

WT1 mutations in T-ALL

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The molecular mechanisms involved in disease progression and relapse in T-cell acute lymphoblastic leukemia (T-ALL) are poorly understood. We used single nucleotide polymorphism array analysis to analyze paired diagnostic and relapsed T-ALL samples to identify recurrent genetic alterations in T-ALL. This analysis showed that diagnosis and relapsed cases have common genetic alterations, but also that relapsed samples frequently lose chromosomal markers present at diagnosis, suggest-

ing that relapsed T-ALL emerges from an ancestral clone different from the major leukemic population at diagnosis. In addition, we identified deletions and associated mutations in the WT1 tumor suppressor gene in 2 of 9 samples. Subsequent analysis showed WT1 mutations in 28 of 211 (13.2%) of pediatric and 10 of 85 (11.7%) of adult T-ALL cases. WT1 mutations present in T-ALL are predominantly heterozygous frameshift mutations resulting in truncation of the C-terminal zinc finger domains of

this transcription factor. WT1 mutations are most prominently found in T-ALL cases with aberrant rearrangements of the oncogenic TLX1, TLX3, and HOXA transcription factor oncogenes. Survival analysis demonstrated that WT1 mutations do not confer adverse prognosis in pediatric and adult T-ALL. Overall, these results identify the presence of WT1 mutations as a recurrent genetic alteration in T-ALL. (Blood. 2009;114:1038-1045)

Introduction

T-lineage acute lymphoblastic leukemia (T-ALL) accounts for 10% to 15% of pediatric and 25% of adult ALL cases.¹ Once associated with a dismal prognosis, the introduction of intensive combination chemotherapy protocols has led to remarkable improvements in survival for this disease. Thus, more than 70% of children and 50% of adult T-ALL patients achieve long-lasting complete remissions.² However, in contrast with the favorable response to therapy in patients at diagnosis, the prognosis of T-ALL patients with relapsed leukemia remains poor, underscoring the need to identify molecular mechanisms responsible for disease progression and to develop more effective antileukemic drugs active against relapsed T-ALL.

WT1 was originally identified as a tumor suppressor gene in patients with the WAGR (Wilms tumor, aniridia, genitourinary abnormalities, mental retardation) tumor predisposition syndrome, caused by inherited germline deletions in the chromosomal band 11p13.³ Germline mutations in WT1 are also present in patients affected with the Denys-Drash syndrome (DDS), a related tumor predisposition syndrome characterized by pseudohermaphroditism, nephropathy, genital abnormalities,

and an increased risk of Wilms tumors.⁴ Moreover, biallelic somatic mutations in WT1 account for 15% of sporadic Wilms tumors.⁵ In addition to its prominent role in the pathogenesis of Wilms tumors, WT1 mutations have been reported in 10% of cases of acute myeloid leukemia (AML), 20% of biphenotypic leukemias, and sporadic cases of T-ALL.⁶⁻⁹

In this study, we aimed to characterize the mechanisms of disease progression and relapse in T-ALL via single nucleotide polymorphism (SNP) array analysis of paired diagnostic and relapsed T-ALL samples. Our results show a remarkable lack of genomic instability in most high-risk T-ALL cases during progression from diagnosis to relapse. In addition, we identified the presence of recurrent chromosomal deletions involving the WT1 locus in T-ALL. Subsequent mutation analysis revealed the presence of WT1 mutations in approximately 10% of pediatric and adult T-ALL samples at diagnosis. WT1 mutations in T-ALL primarily consist of heterozygous frameshift insertions and deletions encoding truncated proteins devoid of the C-terminal zinc finger domains of WT1. Notably, WT1 mutations were characteristically associated with aberrant expression of the

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TLX1, *TLX3*, and *HOXA* transcription factor oncogenes. These results identify *WT1* mutations as a recurrent genetic abnormality associated with aberrant HOX gene expression in T-ALL.

Methods

Patient samples and cell lines

Leukemic DNA and cryopreserved lymphoblast samples were provided by collaborating institutions in the United States (Eastern Cooperative Oncology Group [ECOG], Pediatric Oncology Group), United Kingdom (Medical Research Council [MRC]), The Netherlands (Dutch Childhood Oncology Group [DCOG]), Germany (German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia [COALL]), and Italy (Hemato-Oncology Laboratory, Department of Pediatrics, University of Padua, Padua, Italy). All samples were collected in clinical trials with informed consent and under the supervision of the Institutional Review Board committees of all participating institutions. Consent was obtained from all patients at trial entry according to the Declaration of Helsinki. T-cell phenotype was confirmed by flow cytometry.

CCRF-CEM, Jurkat, RPMI8402, and PF382 were obtained from ATCC. The P12-ICHIKAWA, ALL-SIL, and HPB-ALL cell lines were from the DSMZ repository. KOPTK1 and DND41 cell lines were a gift from Dr A. T. Look (Dana-Farber Cancer Institute). The CUTLL1 has been described before.¹⁰ T-ALL cell lines were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere less than 5% CO₂. HeLa and 293T cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere less than 5% CO₂.

SNP array analysis

DNA samples from pediatric T-ALL patients (n = 9) obtained at diagnosis, remission, and relapse were analyzed with the GeneChip Human Mapping 250K *Sly* Array (Affymetrix), which contains 23 800 SNPs. Copy number and loss of heterozygosity (LOH) analysis was performed with dCHIP. Probe intensity data for each array were normalized to a baseline array with median signal intensity using the “invariant set” model, as previously described.¹¹ We used matched remission samples as a reference for copy number estimation and LOH analysis. LOH was analyzed using a Hidden Markov Model in dChip and an inferred LOH call threshold of 0.5. SNP array data were deposited in the Gene Expression Omnibus (GEO; National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/geo/>) under accession no. GSE15931.

