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ARTICLE

DNMT3A mutation is associated with increased age and adverse outcome in adult T-cell acute lymphoblastic leukemia

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ABSTRACT

The prognostic implications of *DNMT3A* genotype in T-cell acute lymphoblastic leukemia are incompletely understood. We performed comprehensive genetic and clinico-biological analyses of T-cell acute lymphoblastic leukemia patients with *DNMT3A* mutations treated during the GRAALL-2003 and -2005 studies. Eighteen of 198 cases (9.1%) had *DNMT3A* alterations. Two patients also had *DNMT3A* mutations in non-leukemic cell DNA, providing the first potential evidence of age-related clonal hematopoiesis in T-cell acute lymphoblastic leukemia. *DNMT3A* mutation was associated with older age (median 43.9 years *vs.* 29.4 years, $P<0.001$), immature T-cell receptor genotype (53.3% *vs.* 24.4%, $P=0.016$) and lower remission rates (72.2% mutated *vs.* 94.4% non-mutated, $P=0.006$). *DNMT3A* alterations were significantly associated with worse clinical outcome, with higher cumulative incidence of relapse (HR 2.33, 95% CI: 1.05-5.16, $P=0.037$) and markedly poorer event-free survival (HR 3.22, 95% CI: 1.81-5.72, $P<0.001$) and overall survival (HR 2.91, 95% CI: 1.56-5.43, $P=0.001$). Adjusting for age as a covariate, or restricting the analysis to patients over 40 years, who account for almost 90% of *DNMT3A*-mutated cases, did not modify these observations. In multivariate analysis using the risk factors that were used to stratify treatment during the GRAALL studies, *DNMT3A* mutation was significantly associated with shorter event-free survival (HR 2.33, 95% CI: 1.06 – 4.04, $P=0.02$). Altogether, these results identify *DNMT3A* genotype as a predictor of aggressive T-cell acute lymphoblastic leukemia biology. The GRAALL-2003 and -2005 studies were registered at *http://www.ClinicalTrials.gov* as *#NCT00222027* and *#NCT00327678*, respectively.

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Introduction

Mutations in the DNA methyltransferase 3 alpha gene (*DNMT3A*) have been reported in a range of hematologic malignancies, most frequently in myeloid neoplasia, including acute myeloid leukemia (AML),[1-5] myelodysplastic syndromes,[6] myeloproliferative neoplasms[7] and myeloproliferative neoplasm/myelodysplastic overlap syndromes.[8,9] *DNMT3A* alterations in lymphoid malignancies are less common, and reports to date are confined to T-lineage disease.[10-16] In all cases, *DNMT3A* mutations increase in frequency with age, and are extremely rare in children and adolescents.[17-19]

Multiple studies have reported that *DNMT3A* alterations correlate with poor outcome in AML.[1,2,4,20-22] In comparison, the prognostic influence of *DNMT3A* mutation in T-cell acute lymphoblastic leukemia (T-ALL) is poorly characterized. Patients with *DNMT3A* alterations were reported to have shorter survival in three moderately sized (55 to 93 patients) T-ALL cohorts.[9,11,13] *DNMT3A* status did not however independently predict prognosis in the only series for which multivariate analyses were documented, as survival effects were linked to increased rates of *DNMT3A* mutation in poor-risk, phenotypically immature disease.[11] While that study did document a correlation between *DNMT3A* alteration and survival within the immature T-ALL subgroup, this finding was not corroborated in an independent cohort of early thymic precursor (ETP) ALL cases.[12]

The issue of whether *DNMT3A* mutation truly alters the biology of T-ALL is therefore only partially addressed by the currently available evidence. In particular, it is unclear whether the associated poor survival simply reflects the prosaic fact that patients with *DNMT3A* alterations are older,[11,12] and therefore do not tolerate intensive ALL treatment as well as their younger counterparts.

In order to address this question, we used next-generation sequencing (NGS) to evaluate the *DNMT3A* genotype of a large cohort of 198 adult T-ALL patients treated as part of the multinational GRAALL-2003 and -2005 studies. We found that *DNMT3A* mutation strongly correlated with disease relapse and shorter survival, and that these prognostic effects were independent of patients' age. Furthermore, we report the presence of *DNMT3A* mutations in non-leukemic cells in a subset of patients, providing the first evidence of age-related clonal hematopoiesis in T-ALL.

Methods

Patients

Details of the GRAALL-2003 and -2005 studies are provided in the *Online Supplementary Methods*. Informed consent was obtained from all patients before inclusion into the trials. Both studies were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees. The complete study protocols are detailed in the *Online Data Supplement*. Both trials were registered at *http:/www.ClinicalTrials.gov* (NCT00222027, NCT00327678). The criteria for inclusion in the current project were a diagnosis of T-ALL and the availability of diagnostic material for NGS analysis of *DNMT3A* genotype. Survival outcomes of the 198 patients (36 from GRAALL-2003 and 162 from GRAALL-2005) who fulfilled these criteria did not differ from those of the remaining 139 T-ALL patients of the study cohorts. As expected in retrospective studies, initial white blood cell count (WBC) was higher in the study cohort. However, no differences in allogeneic stem cell transplant rate, disease-free survival, event-free survival, or overall survival were found. A full comparison of the clinical features of each group is shown in *Online Supplementary Table S1*.

