well as stem cell transplantation according to a risk-based model with indication for stem cell transplantation in first complete remission for early and mature T-ALL patients; patients with thymic T-ALL were allocated to a standard risk group and treated with intensive chemotherapy. All patients gave written informed consent to participate in the study according to the Declaration of Helsinki. The study was approved by the ethics board of the Goethe University Frankfurt/Main, Germany.

Molecular characterization

Pre-treatment bone marrow (BM) samples were centrally collected, enriched for the blast fraction by density-gradient centrifugation, and stored in liquid nitrogen. Immunophenotyping of fresh samples was centrally performed by flow cytometry in the GMALL reference laboratory at the Charité, Berlin, Germany. Immunophenotyping was carried out as previously described.[23] CD1a positive, cortical (III) stage T-ALL was referred as thymic T-ALL in the GMALL study group.

From pre-treatment bone marrow samples (n=252) sufficient genomic DNA (n=238) and RNA (n=223) were isolated using the Trizol reagent (Invitrogen, Karlsruhe, Germany). Specimens of the 238 patients were studied for *WT1* mutations in exons 7 and 9 by DNA sequencing of amplified PCR products.[5] Mutations were confirmed by cloning the specific PCR products and sequencing up to 14 independent clones. Mutations in the *NOTCH1* and the *FBXW7* genes were determined by direct sequencing of PCR-amplified products.[24,25] *FLT3* mutations [internal tandem duplications (ITD) and mutations in the tyrosine kinase domain (TKD835)] were analyzed using the *FLT3* mutation assay (*InVivo*Scribe Technologies, San Diego, USA). The mRNA expression of *HOX11*, *HOX11L2*, *BAALC*, and *ERG* were determined by real-time RT-PCR.[18] Expression analysis of *WT1* was performed in 223 samples by a comparative real-time RT-PCR assay using primers WT1F CAGGCCAGGATGTTTCCTAA and WT1R AATGAGTGGTTGGGGAACTG with a WT1-probe 5´FAM-CCGCTATTCGCAATCAGGGTTACA-TAMRA. Multiplex PCR was performed with *beta-glucuronidase* (*GUS*) as a housekeeping gene in duplicates.[26] PCR conditions were as follows: initial denaturation with 95°C for 10 min, annealing at 60°C and extension at 72°C. *GUS* and *WT1* were coamplified using 2 µL cDNA, 1x master mix (IQ Mix, BioRad, Munich, Germany). All reactions were carried out using the Rotor Gene Real-time PCR 3000 Machine (Corbett Research, Qiagen, Germany). The comparative cycle threshold (C_T) method was used to determine the relative expression levels of *WT1*, and the cycle number difference (ΔC_T=*GUS*-*WT1*) was calculated using the mean of ΔC_T from the two replicates, that is $\mu(\Delta C_T)$, and expressed as $2^{\mu(\Delta CT)}$. In all samples, amplification of *GUS* reached the threshold within 30 cycles. For samples without detectable *WT1* amplification within 60 cycles, *WT1* expression values were set at 0. A calibrator (cDNA from the cell line KG1a) included in each run was used for standardization between runs. Positive and negative controls were included in all assays.

Statistical analyses

Comparisons of baseline clinical variables across groups were made using the χ^2 Fisher's exact test for categorical data; the non-parametric Mann-Whitney *U* test was applied for quantitative variables. A *P* value ≤0.05 (two-sided) was considered to indicate a significant difference. Clinical follow-up data were available from 215 T-ALL patients with a median follow-up time of 20.5 months (range:

0.5 to 81.2 months). Complete remission was assessed after completion of induction chemotherapy. Overall survival and relapse-free survival were calculated using the Kaplan-Meier method and the log-rank test was used to compare differences between survival curves. Overall survival was measured from the protocol on-study date until the date of death of any cause. Relapse-free survival was measured from time of complete remission date until the date of relapse.[18] For outcome analyses patients were censored at the time of stem cell transplantation.

Three *WT1* expression groups were defined as follows: after categorizing *WT1* expression levels into quintiles, a logistic regression analysis with relapse as the dependent and *WT1* grouping as the independent variable was performed. In this model, T-ALL patients with the highest quintile (*WT1* high, n=45; median expression: 0.04, range: 0.01-0.4) and patients with no detectable *WT1* expression (*WT1* negative, n=97) differentiated substantially with respect to relapse (i. e. the differences in regression coefficients had the magnitude of about two standard errors) compared to the remaining patients with intermediate *WT1* expression levels (*WT1* intermediate, n=81; median expression: 0.0001, range: 2×10^{-9} to 10^{-2}). The follow-up time and censoring was equivalent between the three expression groups.