Karyotype and fluorescence in situ hybridization analysis

Interphase preparations from T-ALL cells obtained at diagnosis were subjected to fluorescence in situ hybridization (FISH) analysis using PAC clones RP11-299P16, RP1-259N9, and RP1-74J1. DNA from BACS RP11-299P16 and RP1-259N9 located telomeric and centromeric to the *WT1* locus, respectively, were labeled using spectrum orange dUTP fluorochrome (Vysis). BAC RP1-74J1, expanding the *WT1* gene, was labeled with spectrum green-dUTP fluorochrome (Vysis). FISH was performed by standard methods as previously described.¹⁰ Hybridization signals were scored on at least 500 interphase nuclei on 4,6-diamidino-2-phenylindole-stained slides using the Cytovision Imaging system attached to a Nikon Eclipse 600 microscope (Applied Imaging).

Molecular characterization of T-ALL samples

WT1 mutations were analyzed by polymerase chain reaction (PCR) amplification of *WT1* exons 1 to 10 followed by direct bidirectional DNA sequencing. DNA samples from T-ALL patients enrolled in the UKALLXII protocol were screened for mutations by denaturing high performance liquid chromatography (DHPLC) using the WAVE system (Transgenomic Inc) before DNA sequencing. Briefly, *WT1* exons 2 to 10 were amplified by

PCR using the proof-reading enzyme Optimase (Transgenomic Inc) according to manufacturer’s specifications, whereas exon 1 was amplified using BioTAQ DNA polymerase (Biolone). For exons 1 to 3, 1 M betaine was added to the PCR buffer to enhance the amplification of these high GC-rich amplicons. Samples with abnormal DHPLC mobility profiles were selected for bidirectional DNA sequence analysis. To ensure adequate detection of hemizygous or homozygous mutations by DHPLC, each PCR product was combined with an amplicon generated from a known *WT1*-wild-type template.

NOTCH1 mutation analysis was performed by PCR amplification and direct DNA sequencing of *NOTCH1* exons 26, 27, 28, and 34 as previously described.^{12,13} *FBXW7* mutation analysis was performed by PCR amplification and direct DNA sequencing of *FBXW7* exons 9 and 10 as previously described.¹⁴

Expression analysis of T-ALL transcription factor oncogenes, including *TLX1/HOX11*, *TLX3/HOX11L2*, *HOXA9*, *TAL1*, *LMO1*, *LMO2*, *SET-NUP*, *MLL-ENL*, and *CALM-AF10*, has been described before.^{15,16}

Methylation analysis of the *WT1* locus

Analysis of the methylation status of regulatory sequences in the *WT1* locus was performed by combined bisulfite restriction analysis.¹⁷ Genomic DNA was bisulfite converted using the CpGenome DNA Modification kit (Chemicon International) and amplified by PCR using primers WT1 bisulfite FW 5'-AATTTTAAAGGTAAGGAGAAG-3' and WT1 bisulfite RV 5'-CACATAAATTTAACTAAAAAACT-3'. Each PCR reaction contained 150 ng of bisulfite converted DNA, 60 pmol of each amplification primer, 10 nM dNTPs, 2.5 U of Taq polymerase (AmpliAq gold, Applied Biosystems), 5 µL of 10 × PCR Gold Buffer and PCR boost buffer (Biomatrix) up to a total volume of 50 µL. PCR amplification reactions consisted of an initial denaturing step of 10 minutes at 95°C followed by 35 cycles of 30 seconds at 96°C, 30 seconds at 52°C, and 30 seconds at 72°C, plus a final elongation step of 10 minutes at 72°C. PCR products were purified using the QIAquick Gel extraction Kit (QIAGEN), digested with the *Bst*II restriction enzyme overnight at 60°C and subsequently analyzed by gel electrophoresis.

Statistical analysis

Therapeutic outcome was analyzed in pediatric patients treated in DCOG (n = 72) and COALL (n = 74) trials and in adult T-ALL cases treated in the UKALLXII/ECOG2993 (n = 85) protocols according to overall survival and event-free survival. Kaplan-Meier curves were used to assess survival, and differences between groups compared by the log-rank test.

Results

Genetic alterations in diagnostic and relapsed T-ALL

Recent studies have illustrated the power of SNP array-based copy number analysis to identify important cancer-related genes associated with the pathogenesis of pediatric and adult acute lymphoblastic leukemia.¹⁸⁻²⁰ To analyze genomic alterations involved in the pathogenesis of high-risk T-ALL and chromosomal changes associated with disease progression at relapse, we analyzed matched diagnostic, remission, and relapsed DNA samples from 9 pediatric T-ALL patients using Affymetrix 250K *Sly* SNP arrays. This analysis demonstrated the presence of known recurrent chromosomal alterations associated with the pathogenesis of T-ALL in each of the cases at diagnosis (Table 1). Most prominently, 6 of 9 cases (samples IId,r; IIId,r; Vd,r; VIId,r; VIIId,r; and VIIIId,r) showed homozygous deletions in the short arm of chromosome 9 centered on band 9p21, which result in loss of the *CDKN2A/p16INK4A* and *p14ARF* tumor suppressor genes. Similarly, 2 patients (samples IIId and IVd) showed discrete deletions at chromosome 4q31.2-31.3, with a common deleted region centered on