Next-generation sequencing

Nextera XT (Illumina) DNA Libraries were prepared according to the manufacturer's instructions and sequenced using the Illumina MiSeq sequencing system. The custom NGS panel comprised genes coding for factors involved in molecular pathways known to be mutated in T-ALL, namely cytokine receptor and RAS signaling (*NRAS, KRAS, JAK1, JAK3, STAT3, STAT5B, IL7R, BRAF, NF1, SH2B3, PTPN11*), hematopoietic development (*RUNX1, ETV6, GATA3, IKZF1, EP300*), chemical modification of histones (*SUZ12, EED, EZH2, KMT2A, KMT2D, SETD2*) and DNA methylation (*DNMT3A, IDH1, IDH2, TET2, TET3*). This panel was originally inspired by the repertoire of genes found to be preferentially altered in pediatric ETP-ALL[23] and we have reported a subset of the results described in the current paper in a previous clinico-biological and genetic analysis of adult ETP-ALL.[24] Sequencing reads were analyzed using in-house software (Polyweb, Institut Imagine, Paris, France), and additional in-house custom filtering criteria (comprising minimum read counts and variant allele frequencies, and reference to external reference databases) were applied to minimize false-positive rates. Primers used to confirm mutations by direct sequencing are listed in *Online Supplementary Table S2*.

Outcome analyses

Comparisons between groups were performed with the Fisher exact and Mann-Whitney tests for categorical and continuous variables respectively. Corticosteroid sensitivity was defined as clearance of peripheral blood circulating blasts (<1 x 10^9/L) following steroid prophase treatment. Complete remission was defined as clearance of bone marrow blasts (<5%) following induction treatment. Overall survival was calculated from the date of inclusion in the trial to the last follow-up date, censoring patients alive at that date. Event-free survival was calculated from date of inclusion in the trial to the date of induction failure, relapse, or death, censoring patients alive in first complete remission without relapse at the last follow-up date. Cumulative incidence of relapse was calculated in patients who attained complete remission, from the date of achieving the complete remission to the date of relapse, with death in first complete remission being considered as a competing event. Univariate and bivariate analyses assessing the impact of *DNMT3A* mutations and age were performed with a Cox model. Variables that were significantly associated with outcome in univariate analysis were considered as covariates in multivariate Cox models. The proportional-hazards assumption was checked before conducting multivariate analyses. Statistical analyses were performed with STATA software (STATA 12.0 Corporation, College Station, TX, USA). All *P* values were two-sided, with $P<0.05$ denoting statistical significance.

Results

Analysis of *DNMT3A* genotype in patients with T-cell acute lymphoblastic leukemia in the GRAALL studies

We performed targeted NGS of a panel of genes, including *DNMT3A*, which have been described to be recurrently mutated in T-ALL. This panel included all exons of *DNMT3A*, thereby providing a comprehensive picture of

the spectrum of alterations across this gene in T-ALL. Diagnostic DNA was available for 198 patients treated during the GRAALL-2003 and -2005 studies. A partial analysis of a subgroup of this cohort has been reported previously.[24] We detected 21 *DNMT3A* mutations in 18 patients (9.1%). Most alterations occurred in regions coding for defined protein functional domains, including six mutations at the R882 hotspot[1] (Figure 1A). Further details of patient-specific alterations are shown in *Online Supplementary Table S3*. Of note, the vast majority of detected mutations are predicted to be significantly damaging to protein function.

In keeping with previous reports of *DNMT3A*-mutated T-ALL which cited high rates of either compound heterozygosity or homozygosity,[9,11] a significant proportion of cases (8/18) had either two separate alterations, or high variant allele frequencies that were suggestive of either homozygous mutation, concomitant deletion of the wild-type (WT) allele or copy-neutral loss of heterozygosity. Comparative genomic hybridization analyses were available for 85 of the cases in this study, including 6/18 patients with *DNMT3A* mutations. We detected only two deletions of the *DNMT3A* locus, which in each case were associated with concomitant *DNMT3A* mutation and elevated variant allele frequencies (cases 11 and 12 in *Online Supplementary Table S3*).

