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

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Running Head: DNMT3A mutation predicts adverse outcome in adult T-ALL.

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Abstract:

The prognostic implications of *DNMT3A* genotype in T-ALL are incompletely understood. We performed comprehensive genetic and clinicobiological analyses of T-ALL 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-ALL. *DNMT3A* mutation was associated with older age (median 43.9 years v 29.4 years, $p < 0.001$), immature T-receptor genotype (53.3% v 24.4%, $p = 0.016$) and lower remission rates (72.2% mutated v 94.4% non-mutated, $p = 0.006$). *DNMT3A* alterations were significantly associated with worse clinical outcome, with higher cumulative incidence of relapse (CIR, HR 2.33, 95% CI 1.05-5.16, $p = 0.037$) and markedly poorer event-free survival (EFS, HR 3.22, 95% CI 1.81-5.72, $p < 0.001$) and overall survival (OS, 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 EFS (HR 2.33, 95% CI 1.06 – 4.04, $p = 0.02$). Altogether, these results identify *DNMT3A* genotype as a predictor of aggressive T-ALL biology. The GRAALL-2003 and -2005 studies were registered at http://www.clinicaltrials.gov as #NCT00222027 and #NCT00327678, respectively.

Introduction:

Mutations in the *DNMT3A* (DNA methyltransferase 3 alpha) gene have been reported in a range of hematological malignancies, and most frequently in myeloid neoplasia, including acute myeloid leukemia (AML), [1-5] myelodysplastic syndromes (MDS), [6] myeloproliferative neoplasms (MPN) [7] and MPN/MDS 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] The prognostic influence of *DNMT3A* mutation in T-acute lymphoblastic leukemia (T-ALL) is poorly characterized by comparison. 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 documented 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 patient 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:

Patient inclusion: Details of the GRAALL-2003 and -2005 studies are provided in the Supplementary Methods. Informed consent was obtained from all patients before inclusion into trial. 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 Data Supplement. Both trials were registered at 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 next generation sequencing (NGS) analysis of *DNMT3A* genotype. Survival outcomes of the 198 patients (36 GRAALL-2003 and 162 GRAALL-2005) who fulfilled these criteria did not differ from the remaining 139 T-ALL patients of the study cohorts. As expected in retrospective studies, initial WBC was higher in the study cohort. However, no differences in any of allogeneic stem cell transplant (allo-SCT) rate, disease-free survival, event-free survival (EFS), or overall survival (OS) were found. Full comparison of the clinical features of each group is shown in 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* and *PTPN11*), hematopoietic development (*RUNX1*, *ETV6*, *GATA3*, *IKZF1*, *EP300*), chemical modification of histones (*SUZ12*, *EED*, *EZH2*, *KMT2A*, *KMT2D* and *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 clinicobiological and genetic analysis of adult ETP-ALL. [24] Sequencing reads were analyzed using in-house software (Polyweb, Institut Imagine, Paris), 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 in Supplementary Table S2.

Outcome analyses: Comparison between groups was performed with Fisher's exact and Mann-Whitney test for categorical and continuous variables respectively. Corticosensitivity was defined as clearance of peripheral blood circulating blasts (< 1 x 10^9/L) following steroid prophase. Complete Remission (CR) was defined as clearance of bone marrow blasts (<5%) following induction treatment. Overall survival (OS) was calculated from date of inclusion to the last follow-up date, censoring patients alive at that date. Event-free survival (EFS) was calculated from date of inclusion to date of induction failure, relapse, or death, censoring patients alive in first CR without relapse at the last follow-up date. Cumulative incidence of relapse (CIR) was calculated in patients who attained CR, from date of CR to date of relapse, with death in first CR being considered as a competing event. Univariate and bivariate analysis 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. Proportional-hazards assumption was checked before conducting multivariate analyses. Statistical analyses were performed with STATA software (STATA 12.0 Corporation, College Station, TX). All p-values were two-sided, with $p < 0.05$ denoting statistical significance.

Results:

Analysis of DNMT3A genotype in T-ALL patients in the GRAALL studies

We performed targeted next generation sequencing (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. Partial analysis of a subgroup of this cohort has previously been reported. [24] We detected 21 *DNMT3A* mutations in 18 patients (9.1%). Most alterations occurred in regions coding for defined protein functional domains, including 6 mutations at the R882 hotspot [1] (Figure 1A). Further details of patient-specific alterations are shown in 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 (VAFs) that were suggestive of either homozygous mutation, concomitant deletion of the wild-type allele or copy-neutral loss of heterozygosity. Comparative genomic hybridization (CGH) 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 VAFs (Cases 11 and 12 in Supplementary Table S3).

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 v 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 v 41.7% *DNMT3A* WT, p = 0.113), hematopoiesis (38.5% *DNMT3A* mutated v 12.8% *DNMT3A* WT, p = 0.082) and chemical modification of histones (50.0% *DNMT3A* mutated v 30.0% *DNMT3A* WT, p = 0.082). However, we did observe significant co-occurrence of *DNMT3A* alterations and *IDH2* mutations (27.8% *DNMT3A* mutated v 2.2% *DNMT3A* WT, p < 0.001). This association has previously been described in both AML [25] and MDS [26].

Evidence of possible clonal hematopoiesis in DNMT3A-mutated T-ALL

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 3 of the 18 patients with *DNMT3A* mutations. While 2 of these samples had a wild-type *DNMT3A* genotype (Supplementary Figure S1), one interesting case had evidence of *DNMT3A* alteration in non-leukemic bone marrow cells. At diagnosis, this patient (Case 6 in 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 2 further diagnostic bone marrow samples, and extracted DNA from both the leukemic and the minor residual non-leukemic fractions. We detected mutation in non-leukemic DNA in one patient (Case 4 in 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 wild-type *DNMT3A* genotype (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

Clinicobiological 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 v 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 v 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 v 20.3% non-mutated, $p = 0.184$).

DNMT3A mutation was notably associated with poor initial treatment response. We observed trends towards early corticoresistance (66.7% mutated v 43.3% non-mutated, $p = 0.081$) and induction failure (13.3% v 2.9%, $p = 0.096$), and patients with *DNMT3A* mutations had significantly higher rates of induction death (16.7% vs 2.8%, $p = 0.027$), and lower attainment of CR (72.2% mutated v 94.4% non-mutated, $p = 0.006$). As only four patients with mutations had minimal residual disease evaluation, 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-ALL

The median follow-up of the cohort was 5.5 years. Prognostic analyses revealed that *DNMT3A* mutation was associated with an increased cumulative incidence of relapse (5 year CIR 53.9% mutated v 28.7% non-mutated, p = 0.037, Figure 3A) and with reduced EFS (5 year EFS 27.8% mutated v 61.0% non-mutated, HR 3.22, 95% CI 1.81-5.72, p < 0.001, Figure 3B). Patients with *DNMT3A* mutations also displayed a markedly inferior overall survival (5 year OS 38.8% mutated v 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-ALL 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 Supplementary Table S4. In each case, *DNMT3A* genotype was still associated with significantly increased CIR (HR 2.80, 95% CI 1.12 - 6.97, p = 0.034), and shorter EFS (HR 2.62, 95% CI = 1.45 – 5.06, p = 0.004) and OS (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 years subgroup, which constituted a quarter of the total patient cohort (50/198, 25.3%). Consistent with the results of the bivariate analyses, *DNMT3A* mutation was associated

with significantly worse CIR (5 year CIR 58.3% mutated v 21.7% non-mutated, HR 3.90, 95% CI 1.30 - 11.68, p = 0.015, Figure 4A), EFS (5 year EFS 25.0% mutated v 56.7% non-mutated, HR 2.95, 95% CI 1.37 - 6.32, p = 0.005, Figure 4B), and OS (5 year OS 37.5% mutated v 62.1% non-mutated, HR 2.35, 95% CI 1.05 - 5.26, p = 0.038, Figure 4C).