In order to identify independent prognostic factors and effect modifiers (i. e. interactions between different factors), Cox's proportional hazards models were constructed. The following covariates were included into the full model: *BAALC* expression (low versus high), *ERG* expression (low versus high), white blood count (WBC as continuous), age (< 35 versus > 35 years), immunophenotype (thymic versus early/mature). The impact of effect modifiers was verified by the inclusion of interaction terms into the multiple Cox's regression analyses. Stepwise forward and backward selections were performed. All calculations were performed using the SPSS software, version 17 (SPSS Inc., Chicago, IL, USA).

Table 1. Clinical and molecular characteristics of T-ALL patients with respect to the *WT1* mutation status.

		WT1 WT (n=218)	*WT1* MUT (n=20)	*P* Value
WBC ($\times10^9$/L)	median	39.7	35.3	0.94
	range	0.8-853	1-666	
Age (years)	median	33	28	0.09
	range	16-70	15-63	
Early T-ALL	n	55	9	
	%	25	45	
Thymic T-ALL	n	125	7	0.10
	%	57	35	
Mature T-ALL	n	38	4	
	%	17	20	
CD34 expression (%)	mean	20	37	0.02
	range	0-97	0-95	
CD13 expression (%)	mean	13	26	0.03
	range	0-93	0-97	
WT1 expression	n	191	18	
	median	0	0.052	<0.001
	range	0-0.148	0-0.395	
HOX11L2 expression	positive/total	13/178	8/17	<0.001
	%	7	47	

Results

WT1 *mutations in adult T-ALL*

WT1 mutations (*WT1mut*) were detectable in 20 (8%) of the 238 T-ALL patients. Mutations in exon 7 (*WT1mut7*) were identified in all 20, with 2 patients having coexisting mutations in exon 9 (*WT1mut9*). *WT1mut7* were frameshift or nonsense mutations predicted to result in a truncated WT1 protein, whereas *WT1mut9* were missense mutations leading to single amino-acid substitutions (Online Supplementary Table S1). *WT1* wild-type amplicons were present in the majority of samples; thus it is likely that mutations were heterozygous, though the presence of residual normal cells or subpopulations of leukemic cell clones without *WT1* mutations cannot be excluded. We focused on *WT1* exons 7 and 9 as these regions have previously been recognized as mutational hot spots in AML. However, we cannot exclude that mutations in other regions also exsist.[4,17]

Association of WT1 *mutations with clinical and molecular characteristics*

There was no significant difference between *WT1mut* and *WT1* wild-type (*WT1wt*) patients with respect to clinical parameters at diagnosis such as WBC, age, sex, medi-

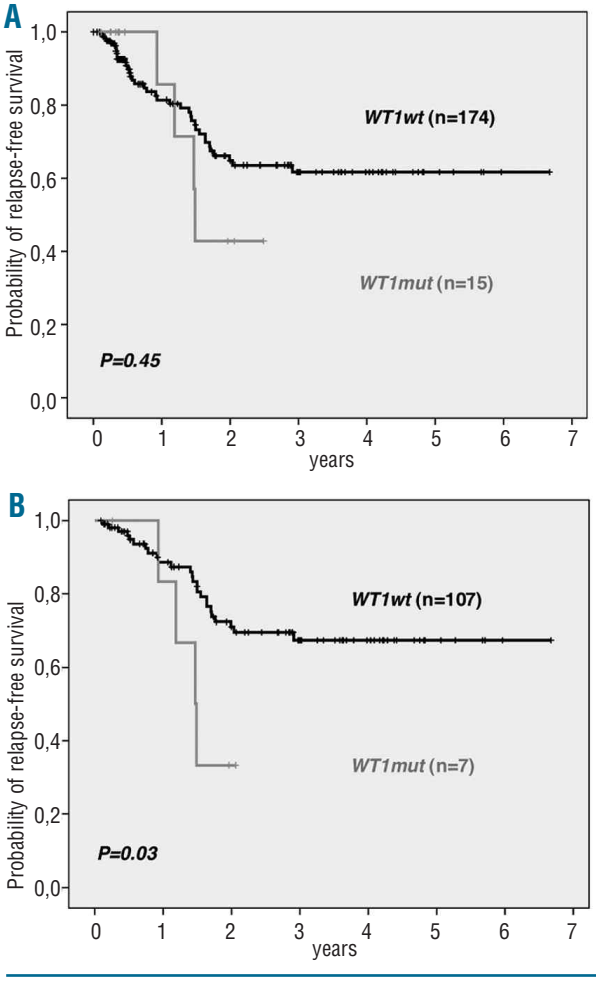

Figure 1. Relapse-free survival of adult T-ALL patients with respect to the *WT1* mutation status. (A) Overall T-ALL cohort. (B) Thymic T-ALL subgroup.

