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The combination of NPM1, DNMT3A, and IDH1/2 mutations leads to inferior overall survival in AML

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Abstract

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with a clinical course predicted by recurrent cytogenetic abnormalities and/or gene mutations. The NPM1 insertion mutations define the largest distinct genetic subset, ~30% of AML, and is considered a favorable risk marker if there is no (or low allelic ratio) FLT3 internal tandem duplication (FLT3 ITD) mutation. However, ~40% of patients with mutated NPM1 without FLT3 ITD still relapse, and the factors that drive relapse are still not fully understood. We used a next-generation sequencing panel to examine mutations at diagnosis; clearance of mutations after therapy, and gain/loss of mutations at relapse to prioritize mutations that contribute to relapse. Triple mutation of NPM1, DNMT3A and IDH1/2 showed a trend towards inferior overall survival in our discovery dataset, and was significantly associated with reduced OS in a large independent validation cohort. Analysis of relative variant allele frequencies suggests that early mutation and expansion of DNMT3A and IDH1/2 prior to acquisition of NPM1 mutation leads to increased risk of relapse. This subset of patients may benefit from allogeneic stem cell transplant or clinical trials with IDH inhibitors.

1 | INTRODUCTION

Acute myeloid leukemia is a genetically heterogeneous disease, with numerous cytogenetic and molecular abnormalities. Genetic data is used to stratify patients into three risk groups: favorable, intermediate and adverse.[1] Favorable risk AML patients are often cured with chemotherapy alone, whereas intermediate and poor risk AML patients are at higher risk of relapse, and are therefore referred for allogeneic hematopoietic stem cell transplant (HSCT) in first remission (CR1). However, even among patients with favorable risk AML, approximately 40% will still relapse within 2 years.[2] Of the favorable risk AML patients that do relapse, some are still able to attain a second

remission (CR2), but transplant outcomes in CR2 are inferior to transplant in CR1. Improved ability to risk stratifies favorable risk patients, which will help identify which of these patients is more likely to benefit from alternative therapies or transplant in CR1.

Minimal residual disease (MRD) predicts relapse in some cases[3,4]; however, the ideal methodology, timing and genetic abnormalities to assay for MRD are not clear, and sensitivity of detection varies widely (10^{-2} to 10^{-6}) across different assays.[5] Moreover, patients with negative MRD still relapse, and MRD does not have a good negative predictive value. Given the limitations of MRD in clinical practice additional approaches to risk stratify patients are necessary. Recent large genetic datasets in AML have begun to uncover combinations of mutations that appear to further refine known prognostic markers. As an example, the combination of NPM1, FLT3 ITD and DNMT3A

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mutations was recently reported to confer much higher risk of relapse, than NPM1 and FLT3 ITD mutations without DNMT3A.[6]

In this study, we sought to investigate factors that predict inferior outcome in a uniformly treated and well-characterized population of patients at our institution. Favorable risk AML patients with normal cytogenetics and mutated NPM1, without FLT3 ITD mutations, were selected for analysis. Using a targeted next-generation sequencing (NGS) panel developed at our institution, we analyzed clearance of mutations after therapy, clonal evolution at relapse, and combinations of mutations. We found that the combination of NPM1, DNMT3A and IDH1/2 had a trend towards inferior overall survival (OS). To validate the results of our study, we took advantage of a large publically available genetic dataset with 1540 AML patients and found that the triple combination of mutations had statistically significant inferior OS.[7]

2 | MATERIALS AND METHODS

2.1 | Patient selection

Genetic and clinical data was obtained under an Institutional Research Board approved protocol, IRB10788. Patients with cytogenetically normal AML, harboring an NPM1 mutation and no FLT3 ITD, diagnosed between 2009-2017, with available diagnostic bone marrow aspirates were identified. Most patients from 2013-2017 had next-generation sequencing performed as part of the routine diagnostic workup. For those diagnosed prior to 2013, sequencing was performed retrospectively on archived paraffin embedded tissue from bone marrow clot/core biopsies.