Table 1. Chromosomal abnormalities identified in T-ALL by SNP array analysis

Cases	Gain/loss	Chromosome	Cytogenetic location	Size, Mb	No. of genes	Candidate genes
VIII _{d,r}	Loss	1	p21.1-31.1	28	>50	<i>LMO4</i>
VIII _{d,r}	Loss	1	p13.1-2	2.8	30	
III _r	Loss	1	q32.3-44	35	>50	<i>WNT9A</i>
IV _d	Loss	2	q21.1-31.2	20	>50	<i>TANK</i>
IV _d	Loss	3	q12.1-21.3	34	>50	<i>mir-198</i>
VIII _r	Loss	3	q26.3-27.1	8.6	50	<i>SOX2</i>
IX _d	Loss	4	q21.1	0.06	0	
III _d , IV _d	Loss	4	q31.2-31.3*	1	5	<i>FBXW7</i>
IV _d , IX _d	Loss	5	q23.3-32*	16	>50	<i>CTNNA1</i>
Id, VI _r , VII _{d,r}	Loss	6	q13-16.3*	27	>50	
VIII _d	Gain	6	q15-27	84	>50	
VIII _r	Loss	7	p11.2-12.3	7.5	20	<i>IKZF1</i>
VIII _r	Loss	7	p21.3	1.6	3	
VIII _r	Loss	7	q31.3	0.8	4	
VIII _d	Gain	8	p11.2-23	43	>50	
II _{d,r} ; III _{d,r} ; V _{d,r} ; VI _{d,r} ; VII _{d,r} ; VIII _{d,r}	Loss	9	p21.3*	0.16	2	<i>p16/INK4A; p14/ARF</i>
V _{d,r}	Gain	9	q34.12	0.6	2	<i>NUP214-ABL1</i>
V _{d,r}	Loss	9	q34.13	0.5	6	
VIII _{d,r}	Gain	9	q22.2-34.3	39	>50	
IV _d	Loss	10	q26.2	1.5	1	<i>MGMT</i>
V _{d,r}	Loss	11	p13-14.1	2.8	18	<i>WT1, PAX6</i>
Id, IV _d , IX _d	Loss	12	p12.3-13.31*	10	>50	<i>ETV6, CDKN1B, BCL2L14</i>
Id	Gain	14	q13.1-21.3	25	>50	
Id; VIII _{d,r}	Loss	14	q23.1*	1.5	10	
Id; VIII _{d,r}	Loss	14	q23.2*	1.8	8	
Id; VIII _{d,r}	Loss	14	q24.4-24.3*	6.3	>50	
Id; V _{d,r} ; VIII _{d,r}	Loss	14	q32.1*	2.2	20	
V _{d,r}	Loss	14	q32.2	1.1	6	
IX _{d,r}	Loss	14	q31.1-1.2	1	3	<i>BCL11B</i>
IX _{d,r}	Gain	14	q32.1-3	17	>50	<i>TRAF3, DICER1</i>
IX _{d,r}	Gain	22	q12.3-13.3	48.5	>50	<i>WNT7B, CERK</i>
VI _{d,r}	Loss	22	q11.2	0.2	1	<i>VPREB1</i>

d indicates diagnostic samples; and r, relapse samples.

*Common minimally deleted regions are indicated.

FBXW7, a tumor suppressor gene encoding an F-box protein responsible for the proteasomal degradation of several oncoproteins involved in the pathogenesis of T-ALL, including NOTCH1, c-MYC, and mTOR.²¹ Larger deletions in chromosome regions 5q23.3-32 and 6q13-16.3, previously described as commonly deleted in T-ALL, were detected in 2 of 9 (22%; samples IV_d and IX_d) and 3 of 9 (33%; samples Id, VI_r and VII_{d,r}) patients, respectively. In addition, we observed a focal area of amplification followed by a small deletion in chromosome band 9q34 in one patient (samples V_{d,r}). This pattern of amplification and deletion corresponds to the rearrangement and amplification of the *NUP214* and *ABL1* loci to generate the *NUP214-ABL1* fusion oncogene present in 5% of T-ALLs.²² In addition to previously described chromosomal alterations, our analysis demonstrated several additional genetic lesions, including focal deletions at chromosome bands 7p21.3 (sample VIII_r), 10q26 (sample IV_d), 14q31.1-31.2 (samples IX_{d,r}), 14q32.2 (sample V_{d,r}), and 22q11.2 (samples VI_{d,r}) as well as broader areas of deletion and genetic amplification (Table 1). Notably, with the exception of patient VIII, whose tumor cells acquired 4 new areas of deletion at the time of relapse, all other relapsed T-ALL samples showed none or only one new chromosomal alteration gained during disease progression. Overall, these results show that that acquisition of overall genomic instability does not seem to be a driver mutagenic effect contributing to relapse in T-ALL.