We finally 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 univariate analysis. Among age, log (WBC), corticosensitivity, early chemosensitivity, and *DNMT3A* genotype, only *DNMT3A* genotype was associated with CIR in univariate analysis (data not shown). As shown in Table 2 and 3, age, log (WBC), corticoresistance along with *DNMT3A* genotype were significantly associated with a poor EFS and OS. In multivariate analysis adjusting for these covariates, *DNMT3A* mutation was still significantly associated with shorter EFS (HR 2.33, 95% CI 1.06 – 4.04, p = 0.02, Table 2), although not with OS (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 patient 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 that 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 detected no 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* alteration in non-leukemic bone marrow suggests that some of these T-ALLs might arise 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 provides 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 notably 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 non-leukemic *DNMT3A* mutation in this study had expression of myeloid cell surface markers as part of an ETP-ALL phenotype, 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 takes place. 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 patient 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 EFS and CIR. *DNMT3A* mutation status also independently predicted EFS and OS in bivariable analyses that incorporated our recently-described oncogenetic risk classifier [37] (Supplementary Table S5). These results suggest that *DNMT3A* mutation is directly linked to aggressive T-ALL biology. As *DNMT3A*-altered T-ALLs had higher mutation rates in other genes included in our targeted sequencing panel, it is also possible that increased genotype complexity may contribute to 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 for allo-SCT in first CR for ETP-ALL, [24] which similarly exhibits high rates of intrinsically treatment-resistant disease. As only 3 of 18 *DNMT3A*-mutated patients in this study had allo-SCT (data not shown), we are unable to estimate the potential benefit in this setting. We have recently reported that treatment-related toxicity in the GRAALL-2005 study increases in proportion to patient age, [38] and further therapy intensification in elderly patients must therefore be considered of questionable benefit. The upper age of this study cohort is 60 years, but it is likely that the rate of mutations in older patients who do not tolerate such intensive chemotherapy is higher. Reported data in 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, investigation of the molecular mechanisms by which *DNMT3A* mutation alters T-ALL biology will hopefully lead to better treatments and improved outcomes for these high-risk cases.

Authorship: JB, LL, GH and NB performed experiments and analyzed data. SL, CG, MB, TL, YH, GG, KB, MH, FH, YC, NC, HD and NB contributed to clinical care. All authors reviewed patient data and the manuscript. JB, HD, VA and NB designed and oversaw conceptual development of the project.

Conflicts of Interest: The authors declare no conflict of interest.

References:

1. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010;363(25):2424-2433.

2. Renneville A, Boissel N, Nibourel O, et al. Prognostic significance of DNA methyltransferase 3A mutations in cytogenetically normal acute myeloid leukemia: a study by the Acute Leukemia French Association. Leukemia. 2012;26(6):1247-1254.

3. Gale RE, Lamb K, Allen C, et al. Simpson's Paradox and the Impact of Different DNMT3A Mutations on Outcome in Younger Adults With Acute Myeloid Leukemia. J Clin Oncol. 2015;33(18):2072-2083.

4. Marcucci G, Metzeler KH, Schwind S, et al. Age-related prognostic impact of different types of DNMT3A mutations in adults with primary cytogenetically normal acute myeloid leukemia. J Clin Oncol. 2012;30(7):742-750.

5. Yan XJ, Xu J, Gu ZH, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet. 2013;43(4):309-315.

6. Haferlach T, Nagata Y, Grossmann V, et al. Landscape of genetic lesions in 944 patients with myelodysplastic syndromes. Leukemia. 2014;28(2):241-247.

7. Stegelmann F, Bullinger L, Schlenk RF, et al. DNMT3A mutations in myeloproliferative neoplasms. Leukemia. 2011;25(7):1217-1219.

8. Jankowska AM, Makishima H, Tiu RV, et al. Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: UTX, EZH2, and DNMT3A. Blood. 2011;118(14):3932-3941.

9. Roller A, Grossmann V, Bacher U, et al. Landmark analysis of DNMT3A mutations in hematological malignancies. Leukemia. 2013;27(7):1573-1578.

10. Van Vlierberghe P, Ambesi-Impiombato A, Perez-Garcia A, et al. ETV6 mutations in early immature human T cell leukemias. J Exp Med. 2011;208(13):2571-2579.

11. Grossmann V, Haferlach C, Weissmann S, et al. The molecular profile of adult T-cell acute lymphoblastic leukemia: mutations in RUNX1 and DNMT3A are associated with poor prognosis in T-ALL. Genes Chromosomes Cancer. 2013;52(4):410-422.

12. Neumann M, Heesch S, Schlee C, et al. Whole-exome sequencing in adult ETP-ALL reveals a high rate of DNMT3A mutations. Blood. 2013;121(23):4749-4752.

13. Van Vlierberghe P, Ambesi-Impiombato A, De Keersmaecker K, et al. Prognostic relevance of integrated genetic profiling in adult T-cell acute lymphoblastic leukemia. Blood. 2013;122(1):74-82.

14. Couronne L, Bastard C, Bernard OA. TET2 and DNMT3A mutations in human T-cell lymphoma. N Engl J Med. 2012;366(1):95-96.

15. Sakata-Yanagimoto M, Enami T, Yoshida K, et al. Somatic RHOA mutation in angioimmunoblastic T cell lymphoma. Nat Genet. 2014;46(2):171-175.

16. Choi J, Goh G, Walradt T, et al. Genomic landscape of cutaneous T cell lymphoma. Nat Genet. 2015;47(9):1011-1019.

17. Ho PA, Kutny MA, Alonzo TA, et al. Leukemic mutations in the methylation-associated genes DNMT3A and IDH2 are rare events in pediatric AML: a report from the Children's Oncology Group. Pediatr Blood Cancer. 2011;57(2):204-209.

18. Huether R, Dong L, Chen X, et al. The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. Nat Commun. 2014;5:3630.

19. Shiba N, Taki T, Park MJ, et al. DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia. Br J Haematol. 2012;156(3):413-414.

20. Shen Y, Zhu YM, Fan X, et al. Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. Blood. 2011;118(20):5593-5603.

21. Ribeiro AF, Pratcorona M, Erpelinck-Verschueren C, et al. Mutant DNMT3A: a marker of poor prognosis in acute myeloid leukemia. Blood. 2012;119(24):5824-5831.

22. Tie R, Zhang T, Fu H, et al. Association between DNMT3A mutations and prognosis of adults with de novo acute myeloid leukemia: a systematic review and meta-analysis. PloS One. 2014;9(6):e93353.

23. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature. 2012;481(7380):157-163.

24. Bond J, Graux C, Lhermitte L, et al. Early Response-Based Therapy Stratification Improves Survival in Adult Early Thymic Precursor Acute Lymphoblastic Leukemia: A Group for Research on Adult Acute Lymphoblastic Leukemia Study. J Clin Oncol. 2017;35(23):2683-2691.

25. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. N Engl J Med. 2016;374(23):2209-2221.