2.2 | Targeted sequencing panel

We used a previously described custom-targeted sequencing panel of 42 leukemia associated genes[8] (Table S1). The leukemia panel was designed with the highly multiplexed Ion AmpliSeq Designer (Life Technologies) software. The AmpliSeq amplicon libraries and template preparation were performed as previously described.[9] Twenty ng of DNA was used for each sample. Sequencing was performed on a PGM sequencer (Life Technologies) using the Ion PGM 200 Sequencing kit, according to the manufacturer's protocol. Two or four barcoded samples were multiplexed on an Ion 318 chip. The Torrent Suite Analysis Pipeline version 3.2.1 (Thermo Fisher Scientific), processed raw acquisition data from the PGM sequencer runs. It also produced read files for subsequent base quality scoring, IonXpress barcode parsing, reads alignment to the reference genome, and reports generation on run metrics and quality controls. Target coverage was evaluated with Coverage Analysis software version 3.2.1 (Thermo Fisher Scientific). Variants were identified with Variant Caller software version 3.2.1 (Thermo Fisher Scientific), by using customized parameters. Raw sequence reads were visualized and investigated with an Integrative Genomics Viewer (Broad Institute, Cambridge, MA). A laboratory-developed algorithm was used for variant annotation against public databases such as COSMIC (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).

At diagnosis, gene variants were reported using our variant caller software with a lower limit of detection of 5% allele frequency. In subsequent samples, the raw sequence reads were manually inspected using Integrative Genomics Viewer (Broad Institute). In cases where new mutations were detected after therapy or at relapse, the sequence reads from the diagnostic specimen were also manually reviewed at these loci. The limit of detection in manually reviewed specimens was 0.5%, with a sequencing depth of 1000-2000 nucleotides.

2.3 | FLT3-internal tandem duplication assay

A separate PCR/capillary electrophoresis-based assay was used to confirm the absence of FLT3 ITD as NGS may not reliably detect large FLT3 internal tandem duplications (ITD). The region flanking exons 14 and 15, and their intervening intron of the FLT3 gene, were PCR amplified using the following primer set: 11F (50 -GCAATTTA GGTATGAAAGCCAGC-30) and 12R (50 -CTTTCAGCA TTTTGACGGCAACC-30). The forward primer was fluorescently labeled with fluorescein amidite at its 5′ site. The 50-μL PCR mixture contained 100 ng genomic DNA, 25 PMol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 10% DMSO, and 2.5 units of AmpliTaq Gold DNA polymerase (Life Technologies). The PCR thermal cycling steps were performed on a GeneAmp PCR system 9700 (Perkin Elmer, Waltham, MA) at 95°C for 9 minutes. That was followed by 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 2 minutes, with a final extension at 72°C for 15 minutes. One microliter of amplified product was mixed with deionized formamide and GeneScan 500 ROX size standard, according to the manufacturer's protocol, heated to 95°C for 2 minutes. It was then placed on ice for 5 minutes before capillary electropheresis analysis on the ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The FLT3-ITD mutations result in PCR products that are longer than that of wild type, which is 330 base pairs.

2.4 | Statistical analysis

Overall survival (OS) was calculated from the date of disease diagnosis until date of death. Findings from the discovery cohort (N = 40) were validated in an orthogonal AML dataset (N = 417).[7] To maximize the similarities between cohorts, we restricted survival analysis of the validation set to a follow-up of 5 years. Survival curves were estimated by the Kaplan-Meier method and OS compared using the log rank test with GraphPad Prism Software (cityi). Categorical variables were compared with a paired Students t-test.

2.5 | Fish plots

Clonality was assigned based on allele frequency of pathological variants, as reported from the targeted sequencing panel. Visualization was generated using the fishplot package in R version 3.4.0.[10]

3 | RESULTS

3.1 | Patient and disease characteristics

Forty patients with normal cytogenetics, mutated NPM1, FLT3-ITD negative and sequential samples for analysis were identified. The

median age of the cohort was 60 years, with a range of 34-79 years. All patients were treated with 7 + 3 induction chemotherapy (idarubicin 12 mg/m^2 and cytarabine 100 mg/m^2 continuous IV infusion), as per institutional standard. The majority of patients were treated with high dose cytarabine consolidation chemotherapy (n = 36) with curative intent, although four patients were transplanted in CR1 per clinician discretion. For 20/40 patients (50%), they eventually suffered a relapse: one relapsed after being transplanted in CR1, and 19 relapsed after consolidation chemotherapy.