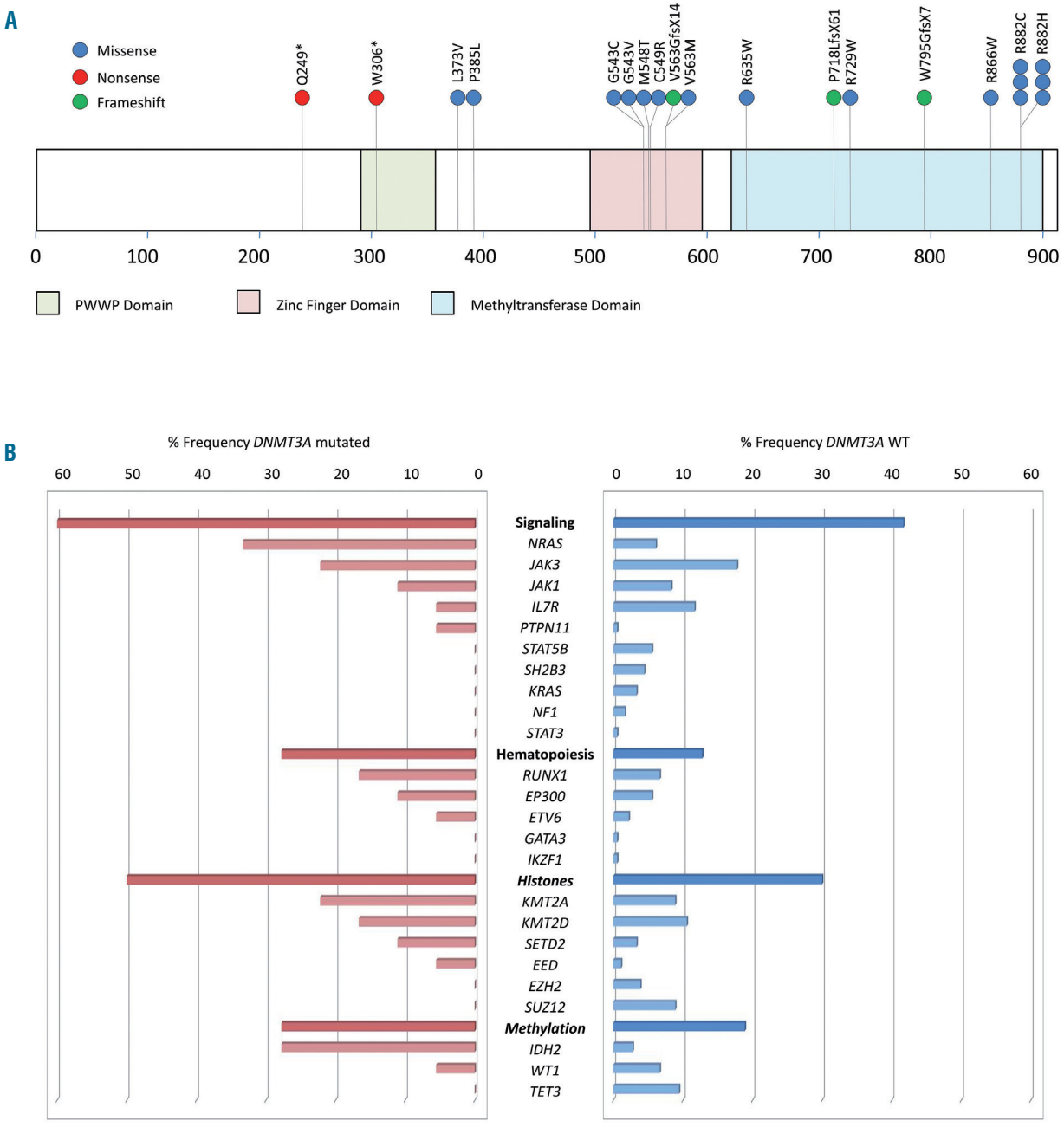

Figure 1. *DNMT3A* mutations in T-cell acute lymphoblastic leukemia. (A) Schematic representation of the 21 mutations detected in this study. Further patient-specific details are provided in *Online Supplementary Table S3*. (B) Comparison of the mutational genotypes of *DNMT3A* altered (n=18) and *DNMT3A* wild-type (n=180) T-cell acute lymphoblastic leukemia. Percentage frequencies in each group are depicted. Functional categories are listed in bold.

The prevalence of other mutations detected by NGS is shown in Figure 1B. *DNMT3A*-altered cases had an increased frequency of alterations in other genes included in the NGS panel, compared with the rest of the cohort (88.9% *DNMT3A* mutated *vs.* 64.4% *DNMT3A* WT, *P*=0.036). There were no statistically significant differences in the prevalence of mutations in any specific functional gene category, namely factors involved in cytokine receptor and RAS signaling (61.1% *DNMT3A* mutated *vs.* 41.7% *DNMT3A* WT, *P*=0.113), hematopoiesis (38.5% *DNMT3A* mutated *vs.* 12.8% *DNMT3A* WT, *P*=0.082) and chemical modification of histones (50.0% *DNMT3A* mutated *vs.* 30.0% *DNMT3A* WT, *P*=0.082). However, we did observe significant co-occurrence of *DNMT3A* alterations and *IDH2* mutations (27.8% *DNMT3A* mutated *vs.* 2.2% *DNMT3A* WT, *P*<0.001). This association has been described previously in both AML[25] and myelodysplastic syndromes.[26]

Evidence of possible clonal hematopoiesis in *DNMT3A*-mutated T-cell acute lymphoblastic leukemia

DNMT3A is the most commonly altered gene in age-related clonal hematopoiesis[27-29] and *DNMT3A* mutations have been detected in non-malignant cells in AML[30,31] and peripheral T-cell lymphoma.[14] We therefore tested whether *DNMT3A* mutations were present in non-leukemic hematopoietic cells in T-ALL patients.

DNA from remission bone marrow was available for only three of the 18 patients with *DNMT3A* mutations. While two of these samples had a *DNMT3A* WT genotype (*Online Supplementary Figure S1*), one interesting case had evidence of *DNMT3A* alterations in non-leukemic bone marrow cells. At diagnosis, this patient (case 6 in *Online Supplementary Table S3*) had mutations in exons 14 and 15 of *DNMT3A*, a *NOTCH1* PEST domain insertion, and an *NRAS* G12D substitution. Sequencing of remission DNA revealed mutation of *DNMT3A* exon 14 in non-leukemic cells, while *NOTCH1*, *NRAS* and *DNMT3A* exon 15 all presented wild-type genotypes (Figure 2A). We confirmed that the exon 14 mutation has never been reported as a polymorphic variant, while SIFT analysis (*http://sift.jcvi.org/*) predicted this M548T substitution to be highly deleterious to protein function, with a SIFT score of 0. These results suggest that this T-ALL may have developed on a background of *DNMT3A*-mutated clonal hematopoiesis, and that the other genetic alterations, including the second *DNMT3A* mutation, were acquired at leukemic transformation.