26. Lin ME, Hou HA, Tsai CH, et al. Dynamics of DNMT3A mutation and prognostic relevance in patients with primary myelodysplastic syndrome. Clin Epigenetics. 2018;10:42.

27. Genovese G, Kahler AK, Handsaker RE, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med. 2014;371(26):2477-2487.

28. Jaiswal S, Fontanillas P, Flannick J, et al. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014;371(26):2488-2498.

29. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. Nat Med. 2014;20(12):1472-1478.

30. Shlush LI, Zandi S, Mitchell A, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014;506(7488):328-333.

31. Corces-Zimmerman MR, Hong WJ, Weissman IL, Medeiros BC, Majeti R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc Natl Acad Sci U S A. 2014;111(7):2548-2553.

32. Asnafi V, Beldjord K, Boulanger E, et al. Analysis of TCR, pT alpha, and RAG-1 in T-acute lymphoblastic leukemias improves understanding of early human T-lymphoid lineage commitment. Blood. 2003;101(7):2693-2703.

33. Coustan-Smith E, Mullighan CG, Onciu M, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. Lancet Oncol. 2009;10(2):147-156.

34. Huguet F, Leguay T, Raffoux E, et al. Pediatric-inspired therapy in adults with Philadelphia chromosome-negative acute lymphoblastic leukemia: the GRAALL-2003 study. J Clin Oncol. 2009;27(6):911-918.

35. Bond J, Marchand T, Touzart A, et al. An early thymic precursor phenotype predicts outcome exclusively in HOXA-overexpressing adult T-cell acute lymphoblastic leukemia: a Group for Research in Adult Acute Lymphoblastic Leukemia study. Haematologica. 2016;101(6):732-740.

36. Eguchi-Ishimae M, Eguchi M, Kempski H, Greaves M. NOTCH1 mutation can be an early, prenatal genetic event in T-ALL. Blood. 2008;111(1):376-378.

37. Trinquand A, Tanguy-Schmidt A, Ben Abdelali R, et al. Toward a NOTCH1/FBXW7/RAS/PTEN-based oncogenetic risk classification of adult T-cell acute

lymphoblastic leukemia: a Group for Research in Adult Acute Lymphoblastic Leukemia study. J Clin Oncol. 2013;31(34):4333-4342.

38. Huguet Fo, Leguay T, Thomas X, et al. The Upper Age Limit for a Pediatric-Inspired Therapy in Younger Adults with Ph-Negative Acute Lymphoblastic Leukemia (ALL)? Analysis of the Graall-2005 Study. 2016:762-762.

39. Metzeler KH, Walker A, Geyer S, et al. DNMT3A mutations and response to the hypomethylating agent decitabine in acute myeloid leukemia. Leukemia. 2012;26(5):1106-1107.

Table 1: Characteristics and outcome 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 (y)[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				
Corticosensitivity	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), ETP immunophenotype (n = 172), HOXA Positivity (n = 170) and Risk classifier based on genotype for *NOTCH1*, *FBXW7*, *PTEN*, *NRAS* and *KRAS* (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.

Table 2: Prognostic impact of *DNMT3A* genotype on event-free survival (EFS).

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
Corticosensitivity	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**

WBC: White blood cell count.
* continuous variable
Statistically significant differences are highlighted in bold.

Table 3: Prognostic impact of *DNMT3A* genotype on overall survival (OS).

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**
Corticosensitivity	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

WBC: White blood cell count.
* continuous variable
Statistically significant differences are highlighted in bold.

Figure Legends:

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

Figure 2: Evidence of *DNMT3A* mutation 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 T-ALL samples. Sequencing of *DNMT3A* and *NOTCH1* in leukemic (left panels) and non-leukemic (right panels) DNA is shown. Cases are numbered according to the listing in Supplementary Table S3.

Figure 3: *DNMT3A* mutation correlates with poor outcome in T-ALL. Comparisons of outcomes for patients with (n = 18) and without (n = 180) mutations are shown for **(A)** Cumulative Incidence of Relapse (CIR) **(B)** Event-Free Survival (EFS) and **(C)** Overall Survival (OS). The 5-year results were as follows: CIR 53.9% mutated v 28.7% non-mutated; EFS 27.8% mutated v 61.0% non-mutated; OS 38.8% mutated v 68.7% non-mutated. P values are indicated.

Figure 4: *DNMT3A* genotype predicts outcome in the patient age group 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 (CIR) **(B)** Event-Free Survival (EFS) and **(C)** Overall Survival (OS). The 5 year results were as follows: CIR 58.3% mutated > 40 years v 21.7% non-mutated > 40 years; EFS, 25.0% mutated > 40 years v 56.7% non-mutated > 40 years; OS 37.5% mutated > 40 years v 62.1% non-mutated > 40 years. P values are indicated.