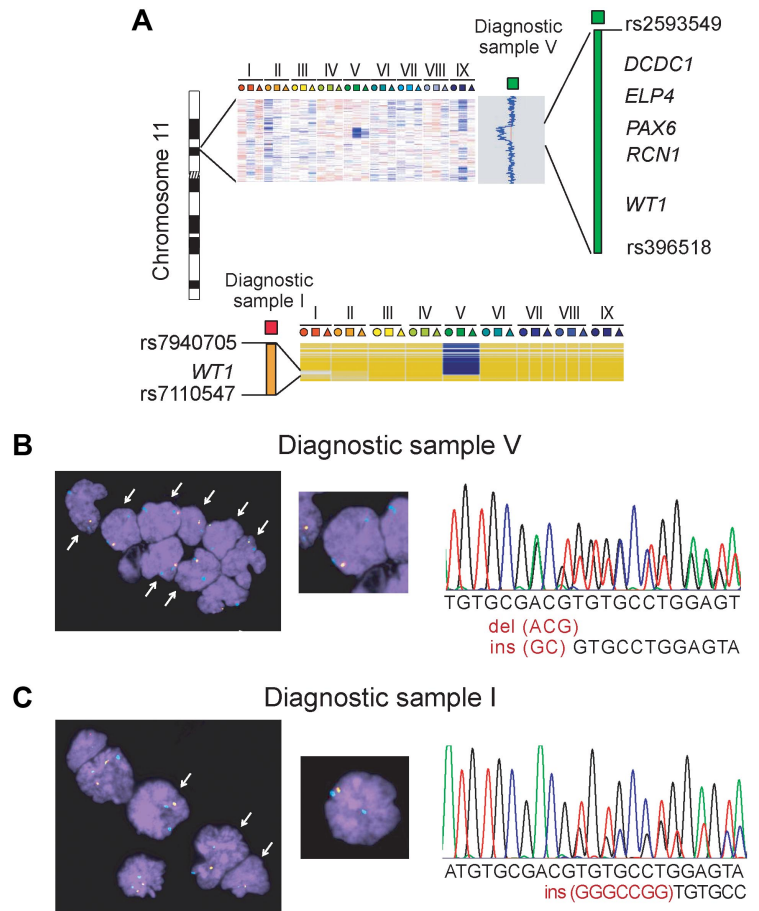
Although most of the chromosomal alterations identified in our SNP array analysis were nonrecurrent, we noted that a region of hemizygous deletion and LOH in chromosome band 11p13 found in one of our patients (patient V) was overlapping with a weak area

of LOH present in a second T-ALL sample. Notably, the overlap between these 2 alterations corresponded to the *WT1* tumor suppressor gene (Figure 1A). These results were confirmed and extended via 2-color FISH analysis using a set of BAC clones overlapping and flanking the *WT1* locus (Figure 1B). Notably, this analysis confirmed the loss of one copy of *WT1* in 80% of the nuclei in the T-ALL sample showing copy number loss and LOH in 11p13 in our SNP array analysis. In addition, this analysis revealed a hemizygous deletion of *WT1* in 39% of the nuclei in patient I at diagnosis (Figure 1C). This subclonal deletion corresponds to the detection of a focal region of weak LOH centered on the *WT1* locus without detectable copy number loss in the SNP array analysis in this sample. Subsequent mutation analysis of *WT1* in these patients revealed frameshift mutations in exon 7 of *WT1* in each of these 2 samples (Figure 1B-C), demonstrating that the LOH in this case also results from an heterozygous deletion centered at the *WT1* locus.

Frequency and types of *WT1* mutations in T-ALL

To address the actual prevalence and potential significance of *WT1* mutations in T-ALL, we extended our mutation analysis of *WT1* to a panel of 294 primary T-ALL samples and 10 T-ALL cell lines. This analysis demonstrated the presence of *WT1* mutations in 28 of 211 (13.2%) pediatric and in 10 of 85 (11.7%) adult T-ALLs (supplemental Tables 1-2, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). The

Figure 1. Hemizygous deletion of *WT1* in T-ALL. (A) Chromosome 11 ideogram and copy number (top) and LOH analysis (bottom) of paired diagnostic (squares), relapsed (triangles), and remission (circles) T-ALL samples. Flanking SNPs and genes included in these alterations are indicated. Roman numerals identify individual patients. (B) FISH and mutation analysis of *WT1* in diagnostic sample V. (C) FISH and mutation analysis of *WT1* in diagnostic sample I. A chromosome 11 centromeric probe is shown in blue. An intact *WT1* locus is marked by an orange signal from a *WT1* BAC clone labeled in green and BAC clones telomeric and centromeric to *WT1* labeled in red. Arrows indicate nuclei with hemizygous deletions of *WT1*. DNA sequence chromatograms focus in the *WT1* exon 7 sequences containing frameshift mutations of *WT1* in each of these samples. Note that, although areas of LOH are marked across diagnostic, remission, and relapsed samples, this corresponds to genetic lesions present in diagnostic and/or relapsed samples compared with the corresponding normal remission DNA.



WT1 gene encompasses 10 exons encoding an N-terminal proline and glutamine-rich domain (exons 1-6) involved in transcriptional regulation followed by 4 C-terminal Kruppel-like DNA-binding zinc fingers (exons 7-10).²³ *WT1* mutations found in T-ALL were primarily frameshift insertions and deletions and occasional nonsense point mutations located in exons 7 (30 of 38) and 8 (1 of 38), which encode prematurely truncated WT1 proteins devoid of the C-terminal zinc finger domains (Figure 2A-C). Splice donor mutations in exons 1 and 7 were found in one T-ALL sample each (Figure 2A,D). Additional frameshift mutations predicted to encode N-terminal truncations in WT1 were detected in exons 1 (4 cases) and 2 (1 case; Figure 2A,E). Two extra cases harbored missense mutations in codon 462 (R462Q and R462P) located in exon 9, which encodes the third zinc finger of the WT1 protein (Figure 2A,F). The functional relevance of these alleles is highlighted by a related *WT1* mutation, R462W, which has been described as the most frequent genetic abnormality in patients with DDS.²⁴ Notably, the R462W mutation has also been identified in a sporadic Wilms tumor sample and is predicted to impair the DNA-binding activity of WT1.²⁵ Three additional *WT1* mutations were identified in the CCRF-CEM, RPMI8402, and ALL-SIL cell lines. CCRF-CEM and RPMI8402 cells showed 2 heterozygous nonsense point mutations in exon 1 of *WT1* encoding truncated proteins of 115 and 188 amino acids, respectively, whereas the ALL-SIL cell line harbored a heterozygous frameshift mutation in exon 3, encoding a truncated WT1 protein of 293 amino acids (supplemental Table 3). Analysis of paired leukemic and remission DNA samples in 10 primary T-ALL cases showed loss of the *WT1* mutant allele at remission, demonstrating that *WT1* mutations are somatically acquired in the leukemic clone (data not shown). In