There was no significant difference in age between the relapsed and non-relapsed group (Table 1). There was a trend towards male gender (P = .04) and higher WBC count at diagnosis (55.8 vs 30.8 P = .0483) in the patients who relapsed, consistent with previous reports.[11,12] All five patients with a WBC count greater than 100 000 cells/dL relapsed (P < .0001).

TABLE 1 Patient characteristics and number of mutations at diagnosis and after therapy in relapsed and non-relapsed cohorts

	Relapsed (n = 20)	Non-relapsed (n = 20)
Median patient age (range)	59 (34-72)	62 (38-79)
WBC at diagnosis (K/cu mm)	55.8 (2.7-169)	30.8 (1.5-83.7)
Gender	14 male, 6 female	7 male, 13 female
Number mutations present at diagnosis	3.45	2.9
Persistent mutations at recovery	Any 12/15 (80%) NPM1 4/15 (26.7%)	Any 12/19 (63.1%) NPM1 4/19 (21.1%)
Persistent mutations after consolidation	Any 12/15 (80%) NPM1 2/15 (13.3%)	Any 11/18 (61.1%) NPM1 0/18

Abbreviation: WBC, white blood cell count.

3.2 | Genes co-mutated with NPM1

The majority (38/40, 95%) of patients harbored additional mutations, with an average total of 3.1 mutations per patient (range 1-6) (Figure 1). After NPM1, DNMT3A was the most commonly mutated gene (21/40, 52.5%) followed by IDH1/2 (16/40, 40%), FLT3 tyrosine kinase domain (FLT3 TKD, 13/40, 32.5%), PTPN11 (10/40, 25%) and N/KRAS (10/40, 25%). Most DNMT3A mutations (71%, 15/21) occurred at codon R882. All IDH1 mutations occurred at codon R132, and all IDH2 mutations at R140; They are collectively referred to as IDH1/2 mutations. Recurrent mutations were also identified in TET2, WT1, monoallelic CEBPA and splicing genes: SRSF2, SF3B1, ZRSR2.

The average number of mutations at diagnosis in the relapse group was 3.45, compared to 2.9 in non-relapsed patients. This difference did not reach significance, but a similar trend between increased number of mutations and reduced OS has been reported.[6] Only two patients harbored solitary NPM1 mutations and neither suffered a relapse. Notably, WT-1 mutations were only present at diagnosis in the relapsed cohort (3/20, 15%). However, there were no statistically significant differences in single additional mutations present in relapsed vs non relapsed groups.

3.3 | Tumor suppressor mutations are frequently gained at relapse, epigenetic modifiers tend to be stable, and RAS/PTPN11 mutations are frequently lost