In order to extend this analysis of non-leukemic *DNMT3A* mutation, we performed immunophenotypic sorting of two further diagnostic bone marrow samples, and extracted DNA from both the leukemic and the minor residual non-leukemic fractions. We detected a mutation in non-leukemic DNA in one patient (case 4 in *Online Supplementary Table S3*). Again, we confirmed that this mutation has not been reported as a polymorphism, and that the resultant P385L substitution is predicted to damage protein function, with a SIFT score of 0.02. Similar to the case with mutated remission DNA, this sample was negative for two *NOTCH1* alterations detected at T-ALL diagnosis, confirming the specificity of *DNMT3A* mutation persistence (Figure 2B). The other tested non-leukemic DNA had a *DNMT3A* WT genotype (*Online Supplementary Figure S2*), giving an overall rate of non-leukemic *DNMT3A* mutant positivity of 2/5 samples from the GRAALL-2003 and -2005 studies. We also tested a further three T-ALL cases not included in this cohort, but found no evidence of non-leukemic *DNMT3A* mutation. This gives an overall incidence of possible clonal hematopoiesis in 2/8 T-ALL samples assessed in our laboratory. It was unfortunately not possible to obtain non-hematopoietic tissue from either of these patients, in order to exclude that these alterations were not constitutional, and to confirm definitively that these results reflect the persistence of a *DNMT3A*-mutated clonal hematopoietic population in these cases.

DNMT3A mutations are associated with older age and treatment resistance

A clinico-biological comparison of cases with and without *DNMT3A* mutations is shown in Table 1. In keeping with previous reports,[11,12] patients with mutations were considerably older than the rest of the T-ALL cohort (median age 43.9 years mutated *vs.* 29.4 years non-mutated, *P*<0.001). In addition, *DNMT3A*-mutated leukemias were more likely to have an immature T-receptor genotype[32] (53.3% mutated *vs.* 24.4% non-mutated, *P*=0.016), although this did not correspond to a significantly higher incidence of an ETP-ALL immunophenotype[33] (35.7% mutated *vs.* 20.3% non-mutated, *P*=0.184).

DNMT3A mutation was notably associated with poor initial treatment response. We observed trends towards early corticosteroid resistance (66.7% mutated *vs.* 43.3% non-mutated, *P*=0.081) and induction failure (13.3% *vs.* 2.9%, *P*=0.096), and patients with *DNMT3A* mutations had significantly higher rates of death during induction (16.7% *vs.* 2.8%, *P*=0.027), and lower attainment of complete remission (72.2% mutated *vs.* 94.4% non-mutated, *P*=0.006). As only four patients with mutations were evaluated for minimal residual disease, we could not verify that molecular remission was similarly compromised.

We found that the type of *DNMT3A* mutation did not significantly correlate with any individual clinico-biological parameter, suggesting that the alterations detected in this study are likely to have broadly similar biological consequences.

DNMT3A mutation correlates with poor outcome in T-cell acute lymphoblastic leukemia

The median follow-up of the cohort was 5.5 years. Prognostic analyses revealed that *DNMT3A* mutation was associated with an increased 5-year cumulative incidence of relapse (53.9% mutated *vs.* 28.7% non-mutated, *P*=0.037) (Figure 3A) and with 5-year event-free survival [27.8% mutated *vs.* 61.0% non-mutated; hazard ratio (HR) 3.22, (95% confidence interval (95% CI): 1.81-5.72, *P*<0.001] (Figure 3B). Patients with *DNMT3A* mutations also had a markedly inferior 5-year overall survival (38.8% mutated *vs.* 68.7% non-mutated, HR 2.91, 95% CI: 1.56-5.43, *P*=0.001) (Figure 3C).

The poor prognosis of *DNMT3A*-mutated T-cell acute lymphoblastic leukemia is age-independent

Our and others' data[11,12] have shown that the incidence of *DNMT3A* mutation in T-ALL increases with age, but previous reports have not documented whether this factor contributes to prognosis. As older patients treated during the GRAALL studies had worse outcomes due to impaired tolerance of intensive chemotherapy,[34] we considered it critical to determine to what extent age was a confounding prognostic variable.

We therefore performed bivariate analyses of the effects of *DNMT3A* mutations and age across a series of outcome measures. These results are shown in *Online Supplementary Table S4*. In each case, *DNMT3A* genotype was still associated with significantly increased cumulative incidence of relapse (HR 2.80, 95% CI: 1.12-6.97, *P*=0.034), and shorter event-free survival (HR 2.62, 95% CI: 1.45–5.06, *P*=0.004) and overall survival (HR 2.05, 95% CI: 1.02-4.12, *P*=0.043).

Since *DNMT3A* alterations were almost exclusively found in patients >40 years (16/18 cases), we also performed survival analyses that were restricted to the >40-year old subgroup, which constituted a quarter of the total cohort of patients (50/198, 25.3%). Consistent with the results of the bivariate analyses, *DNMT3A* mutation was associated with significantly worse 5-year cumulative incidence of relapse (58.3% mutated *vs.* 21.7% non-mutated, HR 3.90, 95% CI: 1.30-11.68, *P*=0.015) (Figure 4A), 5-year event-free survival (25.0% mutated *vs.* 56.7% non-mutated, HR 2.95, 95% CI: 1.37-6.32, *P*=0.005) (Figure 4B), and 5-year overall survival (37.5% mutated *vs.* 62.1% non-mutated, HR 2.35, 95% CI: 1.05-5.26, *P*=0.038) (Figure 4C).