addition to these well-defined *WT1* mutations, we also detected additional nonsynonymous nucleotide substitutions located in exon 1 in 4 T-ALL samples for which no remission DNA was available (supplemental Table 4). Each of these alleles, none of which has been described before, represents 0.002% of the chromosomes analyzed in this study and may constitute somatic mutations or as yet unreported private polymorphisms in the *WT1* gene.

Although our analysis of *WT1* mutations in T-ALL was guided by the identification of *WT1* deletions in our SNP array and FISH analysis, 32 of 38 (84%) of the *WT1* mutations identified in our series showed retention of the wild-type allele. Consistently, FISH analysis of 5 T-ALL samples harboring frameshift mutations in *WT1* demonstrated the absence of deletions or translocations in 11p13 involving the *WT1*-wild-type allele. Notably, each of the 2 T-ALL cases harboring a R462 *WT1* missense mutation showed complete inactivation of WT1 via loss of the *WT1*-wild-type allele in the case of the R462P mutation or the result of an associated frameshift mutation in exon 7 in the case of the R462Q sample (Figure 2F; supplemental Table 1).

Mutation analysis of 10 paired diagnostic and relapsed T-ALL samples showed that *WT1* mutations were present both at diagnosis and relapse in 5 cases (supplemental Table 5). Interestingly, we noted that in one sample the signal of the DNA sequence corresponding to the mutant *WT1* allele was weaker than that of the corresponding wild-type allele at diagnosis, with both alleles becoming equally abundant at relapse. In addition, in 1 additional sample, the *WT1* mutation was present only in the relapse sample. Notably, in the 4 remaining sample pairs, we observed evidence of *WT1* mutations only at diagnosis (supplemental Table 5).

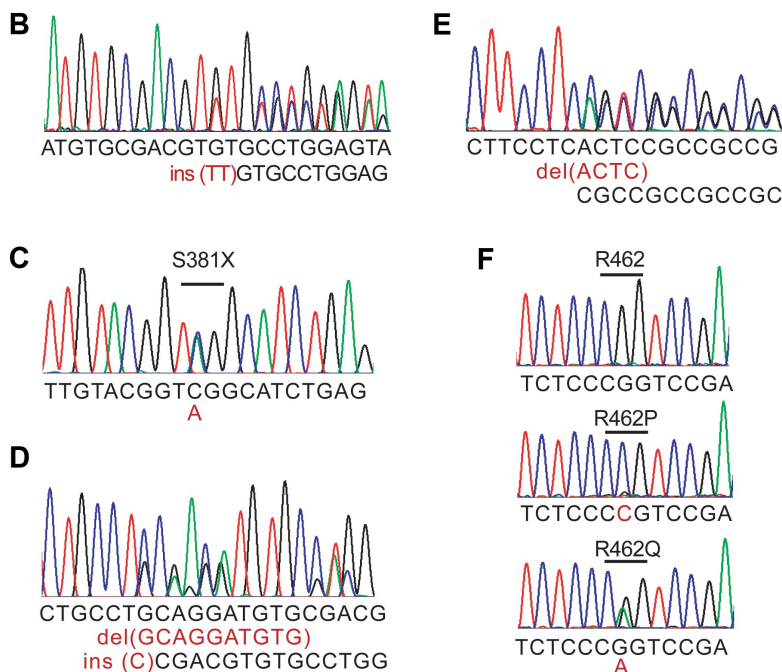
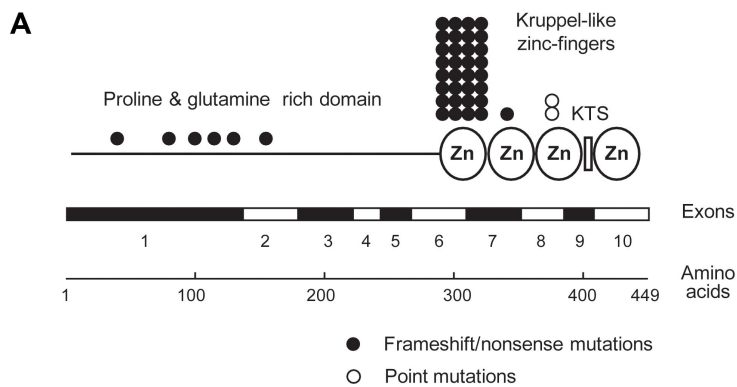


Figure 2. Somatic mutations in *WT1* in T-ALL. (A) Schematic representation of *WT1* mutations identified in primary T-ALL samples. (B-F) DNA sequence chromatograms showing representative examples of different mutations in *WT1*. (B) Frameshift truncating mutation in exon 7 of *WT1*. (C) Nonsense point mutation in exon 7 of *WT1*. (D) Acceptor site mutation affecting exon 7 of *WT1*. (E) Frameshift truncating mutation in exon 1 of *WT1*. (F) Missense mutations involving R462 in the DNA-binding domain of *WT1*.