We compared mutations at diagnosis and relapse (n = 17 with available NGS data) to evaluate clonal evolution during chemotherapy and to stratify the relative importance of different mutations on risk of relapse. Only 2 patients relapsed with the exact same mutations, whereas most patients either gained mutations, lost mutations, or both during therapy (Figure 2A). Specific mutations gained and lost at relapse are shown in Figure 2B, and include the tumor suppressors WT1 (n = 5) and p53
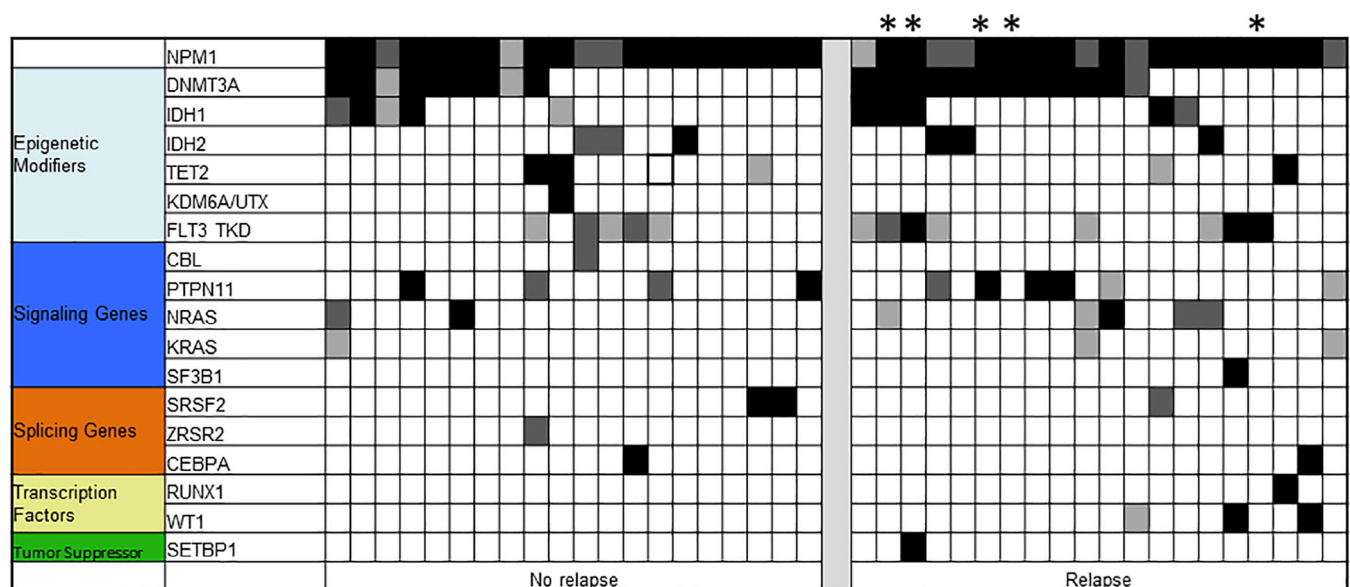

FIGURE 1 NPM1 mutated patients and co-mutations segregated into non-relapsed and relapsed patients. Mutations detected at diagnosis in non-relapsed and relapsed AML patients are shaded according to variant allele frequency (VAF). Black = 40% to 50% VAF, Gray = 20% to 39% VAF and Light Gray = 0.5% to 19% VAF. Asterisk indicate patients with WBC count >100 K/cu mm. AML, acute myeloid leukemia [Color figure can be viewed at wileyonlinelibrary.com]
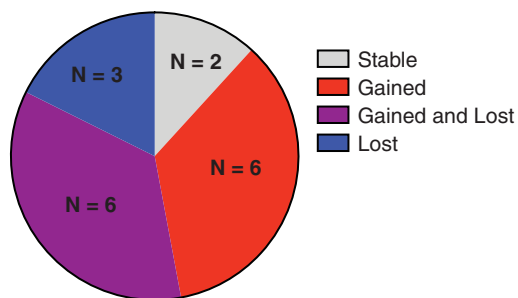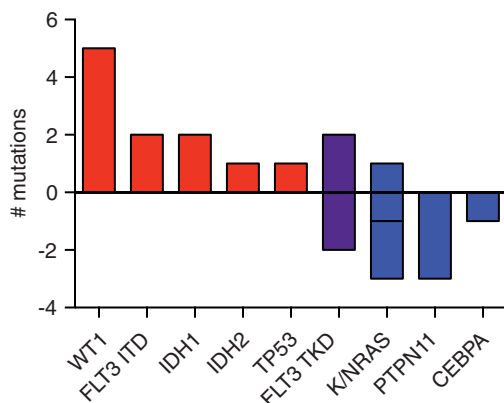
(A) Mutations at relapse compared to diagnosis

(B) Mutations gained/lost at relapse

FIGURE 2 Mutations in NRAS, KRAS and PTPN11 are frequently lost at relapse, whereas tumor suppressors are frequently gained. A, Mutations at diagnosis and at relapse were compared in 18 matched samples and the number with either gained, lost or both gained/lost mutations are plotted as a pie chart. B, The number of mutations in each gene that were either gained or lost at relapse is plotted with positive axis indicating gained mutation and negative axis indicating loss [Color figure can be viewed at wileyonlinelibrary.com]