Figure 1

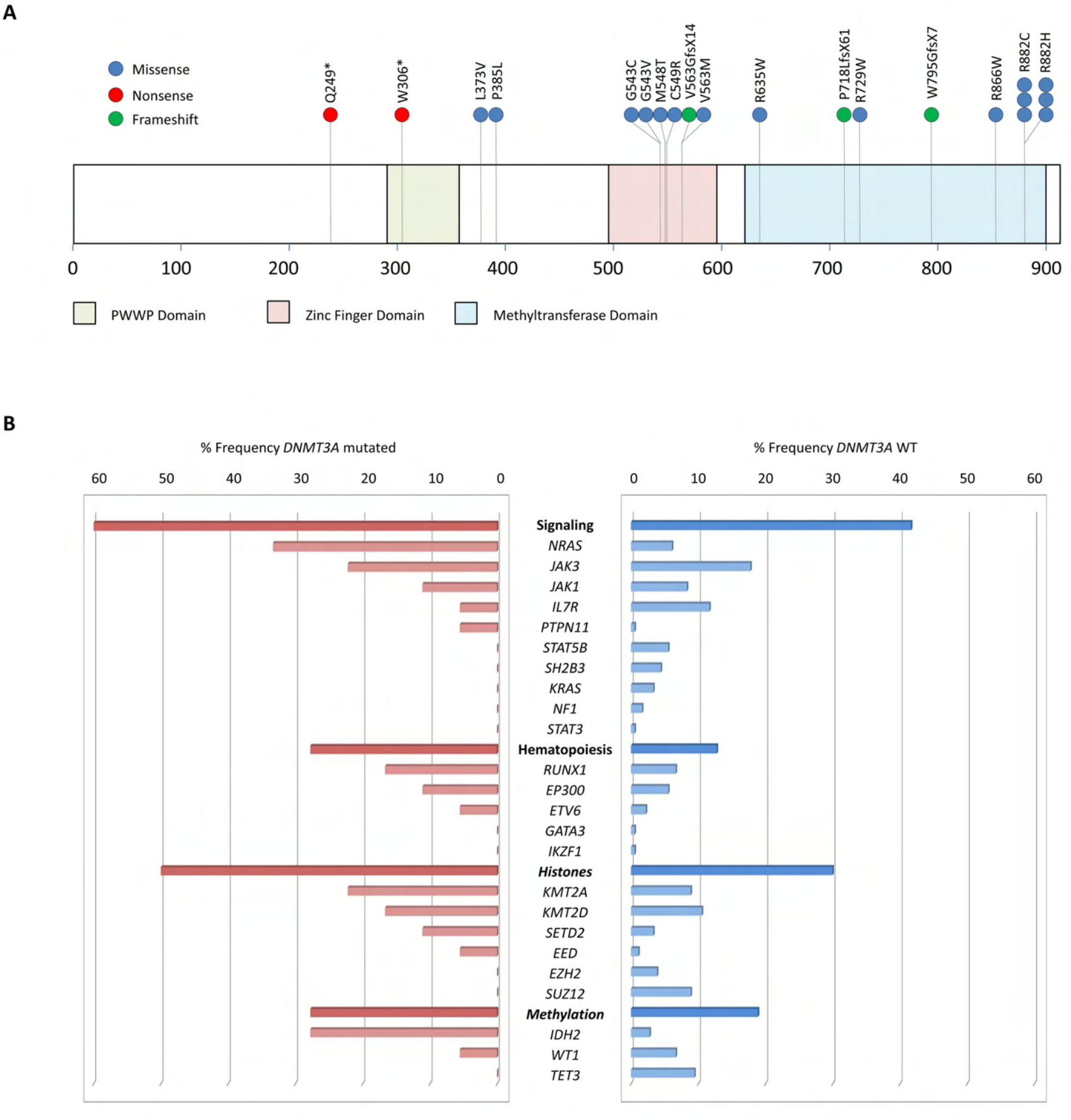
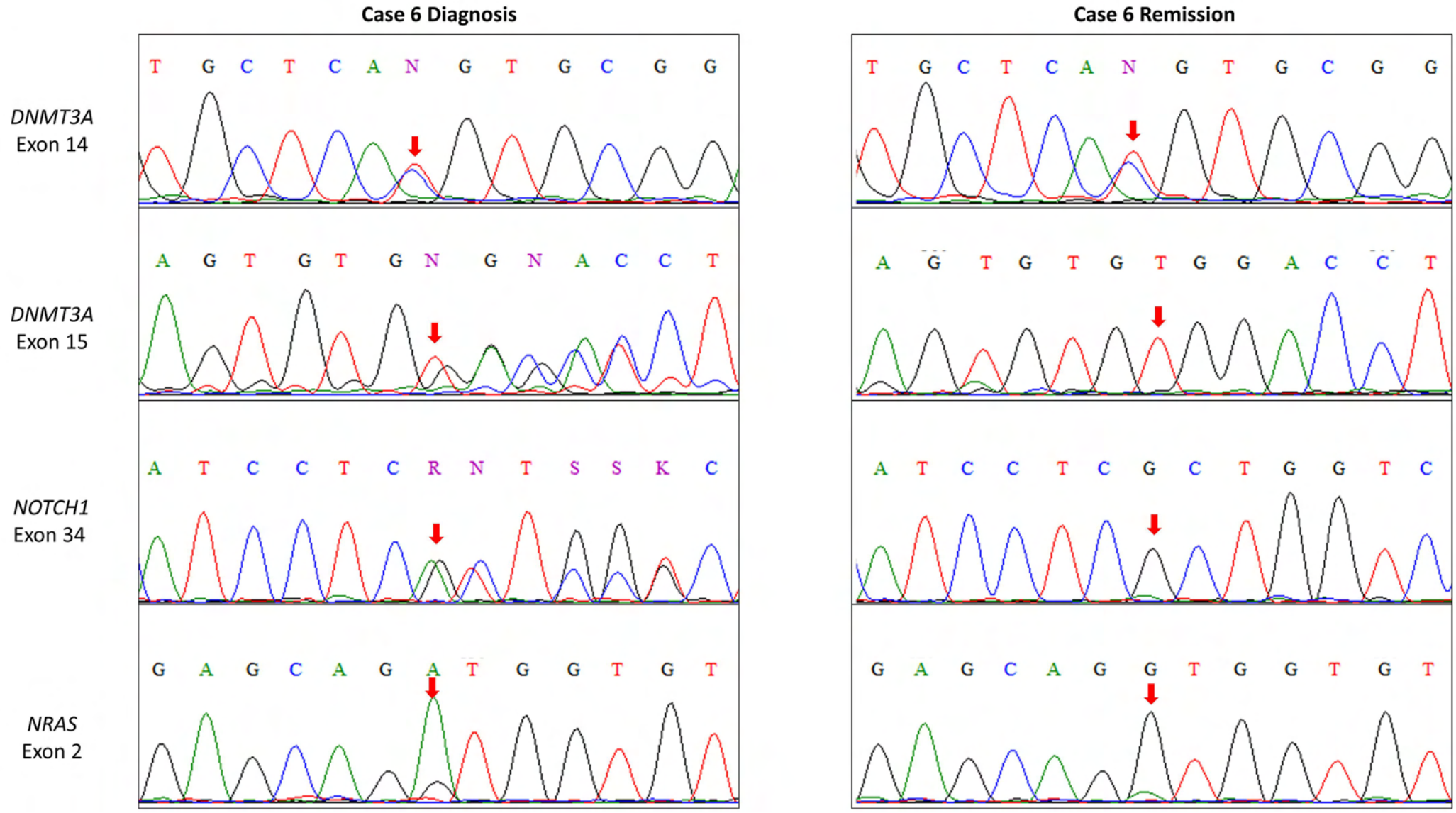

A
Missense
Nonsense
Frameshift
Q249*
W306*
L373V
P385L
G543C
G543V
M548T
C549R
V563GfsX14
V563M
R635W
P718LfsX61
R729W
W795GfsX7
R866W
R882C
R882H
0
100
200
300
400
500
600
700
800
900
PWWP Domain
Zinc Finger Domain
Methyltransferase Domain
B
% Frequency DNMT3A mutated
% Frequency DNMT3A WT
60
50
40
30
20
10
0
Signaling
NRAS
JAK3
JAK1
IL7R
PTPN11
STAT5B
SH2B3
KRAS
NF1
STAT3
Hematopoiesis
RUNX1
EP300
ETV6
GATA3
IKZF1
Histones
KMT2A
KMT2D
SETD2
EED
EZH2
SUZ12
Methylation
IDH2
WT1
TET3

Figure 2

A


Case 6 Diagnosis
Case 6 Remission
DNMT3A Exon 14
DNMT3A Exon 15
NOTCH1 Exon 34
NRAS Exon 2
T G C T C A N G T G C G G
A G T G T G N G N A C C T
A T C C T C R N T S S K C
G A G C A G A T G G T G T
T G C T C A N G T G C G G
A G T G T G T G G A C C T
A T C C T C G C T G G T C
G A G C A G G T G G T G T