astinal mass, CNS involvement (Table 1; *data not shown*). *WT1mut* cases were characterized by immature features such as an early immunophenotype (45% of *WT1mut* showed an early T-ALL as compared to only 25% of *WT1wt*; *P*=0.07). *WT1mut* cases also showed higher CD34 levels as determined by flow cytometry (mean CD34 expression: *WT1mut*: 37% *vs.* *WT1wt*: 20%; *P*=0.02), and more frequently co-expressed the myeloid marker CD13 (*P*=0.03; Table 1). Moreover, *WT1mut* cases showed a higher frequency of aberrant *HOX11L2* expression (47% of *WT1mut vs.* 7% of *WT1wt* expressed *HOX11L2*; *P*<0.001) and *WT1mut* cases had significantly higher *WT1* mRNA expression levels as compared to *WT1wt* cases (*P*<0.001). Expression of both mutated and wild-type *WT1* transcripts were verified by PCR using mutation specific primers in 2 representative cases (*data not shown*). There was no significant correlation between the *WT1* mutation status and expression of the previously characterized molecular risk markers *ERG*, *BAALC*, and *HOX11*.

The presence of coexisting gene mutations was further investigated. Similar to the mutation frequencies observed in *WT1wt* patients, *WT1mut* cases showed gene mutations in *NOTCH1* (10/20, 50% of cases) and *FBXW7* (2/20, 10% of cases). In addition, cases were also analyzed for *FLT3* mutations, given a high frequency of *FLT3* ITD mutations observed in *WT1mut* AML.[4,6] Though *FLT3* mutations are a rare event in T-ALL (found in up to only 3%),[27,28] in *WT1mut* cases, we identified one T-ALL patient with co-existence of an *FLT3* ITD (1/20) and 3 patients with *FLT3* TKD mutations (3/20). Thus 20% of *WT1mut* T-ALL harbored simultaneous *FLT3* mutations (*Online Supplementary Figure S1*). In contrast, only one *WT1wt* patient showed a FLT3 ITD (1/108 *WT1wt* cases) and 2 *WT1wt* patients had an *FLT3* TKD mutation (2 /101 *WT1wt* cases).

WT1 *mutations and outcome*

No differences were observed in the complete remission rates between mutated and wild-type *WT1* patients. We found no significant difference in the relapse-free survival between the *WT1mut* and *WT1wt* patients in the overall cohort (Figure 1A). However, within the standard risk group of thymic T-ALL, the small subgroup of *WT1mut* showed an inferior relapse-free survival as compared to *WT1wt* thymic patients (*P*=0.03; Figure 1B).

WT1 *mRNA expression in adult T-ALL*

Expression levels of *WT1* were analyzed in 223 patients. Three expression groups including *WT1* negative (n=97),

Table 2. Molecular characteristics and outcome with respect to *WT1* expression.

		WT1 negative (n=97)	*WT1* intermediate (n=81)	*WT1* high (n=45)	*P* Value
WT1 genotype	mutated/total	1/94	4/74	13/41	
	%	1	5	29	<0.001
Early T-ALL	n	22	17	25	
	%	23	21	56	overall:
Thymic T-ALL	n	56	52	14	0.001
	%	58	64	31	early *vs.*
Mature T-ALL	n	19	12	6	others: 0.07
	%	20	12	13	
CD34 expression (%)	mean	21	22	29	
	range	0-97	0-93	0-95	0.30
CD13 expression (%)	mean	13	10	25	0.05
	range	0-87	0-87	0-97	
ERG expression group	high/total	48/77	39/76	14/39	
	%	62	51	36	0.03
BAALC expression group	high/total	14/71	16/70	18/40	0.01
	%	20	23	45	
HOX11L2 expression	positive/total	3/76	5/66	14/43	
	%	4	8	33	<0.001
complete remission	n. of pts/total	70/77	71/73	41/44	0.24
	%	91	93	93	
relapse[¶]	n. of pts/total	16/46	11/53	12/18	0.002
	%	35	21	67	
overall group relapse-free survival					
n. of pts		70	70	41	
% relapse-free at 4 years		57.0	77.2	25.8	0.001
SE		8.3	6.3	12.5	
thymic subgroup relapse-free survival					
n. of pts		44	51	13	
% relapse-free at 4 years		59.6	83.9	30.8	0.003
SE		8.8	6.2	14.7	

[¶]*Patients receiving stem cell transplantation in first complete remission and patients who were taken off protocol were not assessed for the relapse rate. SE: standard error.*

WT1 intermediate (n=81), and *WT1* high (n=45) were defined as outlined above (*see Statistical analyses*). These groups did not differ with respect to clinical parameters at diagnosis (including age, WBC, CNS involvement, mediastinal mass; *data not shown*). Patients with high *WT1* expression were characterized by immature features such as an early immunophenotype, high *BAALC* expression levels, and aberrant expression of CD13 and *HOX11L2*. *WT1* negative and *WT1* intermediate cases had predominantly a thymic phenotype (Table 2). A strong correlation was observed between the *WT1* expression and mutation status: *WT1* mutations were predominantly found in the *WT1* high group (13/41; 29% were *WT1mut*) compared to only 1/94 (1%) of *WT1* negative and 4/74 (5%) of *WT1* intermediate cases harboring *WT1* mutations ($P<0.001$). No differences were seen in the frequencies of *NOTCH1* or *FBXW7* gene mutations across the *WT1* expression groups (*data not shown*).