The development of Wilms tumors in patients with WARG syndrome, who harbor germline deletions of the *WT1* locus, is invariably associated with mutational loss of the second *WT1* allele.²⁶ However, 32 of 38 (84%) of the *WT1* mutations found in our T-ALL series were heterozygous, suggesting that epigenetic mechanisms could account for the inactivation of the second copy of this tumor suppressor gene in T-cell lymphoblasts. To test this hypothesis, we performed DNA methylation analysis of regulatory sequences in the *WT1* promoter using a combined bisulfite restriction analysis assay. Notably, this analysis failed to detect any significant methylation in the *WT1* locus in *WT1*-wild-type (n = 6) and mutated (n = 6) T-ALL samples (data not shown). Consistently, direct sequence analysis of reverse-transcribed PCR-amplified *WT1* transcripts in 2 primary T-ALL samples harboring truncating mutations in *WT1* with available cDNA demonstrated biallelic expression of both the wild-type and mutant alleles of *WT1* (data not shown). Overall, only 3 *WT1* mutations found in our series were homozygous, with 3 additional cases showing compound heterozygous *WT1* mutations.

Association of *WT1* mutations with expression of *TLX1*, *TLX3*, and *HOXA* oncogenes

The malignant transformation of T-cell progenitors requires the cooperative action of different oncogenic pathways. Most promi-

nently, aberrant activation of NOTCH signaling resulting from activating mutations in *NOTCH1* or mutational loss of *FBXW7* is found in more than 60% of T-ALL cases.^{12,14,27-29} In addition, aberrant expression of transcription factor oncogenes, such as *TALI*, *LMO1*, *LMO2*, *HOXA*, *TLX1*, and *TLX3*, plays a major role in the pathogenesis of T-ALL and defines distinct molecular groups with prognostic significance.^{15,30-33}

Mutation analysis of *NOTCH1* and *FBXW7* in 85 adult and 37 pediatric T-ALLs showed a similar incidence of these NOTCH-activating lesions in *WT1*-wild-type (63 of 102 [61.7%] for *NOTCH1* and 19 of 102 [18.6%] for *FBXW7*) and *WT1* mutated (11 of 20 [55%] for *NOTCH1* and 5 of 20 [25%] for *FBXW7*) T-ALL cases (Fisher exact test: $P = .62$ for *NOTCH1* and $P = .54$ for *FBXW7*). In contrast, analysis of associated genomic lesions associated with activation of T-ALL transcription factor oncogenes in 127 pediatric T-ALL patients showed that *WT1* mutations were associated with genetic rearrangements resulting in oncogenic activation of *HOX* genes. Thus, 8 of 13 *WT1* mutant cases had an associated *TLX3/HOX11L2* translocation (Fisher exact test: $P < .001$). In addition, 1 patient had a *TLX1/HOX11* translocation and 2 patients demonstrated aberrant activation of *HOXA* genes because of the presence of *inv(7)(p15q34)*, which involves the *HOXA* and *TCRB* loci³² (1 patient), or because of the *SET-NUP214* fusion associated with the *del(9)(q34.11q34.13)*.³³ Overall, 11 of