B

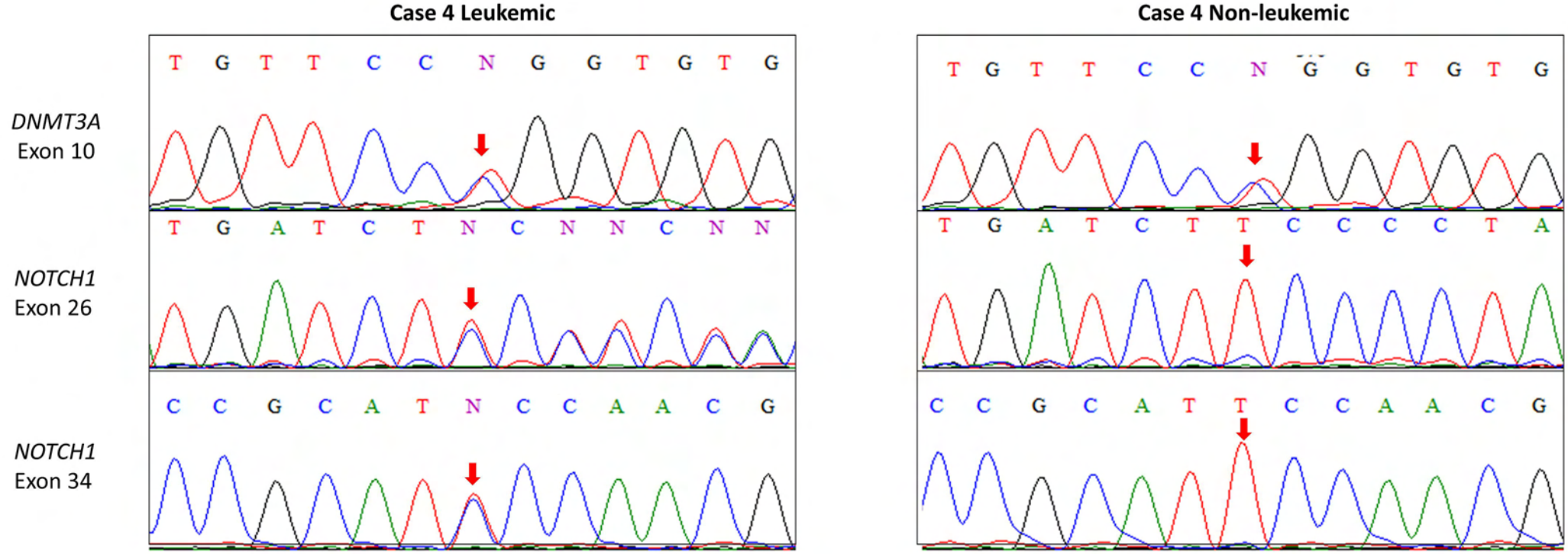
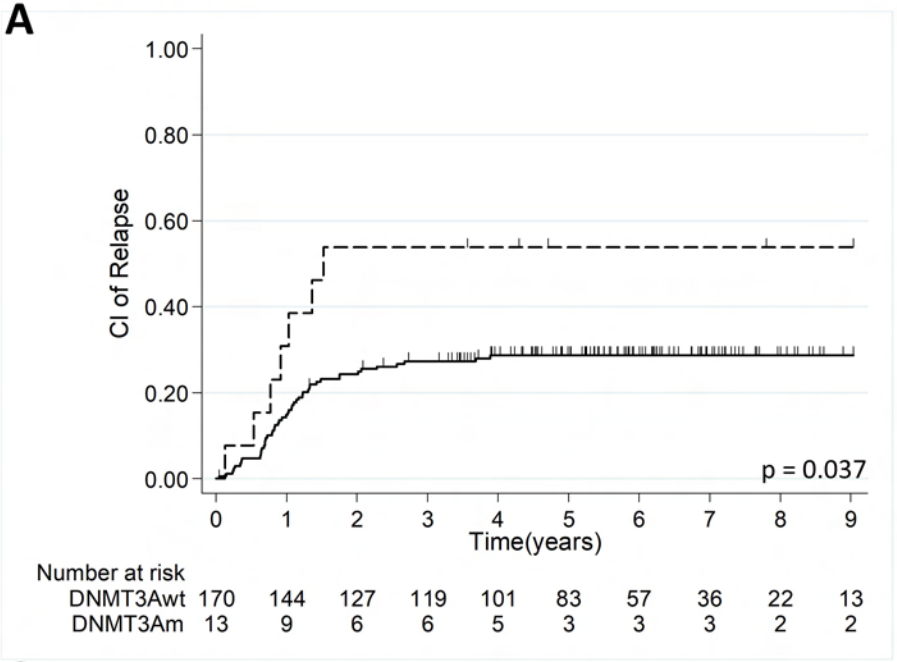
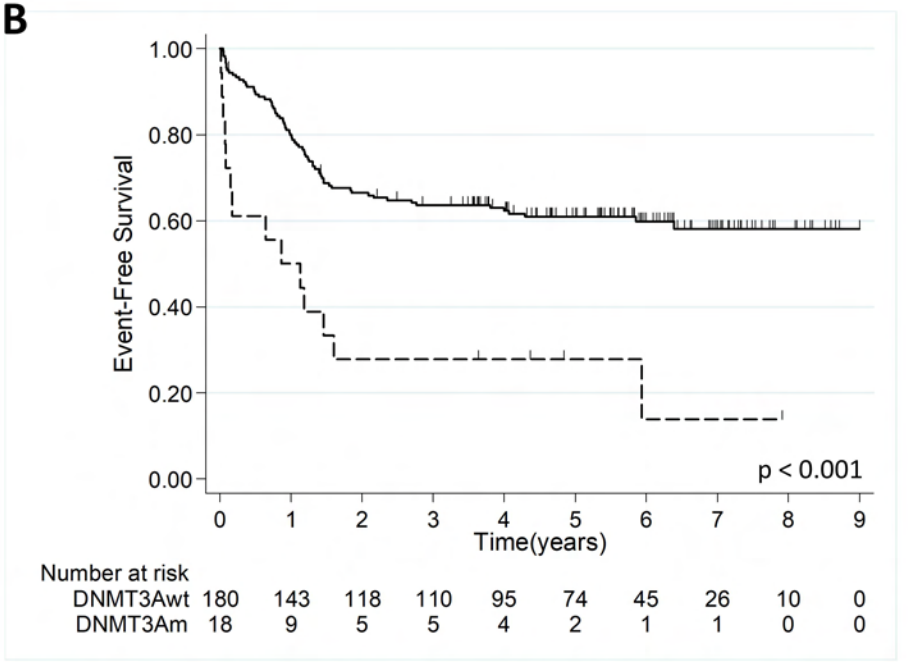

Case 4 Leukemic
Case 4 Non-leukemic
DNMT3A Exon 10
NOTCH1 Exon 26
NOTCH1 Exon 34
T G T T C C N G G T G T G
T G A T C T N C N N C N N
C C G C A T N C C A A C G
T G T T C C N G G T G T G
T G A T C T T C C C C T A
C C G C A T T C C A A C G

Figure 3


A
CI of Relapse
1.00
0.80
0.60
0.40
0.20
0.00
p = 0.037
0 1 2 3 4 5 6 7 8 9
Time(years)
Number at risk
DNMT3Awt 170 144 127 119 101 83 57 36 22 13
DNMT3Am 13 9 6 6 5 3 3 3 2 2


B
Event-Free Survival
1.00
0.80
0.60
0.40
0.20
0.00
p < 0.001
0 1 2 3 4 5 6 7 8 9
Time(years)
Number at risk
DNMT3Awt 180 143 118 110 95 74 45 26 10 0
DNMT3Am 18 9 5 5 4 2 1 1 0 0