Complete remission rates were similar between patients of the different *WT1* expression groups. However, T-ALL patients with negative or high *WT1* expression relapsed more frequently (35% and 67%, respectively) compared to patients with intermediate *WT1* expression (21%; overall $P=0.002$; Table 2). In the overall cohort, T-ALL patients with high expression showed an inferior outcome with only 25.8% of patients remaining relapse-free at four years as compared to 57.0% of *WT1* negative and 77.2% of *WT1* intermediate patients (overall $P=0.001$; Table 2, Figure 2A). Moreover, in the multivariate analysis (Table 3), *WT1* expression was of independent prognostic significance ($P=0.009$). For T-ALL patients with high *WT1* expression a Hazard Ratio of 4.0 and for patients lacking *WT1* expression a Hazard Ratio of 1.6 were observed.

Prognostic relevance of WT1 *mRNA expression in adult T-ALL subgroups*

We next investigated the prognostic impact of *WT1* expression in the standard risk group of thymic T-ALL. An unfavorable outcome was observed for patients with *WT1* high (relapse-free at four years: 30.8%) or negative expression (relapse-free at four years: 59.6%) as compared to thymic T-ALL with intermediate *WT1* expression (relapse-free at four years: 83.9%; overall $P=0.003$; Table 2; Figure 2B).

Moreover, when patients with negative or high *WT1* expression were combined to a *WT1* high-risk group and compared to the favorable group of patients with intermediate *WT1* expression (*WT1* low-risk group), thymic T-ALL patients within the *WT1* high-risk group showed an inferior overall survival ($P=0.037$; Figure 3A).

In the GMALL protocols, early and mature T-ALLs are defined high risk and allocated to allogeneic stem cell transplantation in first complete remission. For these patients, *WT1* expression was no longer of prognostic relevance as a similar outcome for *WT1* low-risk and *WT1* high-risk patients was observed ($P=0.74$; Figure 3B).

Discussion

We present a study investigating genotype and expression alterations of *WT1* in a large cohort of adult T-ALL. Overall, *WT1* mutations were found in 8% of T-ALL patients at initial diagnosis and associated with a more immature T-ALL subtype; *WT1* mutated patients showed a higher relapse rate. In addition, altered *WT1* expression was of independent prognostic significance with negative or high *WT1* expression levels predicting inferior outcome.

WT1 mutations have previously been reported in about

Table 3. Multivariate analysis for relapse-free-survival.

Endpoint	Variables in the final model	HR	CI	*P* Value
RFS	Immunophenotype			0.01
	early *vs.* thymic	3.81	1.25-11.62	0.02
	mature *vs.* thymic	3.36	1.23-9.15	0.02
	WT1 expression			0.009
	negative *vs.* intermediate	1.57	0.71-3.5	0.27
	high *vs.* intermediate	4.01	1.62-9.91	0.003

RFS, relapse-free survival; HR, hazard ratio; CI: confidence interval. Variables considered for model inclusion: WT1 *expression, age,* WBC, ERG *expression,* BAALC *expression, immunophenotype.*