Finally, we carried out multivariate outcome analyses in the whole cohort using the risk factors that were used to stratify treatment during the GRAALL-2003 and -2005 studies, and which were found to significantly predict prognosis in the univariate analyses. Among age, $\log_{(WBC)}$,

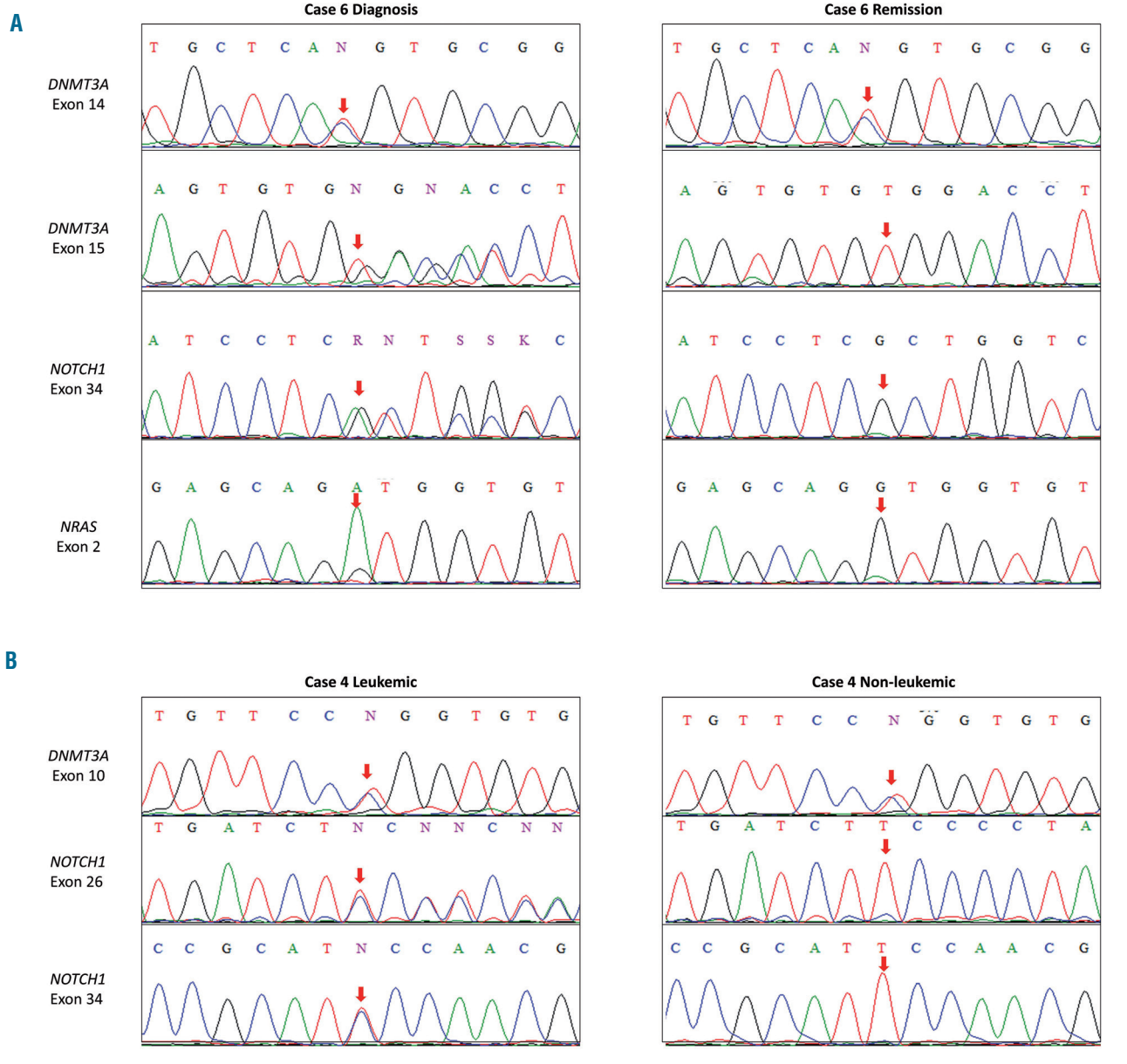

Figure 2. Evidence of *DNMT3A* mutations in non-leukemic DNA. (A) Direct sequencing of *DNMT3A* exons 14 and 15, *NOTCH1* and *NRAS* in diagnostic (left panels) and remission (right panels) samples. (B) Mutational assessment of DNA extracted from leukemic and non-leukemic fractions of samples from patients with T-cell acute lymphoblastic leukemia. Sequencing results of *DNMT3A* and *NOTCH1* in leukemic (left panels) and non-leukemic (right panels) DNA are shown. Cases are numbered according to the listing in *Online Supplementary Table S3*.

corticosteroid sensitivity, early chemosensitivity, and *DNMT3A* genotype, only *DNMT3A* genotype was associated with cumulative incidence of relapse in univariate analysis (*data not shown*). As shown in Tables 2 and 3, age, $\log_{(WBC)}$, corticosteroid resistance along with *DNMT3A* genotype were significantly associated with a poor event-free survival and overall survival. In multivariate analysis adjusting for these covariates, *DNMT3A* mutation was still significantly associated with shorter event-free survival (HR 2.33, 95% CI: 1.06–4.04, *P*=0.02) (Table 2), although not with overall survival (HR 1.66, 95% CI: 0.82–3.37, *P*=0.16) (Table 3).

Taken together, these results provide strong evidence that *DNMT3A* mutation, while mostly observed in older cases, predicts a poor prognosis that is not related to the patient's age.

Discussion

To our knowledge, this is the most extensive study of *DNMT3A*-mutated T-ALL yet reported. Our targeted NGS approach allowed comprehensive assessment of genotype across the entire *DNMT3A* locus, along with the prevalence of co-occurring genetic alterations. Our data additionally benefit from the analysis of a large cohort of patients who were uniformly treated as part of the GRAALL-2003 and -2005 studies, thereby allowing rigorous outcome comparisons between mutated and wild-type cases.