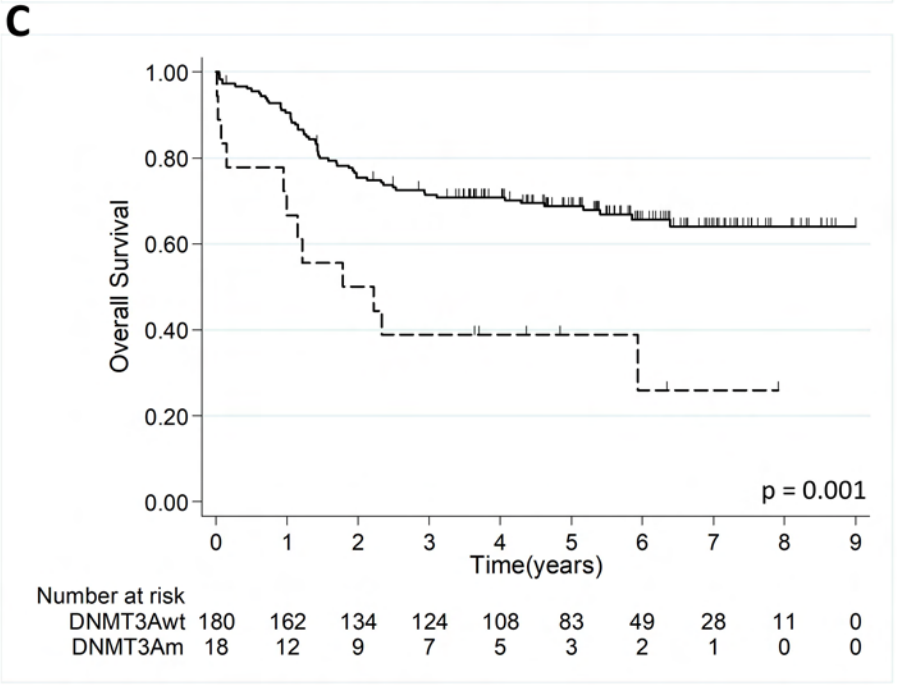
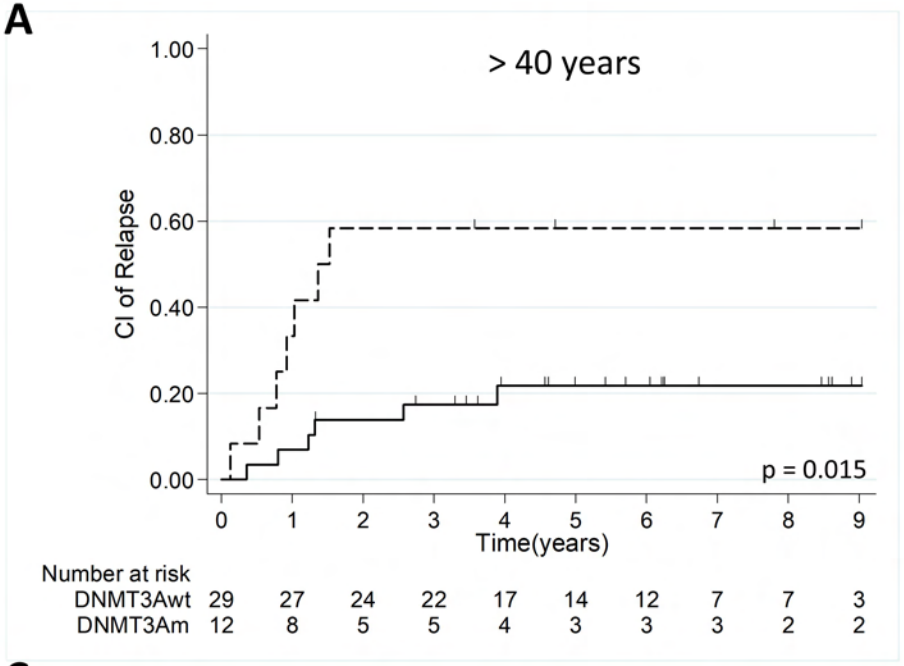
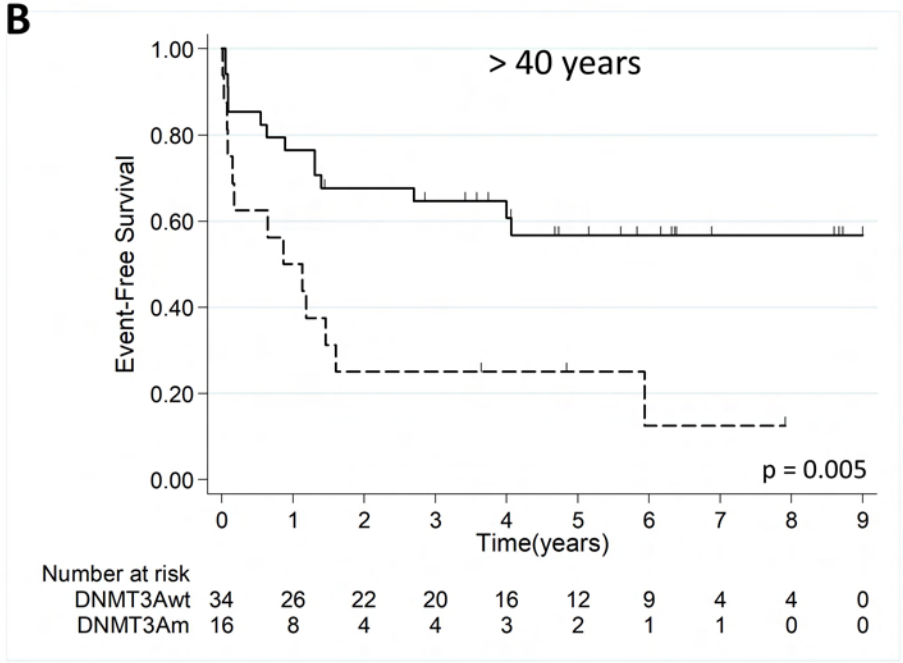
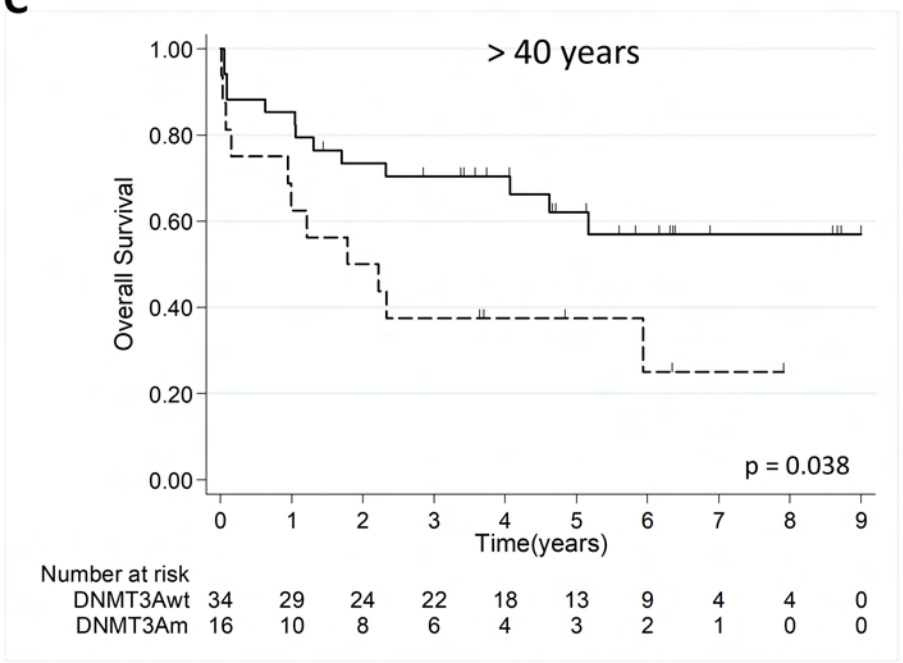

C
Overall Survival
1.00
0.80
0.60
0.40
0.20
0.00
p = 0.001
0 1 2 3 4 5 6 7 8 9
Time(years)
Number at risk
DNMT3Awt 180 162 134 124 108 83 49 28 11 0
DNMT3Am 18 12 9 7 5 3 2 1 0 0