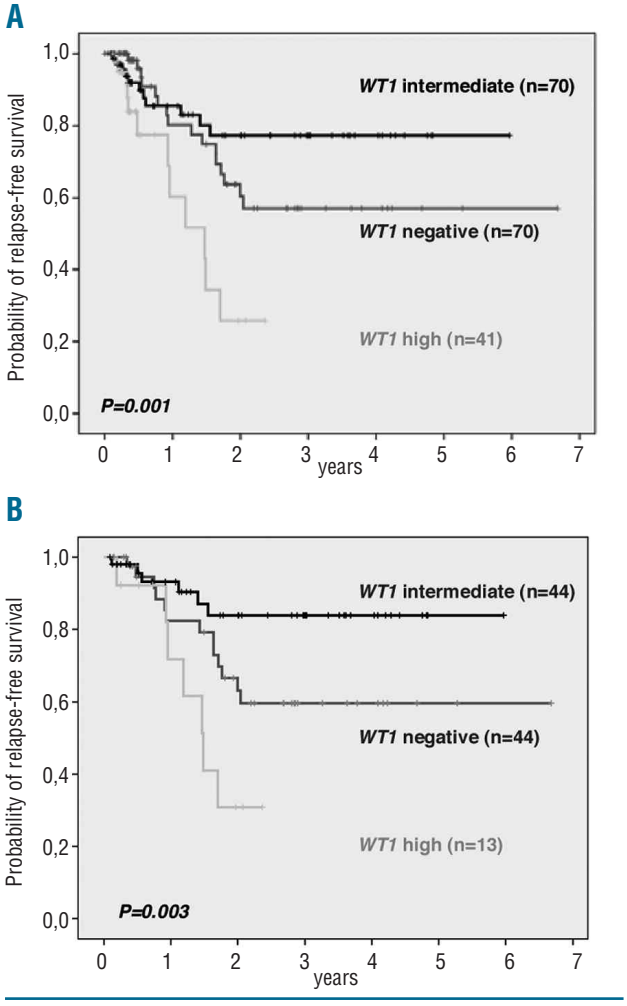

Figure 2. Relapse-free survival of adult T-ALL patients with respect to *WT1* expression. (A) Overall T-ALL cohort. (B) Thymic T-ALL subgroup. The overall *P* values are given. Comparisons between the separate *WT1* expression groups were as follows: 2A: *WT1* intermediate versus *WT1* negative: $P=0.26$; *WT1* intermediate versus *WT1* high: $P=0.003$; *WT1* negative versus *WT1* high: $P=0.002$. 2B: *WT1* intermediate versus *WT1* negative: $P=0.055$; *WT1* intermediate versus *WT1* high: $P=0.054$; *WT1* negative versus *WT1* high: $P=0.001$.

10% of AML patients with normal cytogenetics and were of adverse prognostic significance predicting inferior overall survival, relapse-free survival, as well as response to induction therapy.[3-6] Interestingly, *WT1* mutations were associated with high expression of the progenitor markers *ERG* and *BAALC*, which have been shown to share prognostic significance in AML and T-ALL.[5,18] We, therefore, reasoned that *WT1* mutations might also be of prognostic relevance in T-ALL. Frequency and localization were very similar to the reports in AML. *WT1* mutations in exon 7 are expected to result in a truncated WT1 protein that acts in a dominant negative manner by impaired DNA binding and abolished protein interaction abilities.[2] Thus, WT1 may function as a tumor suppressor not only contributing to myeloid but may also be implicated in T-cell leukemogenesis as the repression of downstream targets is likely impaired in mutated *WT1* leukemic cells. Moreover, the coexistence of gene mutations, 70% (14/20) of T-ALL patients with *WT1* mutations revealed additional mutations in *NOTCH1*, *FBXW7*, or *FLT3*, suggests that loss of WT1 function may act in cooperation with other genetic hits likely affecting differentiation and proliferation.

Whereas *WT1* mutations were not predictive for primary chemotherapy resistance in our T-ALL cohort, in contrast to the observation in AML,[6] *WT1* mutated patients showed a higher relapse rate compared to *WT1wt* T-ALL patients and within the standard risk group of thymic T-ALL, *WT1mut* patients had an adverse relapse-free survival. These outcome analyses suggesting an adverse effect of *WT1* mutations in T-ALL remain limited due to the small number of *WT1* mutated cases. Interestingly, in a recent study by Tosello *et al.* *WT1* mutations were found in a similar frequency of adult T-ALL patients (11.7%); however no significant prognostic impact was observed in the overall T-ALL cohort.[17]

In addition to mutational events, alterations of *WT1* expression (both under- and overexpression) have been described in various malignancies. In acute leukemia, *WT1* expression levels have mainly been studied in AML with inconsistent results with respect to their prognostic impact at initial diagnosis.[15,29] Studies investigating *WT1* expression as marker of minimal residual disease have shown more consistency with rising *WT1* expression predicting relapse.[30,31] However, the prognostic impact of *WT1* expression has not yet been determined in a larger cohort of adult T-ALL. T-ALL patients with high *WT1* expression levels displayed a specific molecular signature as shown by an immature phenotype, aberrant CD13 expression, and positivity for *BAALC* and *HOX11L2* (both markers associated with inferior outcome).[18,31] As it was shown that enforced expression of *WT1* in thymocytes blocked intrathymic differentiation, high expression of *WT1* in T-ALL blasts may reflect the cellular origin of progenitors (e.g. early thymocyte progenitor) that physiologically express *WT1* at high levels.[1,32,33] Importantly, *WT1* expression remained of prognostic significance independently of the other factors including the immunophenotype.[34] Similar to data in pediatric ALL by Boublikova *et al.*, we also observed that in addition to patients with high *WT1* expression, patients with no *WT1* expression showed an inferior outcome compared to patients with intermediate expression levels.[11] In particular, in the standard risk group of thymic T-ALL intermediate *WT1* expression was associated with a relapse-free survival of over 80% at four years, whereas *WT1* negative and *WT1* high expressers did significantly worse (59.6% and 30.8%, respectively).