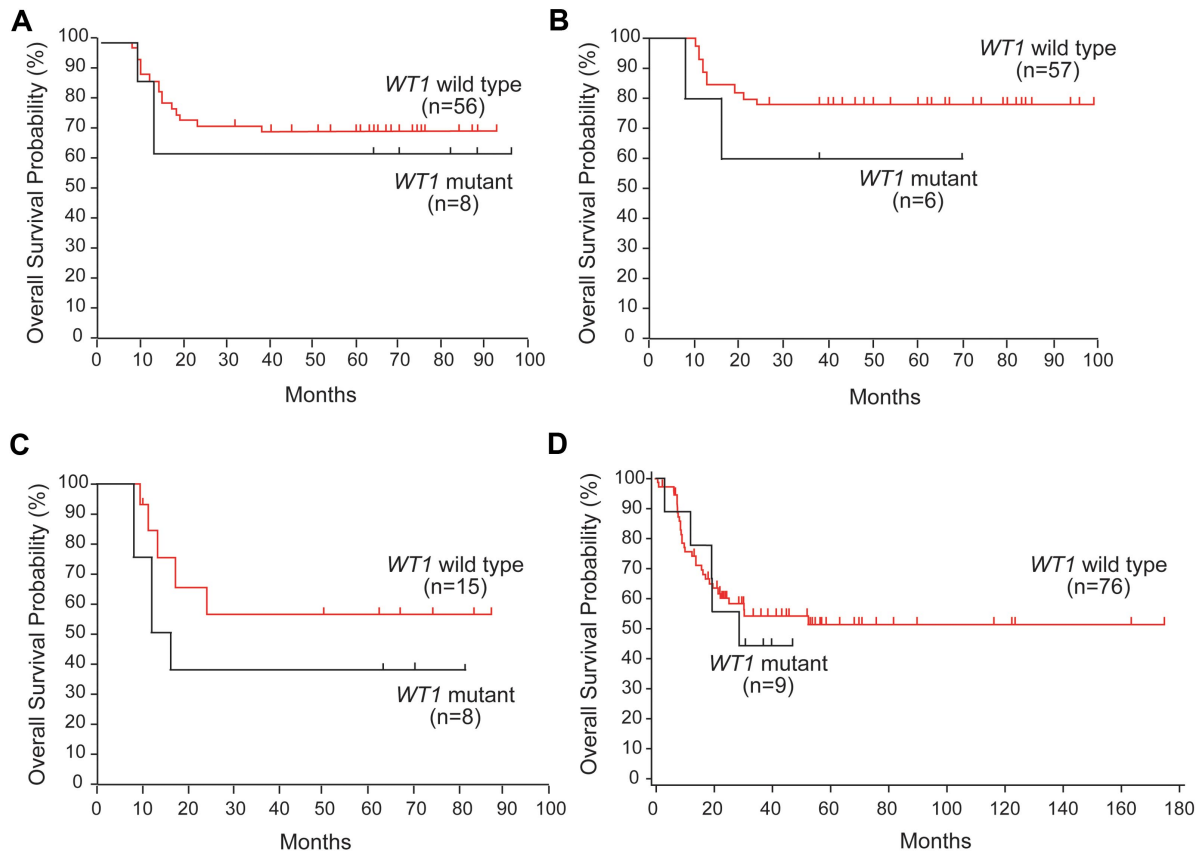


Figure 3. Survival analysis of *WT1* mutations in pediatric and adult T-ALL. (A-B) Kaplan-Meier plots of overall survival for pediatric T-ALL patients treated on DCOG (A) and COALL-97 (B) protocols. (C) Kaplan-Meier plot of overall survival for *TLX3* positive pediatric T-ALL patients. (D) Overall survival analysis in adult T-ALL patients treated in the MRC UKALLXII/ECOG E2993 trial.

13 (85%) *WT1* mutant cases showed aberrant expression of oncogenic *HOX* factor gene. In 1 additional patient, the *WT1* mutation was found in association with an *LMO2* translocation, whereas for 2 other patients no specific translocations were identified.

Prognostic impact of *WT1* mutations in T-ALL

Analysis of the prognostic impact of *WT1* mutations in AMLs has recently demonstrated that AML cases harboring genetic lesions in *WT1* have a poor prognosis.^{34,35} To analyze whether *WT1* mutations are also associated with an adverse clinical outcome in T-ALL, we performed survival analysis in pediatric and adult T-ALL cases.

We investigated the presence of *WT1* mutations in 2 independent cohorts of pediatric T-ALL patients treated on DCOG protocols ($n = 64$)¹⁵ or the COALL-97 protocol ($n = 63$).³⁶ *WT1* mutations were identified in 8 of 64 (12.5%) and 6 of 63 (9.5%) patients treated on DCOG or COALL-97 protocols, respectively. The disease-free survival at 5 years for the DCOG cohort was 62.5% plus or minus 17% versus 70% plus or minus 6% (log-rank: $P = .58$) for *WT1* mutated and wild-type patients, respectively, whereas overall survival at 5 years was 62.5% plus or minus 17% for *WT1* mutated samples compared with 66% plus or minus 6% for *WT1*-wild-type T-ALLs (log-rank: $P = .74$; Figure 3A). For the COALL cohort, the 5-year disease-free survival for *WT1* mutated versus wild-type patients was 60% plus or minus 21% versus 78% plus or minus 6% (log-rank: $P = .29$) and the 5-year overall survival was 60% plus or minus 21% versus 70% plus or minus 8% (log-rank: $P = .54$; Figure 3B). Given the higher prevalence of *WT1* mutations in T-ALL cases characterized by aberrant expression of *TLX1/HOX11*, *TLX3/HOX11L2*, and *HOXA* homeotic

transcription factor oncogenes, we analyzed the specific prognostic significance of *WT1* mutations in a group of 23 *TLX3/HOX11L2*-positive T-ALLs. This analysis showed that the presence of *WT1* mutations did not predict outcome in *TLX3/HOX11L2* group. The 5-year disease-free survival for *TLX3/HOX11L2*-positive *WT1*-mutant cases was 37.5% plus or minus 17% versus 56% plus or minus 15% for *TLX3/HOX11L2*-positive *WT1*-wild-type T-ALLs (log-rank: $P = .29$; Figure 3C).

Next we analyzed the effects of *WT1* mutations on the survival of 85 adult T-ALL patients treated in the MRC UKALLXII/ECOG E2993 trial. *WT1* mutations were present in 9 of 85 (10.5%) T-ALL cases in this series. Complete remission was achieved in 76 of 85 (90%) of the patients, with no difference in therapy response between *WT1*-wild-type and *WT1*-mutant samples in this cohort ($P = .65$). Similarly, log-rank test analysis of overall survival showed no difference between these 2 groups. Overall survival at 2 years was 60% plus or minus 6% in *WT1*-wild-type T-ALLs versus 56% plus or minus 17% in *WT1* mutated cases ($P = .7$; Figure 3D). Overall, these results support that, in contrast with AML, *WT1* mutations are not associated with poor prognosis in T-ALL.

Clonal evolution and disease relapse in T-ALL

Although analysis of clonality was not the primary aim of our study, careful examination of the pattern of genetic alterations observed in our SNP arrays and the pattern of concordance of additional genetic markers such as *NOTCH1* and *WT1* mutations and *TCR* rearrangements between diagnosis and relapse shows that in 9 of 9 cases the relapsed clone shared at least one genetic marker present in leukemic cells at diagnosis (Table 1, supplemental Table

5). However, we also noted that matched diagnostic and relapsed samples were not identical and that most relapsed samples showed the loss of at least one genetic marker present at diagnosis (Table 1, supplemental Table 5). Acquisition of new chromosomal abnormalities and mutations at relapse was more limited, with only 3 of 9 samples showing the acquisition of new genetic lesions at relapse (Table 1, supplemental Table 5). The model that emerges from these data are that relapsed T-ALLs in our series did not derive from the most predominant clone present at diagnosis, but from an ancestral clone, which represents a related (shares some genetic markers) but different (shows loss of genetic markers and in some cases gain of new ones) subpopulation of leukemic cells.