Figure 4


A
> 40 years
CI of Relapse
1.00
0.80
0.60
0.40
0.20
0.00
p = 0.015
0 1 2 3 4 5 6 7 8 9
Time(years)
Number at risk
DNMT3Awt 29 27 24 22 17 14 12 7 7 3
DNMT3Am 12 8 5 5 4 3 3 3 2 2


B
> 40 years
Event-Free Survival
1.00
0.80
0.60
0.40
0.20
0.00
p = 0.005
0 1 2 3 4 5 6 7 8 9
Time(years)
Number at risk
DNMT3Awt 34 26 22 20 16 12 9 4 4 0
DNMT3Am 16 8 4 4 3 2 1 1 0 0


C
> 40 years
Overall Survival
1.00
0.80
0.60
0.40
0.20
0.00
p = 0.038
0 1 2 3 4 5 6 7 8 9
Time(years)
Number at risk
DNMT3Awt 34 29 24 22 18 13 9 4 4 0
DNMT3Am 16 10 8 6 4 3 2 1 0 0

SUPPLEMENTARY MATERIAL

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

Bond *et al*

Contents:

Supplementary Methods:

***The GRAALL-2003 and GRAALL-2005 studies*:** The GRAALL-2003 study was a multicenter Phase II trial, which enrolled 76 adults with T-ALL between November 2003 and November 2005. The multicenter randomized GRAALL-2005 study was the following Phase III trial, with the addition of a randomized evaluation of an intensified sequence of hyper-fractionated cyclophosphamide during induction and late intensification. 261 adults with T-ALL were enrolled in this study between May 2006 and September 2011. Informed consent was obtained from all patients at trial entry. 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 Supplementary File 'GRAALL_2003_2005 supplementary protocol'. Both trials were registered at http://www.clinicaltrials.gov as #NCT00222027 and #NCT00327678, respectively. With a data cut-off date on June 2015, the median follow-up was 5.7 years (6.0 and 5.4 years for GRAALL-2003 and GRAALL-2005 patients, respectively).

Supplementary Table S1: Characteristics of the study patients and the remainder of the T-ALL cohort in the GRAALL-2003 and GRAALL-2005 studies.

	Non-included cohort	Study cohort	p-value
Total, no.	139	198	
GRAALL 2003/05 trial	40/99	36/162	0.025
Sex ratio (Male/ Female)	98/41	141/57	0.903
Median age, y [Q1-Q3]	34.1 [25.5-47.2]	30.5[23.4-40.4]	0.022
Median WBC, 10^9/L [Q1-Q3]	16.7 [7.4-59.9]	32.6 [11.9-103.9]	0.0001
CNS involvement, no. (%)	11 (7.9%)	24 (12.1%)	0.143
CR, no. (%)	132 (95.0%)	183 (92.4%)	0.243
CS, no. (%)	97 (69.8%)	108 (54.5%)	0.003
CHS, no. (%)	76/134 (56.7%)	110/195 (56.4%)	1.000
SCT, no. (%)	39 (28.1%)	72 (36.4%)	0.126
EFS at 5 years, % [95% CI]	54.3% [45.6-62.3]	58.0% [50.6-64.5]	0.606
OS at 5 years, % [95% CI]	61.5% [52.8-69.1]	66.0% [58.8-72.2]	0.474

No: number; Q: Quartile; WBC: white blood cell count; CNS: central nervous system; CR: complete remission; CS: cortico-sensitive; CHS: chemo-sensitive; SCT: stem-cell transplantation; DFS: Disease-Free Survival; EFS: Event-Free Survival; OS: Overall Survival.

Supplementary Table S2: Primers used for direct sequencing of *DNMT3A*.

Exon	Forward Primer	Reverse Primer
7	GAATGCTGTGGAAGAAAACCAG	ATTCTTGTCCCCAGCATCG
8	GCCTCGTGACCACTGTGTAATG	CACTGAGAATTTGCCGTCTCC
10	GAGCCTGACCCATCTGCCTT	CTTCTGGTGGCTCCAGGCC
14	GCTTTCTGGAGTGTGCGTACCA	CAAGGTGTGCTACCTGGAATGG
15	CAGACCCGGTCTTTCCATTCC	CGAAGAACATCTGGAGCCGG
19	CTATGCAGACAGCCCCAGCT	ATCGCGAGATGTCCCTCTTG
23	CTGGTCTGGCCAGCACTCAC	CTTTGTGTCGCTACCTCAGTTTG

Supplementary Table S3: Details of *DNMT3A* mutations detected in this study.

Case	Age (y)	Exon	Type	Mutation	Amino Acid	SIFT score	VAF (%)
1	56.2	7	Nonsense	c.745C>T	p.Q249*	NA	40
1	56.2	14	Missense	c.1627G>T	p.G543C	0.000	46
2	44.9	8	Nonsense	c.918G>A	p.W306*	NA	44
3	40.9	9	Missense	c.1117C>G	p.L373V	0.013	18
4	42.8	10	Missense	c.1154C>T	p.P385L	0.025	46
5	50.0	14	Missense	c.1628G>T	p.G543V	0.000	59
6	40.3	14	Missense	c.1643T>C	p.M548T	0.023	45
6	40.3	15	Frameshift	c.1688_1689delTG	p.V563GfsX14	0.000	43
7	50.9	14	Missense	c.1645T>C	p.C549R	0.017	44
7	50.9	20	Frameshift	c.2383delT	p.W795GfsX7	0.000	41
8	40.5	15	Missense	c.1687G>A	p.V563M	0.197	50
9	42.9	16	Missense	c.1903C>T	p.R635W	0.000	81
10	50.4	18	Frameshift	c.2153delC	p.P718LfsX61	0.006	94
11	41.5	19	Missense	c.2185C>T	p.R729W	0.111	84
12	59.0	22	Missense	c.2596A>T	p.R866W	0.000	78
13	26.7	23	Missense	c.2644C>T	p.R882C	0.000	38
14	40.8	23	Missense	c.2645G>A	p.R882H	0.002	47
15	53.5	23	Missense	c.2644C>T	p.R882C	0.000	46
16	58.1	23	Missense	c.2644C>T	p.R882C	0.000	58
17	53.9	23	Missense	c.2645G>A	p.R882H	0.002	30
18	36.4	23	Missense	c.2645G>A	p.R882H	0.002	89

Age in years is shown. VAF: Variant Allele Frequency. All nucleotide changes were verified to have not been reported as single nucleotide polymorphisms (SNPS) in reference SNP databases, namely dbSNP (https://www.ncbi.nlm.nih.gov/SNP/) and Ensembl (http://www.ensembl.org/info/genome/variation/index.html). SIFT scores were calculated at the Provean website (http://provean.jcvi.org/index.php). All amino acid changes were predicted to be damaging, apart from the V563M and R729W substitutions, both of which have SIFT scores at the lowest range of predicted tolerability.

Supplementary Table S4: Bivariate analysis of CIR, EFS and OS including age and *DNMT3A* genotype as covariates.