The identification of *WT1* mutations acting in a dominant negative fashion as well as the ability of *WT1* to induce growth inhibition and suppress tumorgenicity in mice has supported its role as tumor suppressor.[14,15] We propose that in addition to its mutational inactivation, lack of *WT1* expression may act in a similar manner in T-ALL. So far combined expression and mutation analyses of *WT1* have not yet been performed, but gene expression analyses identified a subgroup of AML patients with very low *WT1* expression and indicated that *WT1* is specifically deregulated in subgroups of leukemia.[35] In fact, the *WT1* negative expression group rarely showed *WT1* mutations (only one T-ALL patient lacking *WT1* expression harbored a *WT1* mutation) suggesting that lack of mRNA might be sufficient for a pathophysiological impact and would not require additional mutational inactivation. Thus, lack of physiological *WT1* function resulting in altered regulation of downstream targets might be implicated in leukemogenesis and may confer chemotherapy resistance similar to mutational inactivation of *WT1*.

On the other hand, an oncogenic role has also been sug-

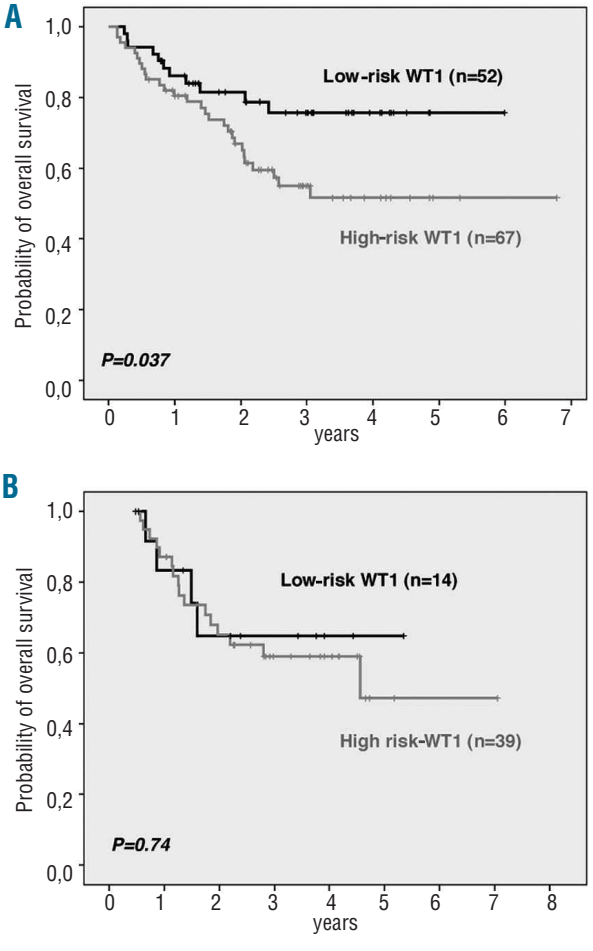

Figure 3. Overall-survival with respect to *WT1* expression in T-ALL subgroups. (A) *WT1* expression risk groups in thymic T-ALL. (B) *WT1* expression risk groups in early/mature T-ALL (including only patients undergoing allogeneic stem cell transplantation as assigned by the protocol).

gested by *WT1* overexpression where enforced expression of *WT1* in murine hematopoietic progenitors resulted in expansion of undifferentiated cells and the addition of a second genetic hit blocking differentiation-induced leukemic transformation.[36] In leukemia, high levels of *WT1* expression may promote proliferation and protect cells from apoptosis as it was shown that *WT1* overexpression is associated with chemotherapy resistance *in vitro* due to repression of the proapoptotic gene *BAK* and induction of *BCL2*.[37,38] In T-ALL, these findings can be recapitulated as leukemic blasts with high level *WT1* expression displaying an immature phenotype and patients with *WT1* high expression showing a higher relapse rate and an inferior survival. The observation that *WT1* mutated cases show frequent overexpression of *WT1* (expression of the *WT1* wild type as well as mutated allele) might be a result of its negative auto-regulation, which is impaired by the mutations affecting the DNA binding domain.[39]

In summary, these data suggest that the transcriptional control of *WT1* resembles Janus-like characteristics with abilities in repression as well as activation of different downstream pathways. Thus altered *WT1* function, either by mutational inactivation or lack of mRNA expression, as well as aberrant overexpression might contribute in different ways. Though the precise alterations of *WT1* downstream pathways in these scenarios will have to be explored in T-ALL, the prognostic implications already ask for validation. Until further studies confirm these findings, immunophenotyping remains at this point the most established factor used for risk adapted treatment stratification.