Some of our results were expected, and the findings that *DNMT3A* mutations are more commonly present in older patients and genotypically immature leukemias are consistent with previously published data.[9,11-13] We did not, however, observe increased rates of ETP-ALL immunophenotype, as might have been predicted. We did not detect a clear association with any other genetically-defined subgroup, and there was no link to increased HOXA expression, which we have previously shown to predict outcome in immature T-ALL.[35]

The detection of *DNMT3A* alterations in non-leukemic bone marrow suggests that some of these cases of T-ALL might have arisen from *DNMT3A*-mutated clonal hematopoiesis. While pre-leukemic *NOTCH1* mutations have been detected in neonatal blood spot samples of pediatric patients with T-ALL,[36] to our knowledge our data provide the first potential evidence of age-related clonal hematopoiesis in T-ALL. As it was not possible to obtain non-hematopoietic tissue from either of the patients with this finding, we cannot definitively exclude that these alterations are constitutional, or might even represent an inherited cancer predisposition. Further work is necessary to investigate the incidence of clonal hematopoiesis linked to alterations in *DNMT3A* and other genes in T-ALL.

Non-leukemic *DNMT3A* mutations have been seen in AML,[30,31] and it has been postulated that *DNMT3A*-altered immature T-ALL might arise from malignant transformation of a multipotent myeloid/lymphoid progenitor cell.[12] In keeping with this, one of the cases with a non-leukemic *DNMT3A* mutation in this study had expression of myeloid cell surface markers as part of an ETP-ALL phe-

Table 1. Characteristics and outcome of the patients according to *DNMT3A* genotype.

	DNMT3A Mutated	*DNMT3A* Wild-type	Total	*P*-value
Total (%)	18 (9.1%)	180 (90.9%)	198 (100%)	
Clinical subsets analyzed				
Male	13 (72.2%)	128 (71.1%)	141 (71.2%)	0.921
Median age (years) [IQR]	43.9 [40.7–53.6]	29.4 [23.2–37.2]	30.5[23.4-40.4]	**<0.001**
WBC (10^9/L, median)	41.1	31.9	32.6	0.491
CNS involvement	3 (16.7%)	21 (11.7%)	24 (12.1%)	0.463
T-cell receptor status				
Immature (IM0, IMD, IMG[#])	8 (53.3%)	38 (24.4%)	46 (26.9%)	**0.015**
αβ lineage	3 (20.0%)	104 (66.7%)	107 (62.6%)	**<0.001**
γδ lineage	4 (26.7%)	14 (9.0%)	18 (10.5%)	**0.033**
ETP immunophenotype[#]	5 (35.7%)	32 (20.3%)	37 (18.7%)	0.184
Oncogenetics				
HOXA positivity[#]	4 (25.0%)	41 (26.6%)	45 (26.5%)	1.000
NOTCH1/FBXW7 mutated	15 (83.3%)	124 (68.9%)	139 (70.2%)	0.282
RAS/PTEN mutated	5 (29.4%)	33 (19.4%)	38 (20.3%)	0.365
Risk classifier, high[#]	8 (44.4%)	74 (42.3%)	82 (42.5%)	1.000
Early treatment response				
Corticosteroid sensitivity	6 (33.3%)	102 (56.7%)	108 (54.5%)	0.081
Complete remission	13 (72.2%)	170 (94.4%)	183 (92.4%)	**0.006**
Induction death	3 (16.7%)	5 (2.8%)	8 (4.0%)	**0.027**
Induction failure	2/15 (13.3%)	5/175 (2.9%)	7/190 (3.7%)	0.097
5-year treatment outcome				
Cumulative incidence of relapse	53.9%	28.7%	30.5%	**0.037**
Event-free survival	27.8%	61.0%	58%	**<0.001**
Overall survival	38.8%	68.7%	66%	**0.001**

[#]T-cell receptor status (n=171), early thymic precursor (ETP) immunophenotype (n=172), HOXA positivity (n=170) and Risk classifier based on *NOTCH1*, *FBXW7*, *PTEN*, *NRAS* and *KRAS* genotypes (n=193) were determined as previously described.[32,33,35,37] For the Risk classifier, numbers categorized as high risk (*NOTCH1/FBXW7* WT *and/or NRAS/KRAS/PTEN* altered) are shown. Statistically significant results are shown in bold.