Discussion

Over the last 2 decades, the study of recurrent chromosomal alterations has led to the identification of numerous oncogenes and tumor suppressor genes involved in the pathogenesis of this disease.³⁷⁻³⁹ More recently, the introduction of molecular cytogenetic techniques in the study of T-ALL has identified new chromosomal rearrangements previously unrecognized using standard cytogenetic assays.^{12,22,31,40-43} However, few studies have addressed the mechanisms of disease progression leading to relapse in T-ALL. Early studies on the role of *TP53* in the pathogenesis of T-ALL revealed *TP53* mutations are rare in T-ALL samples at diagnosis^{44,45} but can be found in approximately 30% of cases at relapse,⁴⁵ suggesting *TP53* inactivation is selected in the leukemic clone as a mechanism to escape the cytotoxic effects of chemotherapy. Given the safeguard function of *TP53* in preserving the integrity of the genome and the mutagenic and genotoxic effects of chemotherapy, one could predict that relapsed leukemias may be characterized by increased genomic instability and harbor an increased number of genetic mutations and chromosomal alterations. However, our SNP array analysis comparing the pattern of chromosomal alterations failed to detect a major increase in chromosomal abnormalities at relapse, suggesting that spontaneous or chemotherapy-induced chromosomal instability may not be a major mutagenic mechanism contributing to therapeutic failure.

After our identification via SNP array and FISH analysis of chromosomal deletions in 11p13 encompassing the *WT1* locus, we have performed an extensive mutation analysis of the *WT1* gene in T-ALL. Our results demonstrate the presence of *WT1* mutations in approximately 10% of pediatric and adult T-ALL cases at diagnosis. Notably, previous reports have shown the absence of *WT1* mutations in pre-B-ALL, suggesting that mutational loss of *WT1* may contribute to transformation of T-cell and myeloid but not B-cell progenitor cells.

Notably, 32 of 38 (84%) of the *WT1* mutated T-ALL samples in our series were heterozygous with integrity of the second *WT1* allele. In addition, we failed to detect epigenetic inactivation of the wild-type *WT1* allele. Consistent with these results, allelic expression analysis in *WT1*-mutated T-ALLs demonstrated the presence of both wild-type and mutant *WT1* transcripts in T-ALL lymphoblasts. Moreover, *WT1* mutations found in T-ALL are typically frameshift insertions and deletions predicted to encode C-terminal truncated *WT1* proteins devoid of DNA-binding activity. The clustering of *WT1* mutations in exon 7 could reflect some dominant-negative effect of the resulting truncated *WT1* protein products. In this regard, one of the original papers on the characterization of

WT1 had already suggested that disruption of the DNA-binding domain of *WT1* could have a dominant-negative effect.⁴⁶ Moreover, *WT1* truncating mutations have been described as dominant-negative alleles associated with the pathogenesis of the DDS.⁴⁷ Nevertheless, the presence of associated deletions in the *WT1*-wild-type allele in some of our cases suggests that *WT1* mutations may only have a partial dominant-negative effect.

Interestingly, *WT1* mutations were particularly prevalent in T-ALL cases harboring chromosomal rearrangements associated with the expression of *TLX1/HOX11*, *TLX2/HOX11L2*, and *HOXA9* transcription factor oncogenes, suggesting that *WT1* loss and aberrant expression of oncogenic homeobox factors may be mechanistically linked in the pathogenesis of T-ALL.

Several studies have reported an association of *WT1* mutations with resistance to chemotherapy and poor prognosis in AML.^{7,34,35,48} However, we failed to detect any significant differences in response to induction therapy and survival between *WT1*-mutated and *WT1*-wild-type T-ALLs. This discrepant result suggests that *WT1* mutations may confer different biologic properties to AML and T-ALL lymphoblasts.

Finally, examination of the pattern of copy number alterations from our SNP array analysis and additional genetic markers, such as *NOTCH1* and *WT1* mutations and *TCR* rearrangements, showed that most T-ALL cases at relapse are not the result of clonal evolution from the predominant leukemic population present at diagnosis. Thus, although all relapsed T-ALLs shared at least one genetic marker with the predominant clone at diagnosis, they also showed frequent loss of copy number alterations and mutations, resulting in a genetically related but distinct disease. These results are in agreement with those reported in an extensive study analyzing the clonality of ALLs at diagnosis and relapse using SNP array analysis.⁴⁹ In that study, most ALLs revealed changes in the pattern of alterations during disease progression. Thus, although 88% of ALLs shared genetic markers between the diagnosis and relapse, 52% of the cases showed loss of at least one genetic marker, demonstrating that clonal evolution from an ancestral clone is a frequent mechanism associated with relapse in this disease.⁴⁹

In conclusion, our results identify the presence of *WT1* mutations as a recurrent genetic abnormality associated with aberrant *HOX* gene expression in T-ALL.

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A complete list of the DCOG and the German COALL participants can be found in the supplemental Appendix.

Authorship

Contribution: V.T., M.R.M., K.B., M.P., M.L.S., S.J., C.G.A., R.E.G., T.P., P.R., and V.M. performed research; X.Y. and S.M.R. performed statistical analysis; D.C.L., A.G., J.R., G.B., P.H.W., E.P., R.P., M.H., and J.P.P.M. provided samples and supervised

clinical studies; A.A.F. designed and supervised the study; and A.A.F. and V.T. wrote the manuscript.

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