	Age			*DNMT3A* genotype		
	HR	**95% CI**	**p**	**HR**	**95% CI**	**p**
CIR	0.99	0.96 - 1.02	0.432	2.80	1.12 - 6.97	**0.027**
EFS	1.01	0.99 – 1.04	0.211	2.62	1.35 – 5.06	**0.004**
OS	1.03	1.00 - 1.05	**0.034**	2.05	1.02 - 4.12	**0.043**

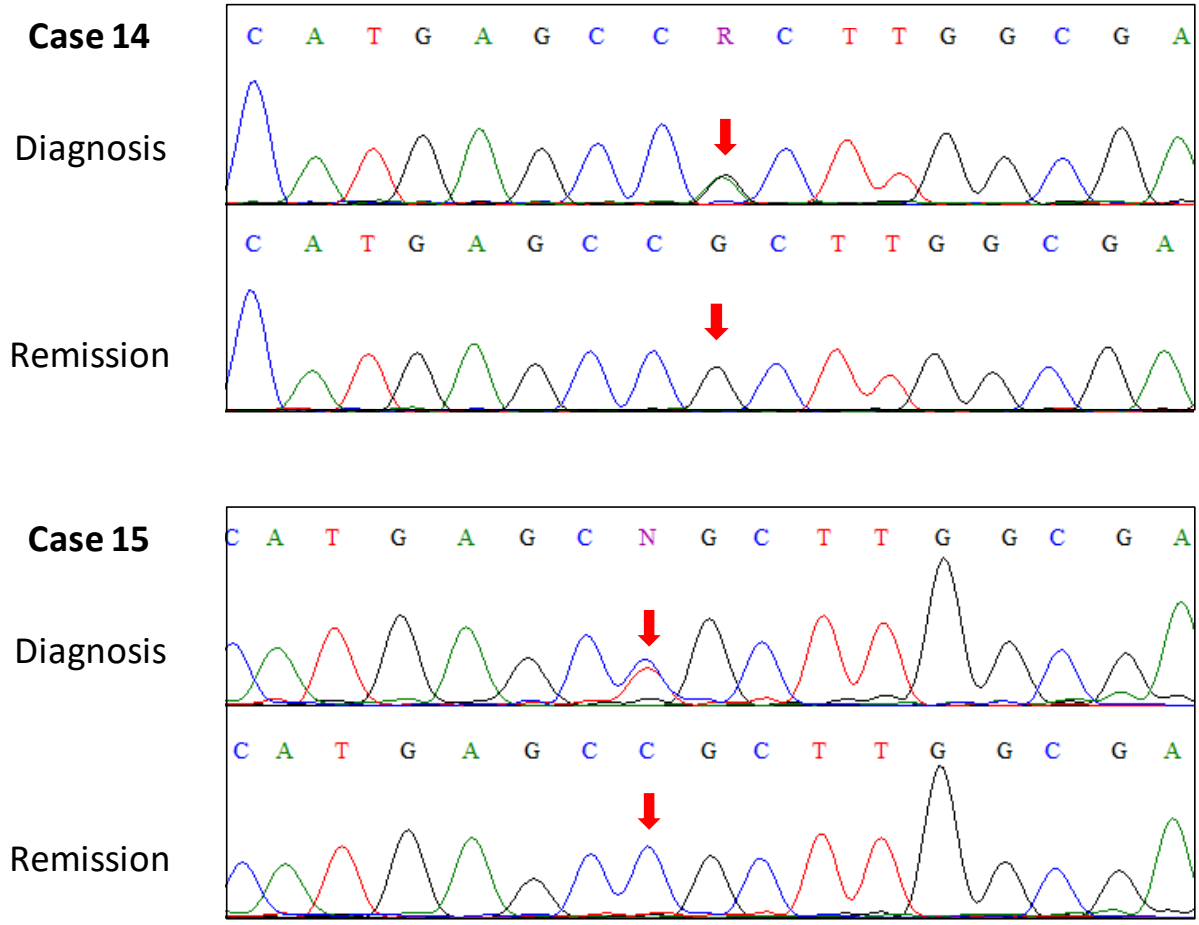
CIR: Cumulative Incidence of Relapse. EFS: Event-free Survival. OS: Overall Survival. HR: Hazard Ratio. CI: Confidence Interval. Statistically significant results are shown in bold.

Supplementary Table S5: Bivariate analysis for prognostic impact of *DNMT3A* genotype and oncogenetic risk classifier on cumulative incidence of relapse (CIR), EFS, and OS.

	CIR			EFS			OS		
	HR	**95% CI**	**p**	**HR**	**95% CI**	**p**	**HR**	**95% CI**	**p**
***DNMT3A* mut.**	2.11	0.95 – 4.69	0.066	2.86	1.60 – 5.10	<0.001	2.90	1.55 – 5.42	0.001
Risk Classifier[#]	2.93	1.68 – 5.10	< 0.001	2.67	1.71 – 4.16	<0.001	3.02	1.84 – 4.94	<0.001

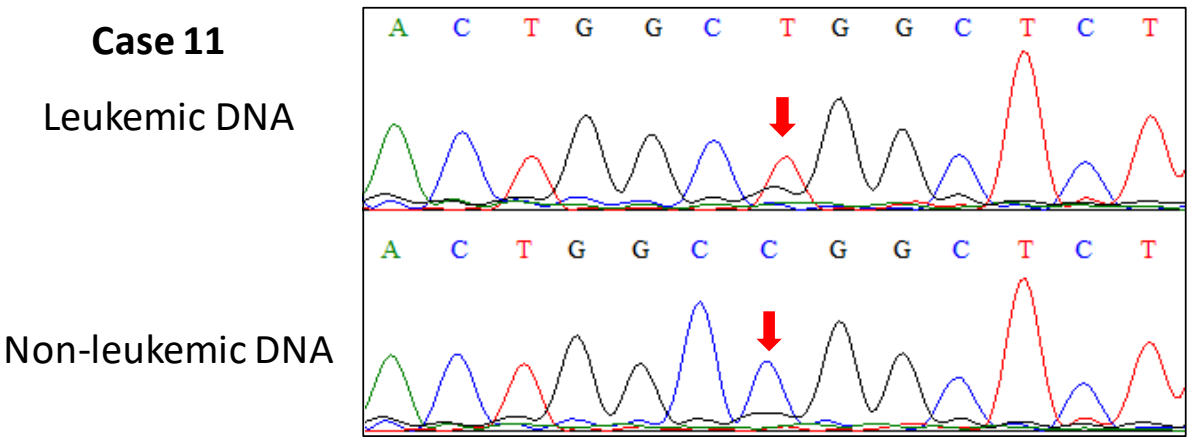
[#] Oncogenetic Risk Classifier based on genotype for *NOTCH1*, *FBXW7*, *PTEN*, *NRAS* and *KRAS,* reported in Trinquand et al J Clin Oncol. 2013 Dec 1;31(34):4333-42.
WBC: White blood cell count.
* continuous variable

Supplementary Figure S1: Analysis of *DNMT3A* mutations in remission samples.



Direct sequencing confirmed the presence of the mutations c.2645G>A (Case 14) and c.2644C>T (Case 15) in *DNMT3A* Exon 23 in diagnostic samples. These mutations were not detected in remission samples in either case. The relevant nucleotides are indicated by red arrows. Patient samples are numbered according to the listing in Supplementary Table S3.

Supplementary Figure S2: Analysis of *DNMT3A* mutations in non-leukemic DNA.



2 diagnostic T-ALL samples were separated into leukemic and non-leukemic fractions by immunophenotypic sorting. 1 case had *DNMT3A* mutations in the non-leukemic fractions (see Figure 2). One sample (Case 11 in Supplementary Table S3) had a probable homozygous c.2185C>T substitution in *DNMT3A* Exon 19 in the leukemic fraction only, as shown here. The relevant nucleotides are indicated by red arrows.

Supplementary Figure S3: Consort Diagram