Authorship and Disclosures

SH conducted the study, performed the laboratory work and wrote the manuscript. NG and DH provided the clinical data and critically reviewed the manuscript. AS participated in the statistical analysis. CS, TB, SS, OB, UK, AB provided molecular data. ET and WKH co-ordinated the research. CDB was the principal investigator, wrote the manuscript, and takes primary responsibility for the paper.

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References

1. Ariyaratana S, Loeb DM. The role of the Wilms tumour gene (WT1) in normal and malignant haematopoiesis. Expert Rev Mol Med. 2007;9(14):1-17.
2. Little M, Wells C. A clinical overview of WT1 gene mutations. Hum Mutat. 1997; 9(3):209-25.
3. 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(8): 2961-8.
4. Summers K, Stevens J, Kakkas I, Smith M, Smith LL, Macdougall F, et al. Wilms' tumour 1 mutations are associated with FLT3-ITD and failure of standard induction chemotherapy in patients with normal karyotype AML. Leukemia. 2007;21(3): 550-1.
5. Paschka P, Marcucci G, Ruppert AS, Whitman SP, Mrózek K, Maharry K, 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(28):4595-602.
6. Virappane P, Gale R, Hills R, Kakkas I, Summers K, Stevens J, et al. Mutation of the Wilms' Tumor 1 gene is a poor prognostic factor associated with chemotherapy resistance in normal karyotype acute myeloid leukemia: The United Kingdom Medical Research Council Adult Leukaemia Working Party. J Clin Oncol. 2008;26(33):5429-35.
7. Menssen HD, Renkl HJ, Rodeck U, Maurer J, Notter M, Schwartz S, et al. Presence of Wilms' tumor gene (wt1) transcripts and the WT1 nuclear protein in the majority of human acute leukemias. Leukemia. 1995; 9(6):1060-7.
8. Schmid D, Heinze G, Linnerth B, Tisljar K, Kusec R, Geissler K, et al. Prognostic significance of WT1 gene expression at diagnosis in adult de novo acute myeloid leukemia. Leukemia. 1997;11(5):639-43.
9. Barragán E, Cervera J, Bolufer P, Ballester S, Martín G, Fernández P, et al. Prognostic implications of Wilms' tumor gene (WT1) expression in patients with de novo acute myeloid leukemia. Haematologica. 2004; 89(8):926-33.
10. Bergmann L, Miething C, Maurer U, Brieger J, Karakas T, Weidmann E, Hoelzer D. High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. Blood. 1997;90(3):1217-25.
11. Boublikova L, Kalinova M, Ryan J, Quinn F, O'Marcaigh A, Smith O, et al. Wilms' tumor gene 1 (WT1) expression in childhood acute lymphoblastic leukemia: a wide range of WT1 expression levels, its impact on prognosis and minimal residual disease monitoring. Leukemia. 2006;20(2):254-63.
12. Chiusa L, Francia di Celle P, Campisi P, Ceretto C, Marmont F, Pich A. Prognostic value of quantitative analysis of WT1 gene transcripts in adult acute lymphoblastic leukemia. Haematologica. 2006;91(2):270-1.
13. Baird PN, Simmons PJ. Expression of the Wilms' tumor gene (WT1) in normal hemopoiesis. Exp Hematol. 1997;25(4):312-20.
14. Svedberg H, Richter J, Gullberg U. Forced expression of the Wilms tumor 1 (WT1) gene inhibits proliferation of human hematopoietic CD34(+) progenitor cells. Leukemia. 2001;15(12):1914-22.
15. Yang L, Han Y, Suarez Saiz F, Minden MD. A tumor suppressor and oncogene: the WT1 story. Leukemia. 2007;21(5):868-76.
16. Ellisen LW, Carlesso N, Cheng T, Scadden DT, Haber DA. The Wilms tumor suppressor WT1 directs stage-specific quiescence and differentiation of human hematopoietic progenitor cells. EMBO J. 2001;20(8): 1897-909.
17. Tosello V, Mansour MR, Barnes K, Paganin M, Sulis ML, Jenkinson S, et al. WT1 mutations in T-ALL. Blood. 2009;114(5):1038-45.
18. Baldus CD, Martus P, Burmeister T, Schwartz S, Gökbuget N, Bloomfield CD, et al. Low ERG and BAALC expression identifies a new subgroup of adult acute T-lymphoblastic leukemia with a highly favorable outcome. J Clin Oncol. 2007; 25(24):3739-45.
19. Gilliland DG, Jordan CT, Felix CA. The molecular basis of leukemia. Hematology Am Soc Hematol Educ Program. 2004:80-97.
20. Van Vlierberghe P, Homminga I, Zuurbier L, Gladdines-Buijs J, van Wering ER, Horstmann M, et al. Cooperative genetic defects in TLX3 rearranged pediatric T-ALL. Leukemia. 2008;22(4):762-70.
21. Brüggemann M, Raff T, Flohr T, Gökbuget N, Nakao M, Droese J, et al. German Multicenter Study Group for Adult Acute Lymphoblastic Leukemia. Clinical significance of minimal residual disease quantification in adult patients with standard-risk acute lymphoblastic leukemia. Blood. 2006;107(3):1116-23.
22. Schwartz S, Rieder H, Schläger B, Burmeister T, Fischer L, Thiel E. Expression of the human homologue of rat NG2 in adult acute lymphoblastic leukemia: close association with MLL rearrangement and a CD10(-)/CD24(-)/CD65s(+)/CD15(+) B-cell phenotype. Leukemia 2003;17(8):1589-95.
23. Bene MC, Castoldi G, Knapp W, Ludwig WD, Matutes E, Orfao A, van't Veer MB. Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL). Leukemia. 1995;9(10): 1783-6.
24. Weng AP, Ferrando AA, Lee W, Morris JP 4th, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269-71.
25. Nowak D, Mossner M, Baldus CD, Hopfer