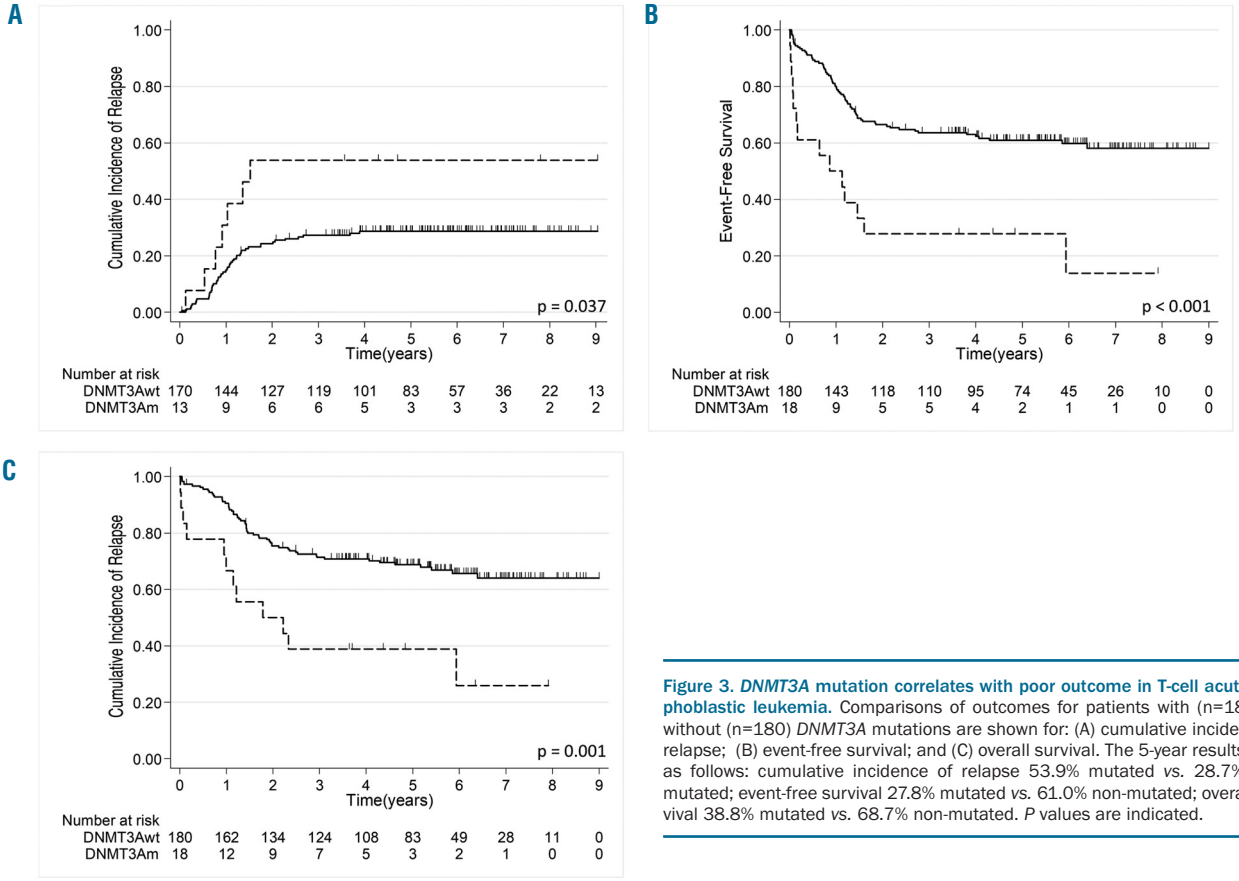

Figure 3. *DNMT3A* mutation correlates with poor outcome in T-cell acute lymphoblastic leukemia. Comparisons of outcomes for patients with (n=18) and without (n=180) *DNMT3A* mutations are shown for: (A) cumulative incidence of relapse; (B) event-free survival; and (C) overall survival. The 5-year results were as follows: cumulative incidence of relapse 53.9% mutated *vs.* 28.7% non-mutated; event-free survival 27.8% mutated *vs.* 61.0% non-mutated; overall survival 38.8% mutated *vs.* 68.7% non-mutated. *P* values are indicated.

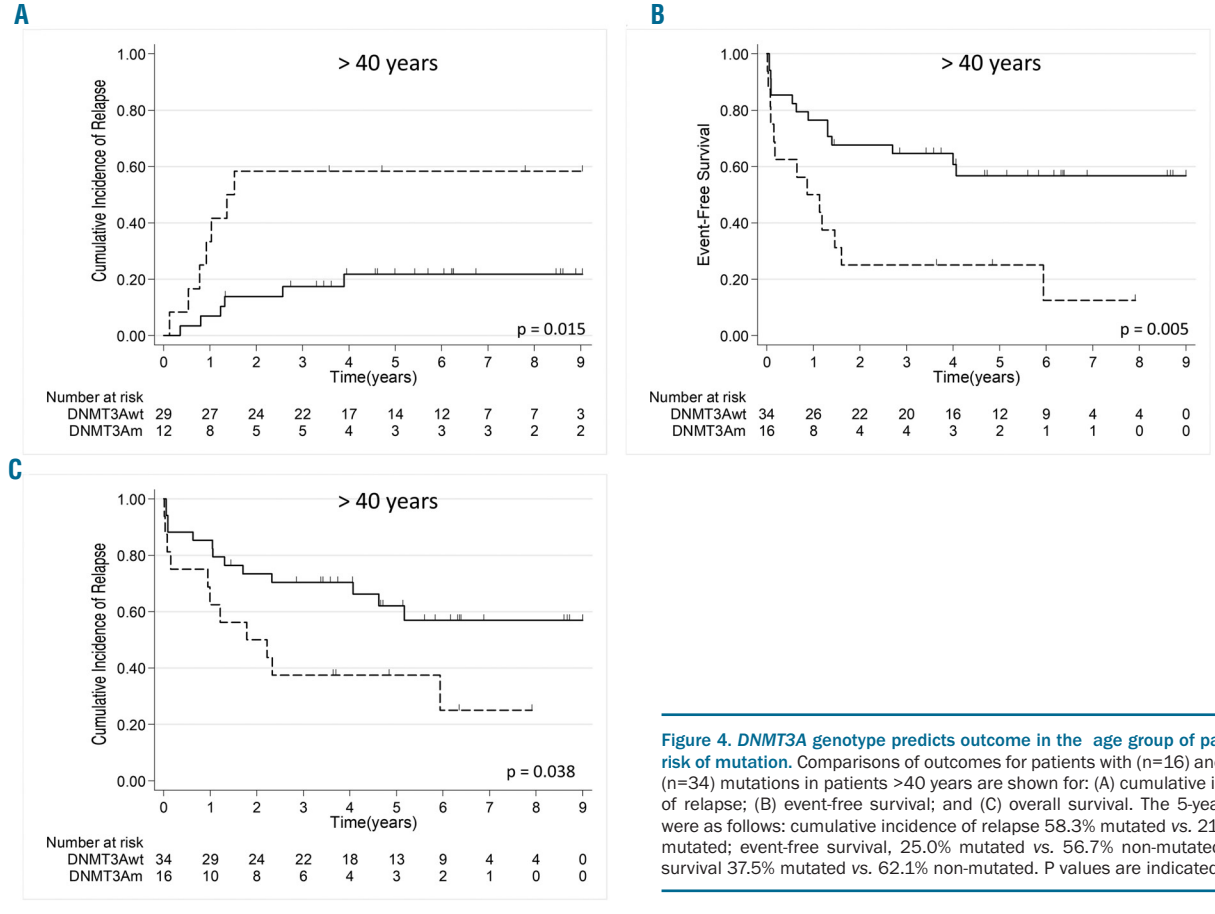

Figure 4. *DNMT3A* genotype predicts outcome in the age group of patients at risk of mutation. Comparisons of outcomes for patients with (n=16) and without (n=34) mutations in patients >40 years are shown for: (A) cumulative incidence of relapse; (B) event-free survival; and (C) overall survival. The 5-year results were as follows: cumulative incidence of relapse 58.3% mutated *vs.* 21.7% non-mutated; event-free survival, 25.0% mutated *vs.* 56.7% non-mutated; overall survival 37.5% mutated *vs.* 62.1% non-mutated. P values are indicated.

Table 2. Prognostic impact of *DNMT3A* genotype on event-free survival.

EFS	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
Age*	1.03	1.01 – 1.05	**0.009**	1.02	1.00 – 1.04	0.071
$Log_{(WBC)}$*	1.62	1.12 – 2.34	**0.011**	1.50	0.98 – 2.29	0.062
Corticosteroid sensitivity	0.52	0.34 – 0.81	**0.003**	0.66	0.41 – 1.07	0.093
Early chemosensitivity	0.90	0.68 – 1.18	0.436	-	-	-
DNMT3A mutation	3.22	1.81 – 5.72	**<0.001**	2.20	1.13 – 4.27	**0.02**

*Continuous variable. Statistically significant differences are highlighted in bold. EFS: event-free survival; HR: hazard ratio; 95% CI: 95% confidence interval; WBC: white blood cell count..

Table 3. Prognostic impact of *DNMT3A* genotype on overall survival.

OS	Univariate			Multivariate		
	HR	95% CI	p	HR	95% CI	p
Age*	1.04	1.01 – 1.06	**0.002**	1.03	1.01 – 1.06	**0.009**
$Log_{(WBC)}$*	1.65	1.10 – 2.46	**0.015**	1.63	1.03 – 2.57	**0.037**
Corticosteroid sensitivity	0.59	0.37 – 0.94	**0.027**	0.79	0.47 – 1.34	0.388
Early chemosensitivity	0.94	0.71 – 1.24	0.640	-	-	-
DNMT3A mutation	2.91	1.56 – 5.43	**0.001**	1.66	0.82 – 3.37	0.160

*Continuous variable. Statistically significant differences are highlighted in bold. OS: overall survival; HR: hazard ratio; 95% CI: 95% confidence interval; WBC: white blood cell count..

notype, while full immunophenotypic assessment was unfortunately not possible for the other patient. The factors that may dictate the acute leukemic phenotype in clonally mutated cases remain to be clarified. For example, this might be influenced by the differentiation capacity of the cell in which the initial *DNMT3A* mutation occurs. In addition, it is tempting to speculate that the acquisition of specific cooperative mutations, such as the *NOTCH1* mutations observed in these T-ALL cases, might act as lineage determinants.

Outcome analyses revealed that *DNMT3A* mutation correlated with poor prognosis independently of the patients' age in bivariate analyses. Multivariable analyses using parameters that were used to stratify treatment in the GRAALL-2003 and -2005 studies showed that *DNMT3A* genotype independently predicted both event-free survival and cumulative incidence of relapse. *DNMT3A* mutation status also independently predicted event-free survival and overall survival in bivariable analyses that incorporated our recently described oncogenetic risk classifier[37] (*Online Supplementary Table S5*). These results suggest that *DNMT3A* mutation is directly linked to aggressive T-ALL biology. As *DNMT3A*-altered T-ALL had higher mutation rates in other genes included in our targeted sequencing panel, it is also possible that increased genotype complexity may contribute to the more aggressive phenotype in these leukemias. This issue may be clarified by more comprehensive genomic assessment in future studies.

The high rates of treatment failure observed in this study suggest that therapeutic intervention is warranted for *DNMT3A*-mutated cases, and that treatment intensification should be considered for the infrequent younger patients with mutations. Indeed, we have previously documented a benefit from allogeneic stem cell transplantation in first complete remission for ETP-ALL,[24] which similarly exhibits high rates of intrinsically treatment-resistant disease. As only three of the 18 *DNMT3A*-mutated patients in this study underwent allogeneic stem cell transplantation (*data not shown*), we are unable to estimate the potential benefit of such treatment in this setting. We recently reported that treatment-related toxicity in the GRAALL-2005 study increased in proportion to the patients' age,[38] and further therapy intensification in elderly patients must therefore be considered of questionable benefit. The upper age of this study cohort was 60 years, but it is likely that the rate of mutations in older patients who do not tolerate such intensive chemotherapy is higher. Data reported for patients with AML suggest that *DNMT3A* mutation confers increased sensitivity to hypomethylating agents,[39] providing a rationale for evaluation of these drugs in *DNMT3A*-mutated T-ALL. In the longer term, it is to be hoped that investigation of the molecular mechanisms by which *DNMT3A* mutation alters T-ALL biology will lead to better treatments and improved outcomes for these high-risk cases.

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