O, Thiel E, Hofmann WK. Mutation analysis of hCDC4 in AML cells identifies a new intronic polymorphism. Int J Med Sci. 2006;3(4):148-51.
26. Beillard E, Pallisgaard N, van der Velden VH, Bi W, Dee R, van der Schoot E, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. Leukemia. 2003;17(12): 2474-86.
27. Paietta E, Ferrando AA, Neuberg D, Bennett JM, Racevskis J, Lazarus H, Dewald G, Rowe JM, Wiernik PH, Tallman MS, Look AT. Activating FLT3 mutations in CD117/KIT(+) T-cell acute lymphoblastic leukemias. Blood. 2004;104(2):558-60.
28. Van Vlierberghe P, Meijerink JP, Stam RW, van der Smissen W, van Wering ER, Beverloo HB, Pieters R. Activating FLT3 mutations in CD4+/CD8- pediatric T-cell acute lymphoblastic leukemias. Blood. 2005;106(13):4414-5.
29. Lapillonne H, Renneville A, Auvrignon A, Flamant C, Blaise A, Perot C, et al. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. J Clin Oncol. 2006;24(10):1507-15.
30. Weisser M, Kern W, Rauhut S, Schoch C, Hiddemann W, Haferlach T, Schnittger S. Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia. Leukemia. 2005;19(8):1416-23.
31. Baak U, Gökbuget N, Orawa H, Schwartz S, Hoelzer D, Thiel E, Burmeister T; German Multicenter ALL Study Group. Thymic adult T-cell acute lymphoblastic leukemia stratified in standard- and high-risk group by aberrant HOX11L2 expression: experience of the German multicenter ALL study group. Leukemia. 2008;22(6): 1154-60.
32. Hosen N, Sonoda Y, Oji Y, Kimura T, Minamiguchi H, Tamaki H, et al. Very low frequencies of human normal CD34+ haematopoietic progenitor cells express the Wilms' tumour gene WT1 at levels similar to those in leukaemia cells. Br J Haematol. 2002;116(2):409-20.
33. Li H, Oka Y, Tsuboi A, Yamagami T, Miyazaki T, Yusa S, et al. The lck promoter-driven expression of the Wilms tumor gene WT1 blocks intrathymic differentiation of T-lineage cells. Int J Hematol. 2003;77(5):463-70.
34. Thiel E, Kranz BR, Raghavachar A, Bartram CR, Löffler H, Messerer D, et al. Prethymic phenotype and genotype of pre-T (CD7+/ER-)-cell leukemia and its clinical significance within adult acute lymphoblastic leukemia. Blood. 1989;73(5):1247-58.
35. Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh van Waalwijk van Doorn-Khosrovani S, Boer JM, et al. Prognostically useful gene-expression profiles in acute myeloid leukemia. N Engl J Med. 2004; 350(16):1617-28.
36. Nishida S, Hosen N, Shirakata T, Kanato K, Yanagihara M, Nakatsuka S, et al. AML1-ETO rapidly induces acute myeloblastic leukemia in cooperation with the Wilms tumor gene, WT1. Blood. 2006;107(8): 3303-12.
37. Ito K, Oji Y, Tatsumi N, Shimizu S, Kanai Y, Nakazawa T, et al. Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway. Oncogene. 2006;25(30):4217-29.
38. Mayo MW, Wang CY, Drouin SS, Madrid LV, Marshall AF, Reed JC, Weissman BE, Baldwin AS. WT1 modulates apoptosis by transcriptionally upregulating the bcl-2 proto-oncogene. EMBO J. 1999;18(14): 3990-4003.
39. Rupprecht HD, Drummond IA, Madden SL, Rauscher FJ 3rd, Sukhatme VP. The Wilms' tumor suppressor gene WT1 is negatively autoregulated. J Biol Chem. 1994;269(8):6198-206.