(n = 1); FLT3 ITD (n = 2); and mutations in IDH1/2 (n = 3). Considered together with the 3 patients with WT1 mutations at diagnosis (one patient developed an additional WT1 mutation at relapse), WT1 mutations were significantly more enriched in relapsed patients (7/20, P = .007), strengthening previous data that reported frequent acquisition of WT mutations in relapsed AML.[13] In addition, cytogenetic abnormalities were also frequently acquired at relapse (7/17, 41%), consistent with loss of tumor suppressor function and genomic integrity (Table S2). In contrast, mutations in NRAS, KRAS and PTPN11 were more often lost at relapse, suggesting that these subclones were relatively more chemo-sensitive. FLT3 TKD mutations were both gained and lost at relapse, sometimes in the same patient. NRAS, KRAS, PTPN11, and FLT3 TKD mutations were also usually present at lower allele frequencies compared to NPM1, consistent with late acquisition during leukemogenesis.[14]

3.4 | The combination of DNMT3A, IDH1/2 and NPM1 mutations leads to reduced OS

The three most frequent mutations at diagnosis and relapse were NPM1, DNMT3A and IDH1/2. We hypothesized that some

combination of these mutations may have a negative effect on OS. In our discovery cohort, the 5-year overall survival OS was 54.8%, and disease free survival rate was 42.8%. We compared NPM1 mutations without DNMT3A or IDH1/2, NPM1 + DNMT3A, NPM1 + IDH1/2, and the triple combination of NPM1 + DNMT3A + IDH1/2 (Figure 3A). The triple combination of NPM1 + DNMT3a and IDH1/2 mutations showed a trend towards reduced OS (Figure 3A,B). Of note, the potential confounders of age and WBC at presentation did not differ significantly for the triple mutated NPM1 + DNMT3A + IDH1/2 patients (Figure S1). To evaluate if this combination was significant, we utilized a publically available European dataset of 1540 AML patients[7] and analyzed OS at 5 years. In this dataset there were 417 NPM1 patients without FLT3 ITD. The patients were treated with a variety of induction and consolidation trials, yet despite the heterogeneity of chemotherapy regimens, NPM1 + DNMT3A + IDH1/2 had significantly worse OS compared to the rest of NPM1 mutated patients, with a 5-year OS of only about 30% (Figure 3C,D), confirming the trend in our discovery cohort.

3.5 | Relative variant allele frequencies (VAF) of DNMT3A, IDH1/2 and NPM1 mutations differ in relapsed vs non relapsed patients

We hypothesized that order of acquisition of mutations may impact the clinical course, as this has been previously described in myeloproliferative neoplasms.[15] To investigate this further, we evaluated the VAF of NPM1, DNMT3A and IDH1/2 at diagnosis, for both non-relapsed and relapsed patients who had all three mutations. The VAF of IDH1/2 was significantly higher at diagnosis in patients that relapsed, vs those that did not (P = .04) Often it was much higher than the NPM1 VAF, suggesting that IDH1/2 mutations occurred early, and clonally expanded prior to acquisition of NPM1 (Figure 4A). The same was true of DNMT3A, although to a lesser extent. The VAF of DNMT3A and IDH1/2 relative to NPM1 for individual patients is shown in Figure S2.

3.6 | Mutation clearance after therapy

Another method to evaluate clonal architecture is the detection of persistent mutations after patients are in remission, a.k.a. MRD. This approach was previously used to demonstrate that DNMT3A mutations are present in the hematopoietic stem cell, and usually precede NPM1 mutations.[16] NGS was performed after completion of consolidation, and mutations in epigenetic modifiers frequently persisted after therapy, including 19/21 DNMT3A, 9/16 IDH and 5/7 TET2 mutations (Figure S3). Persistence of DNMT3A, TET2, or spliceosome mutations alone, did not correlate with increased relapse consistent with previous reports.[17] Only two patients had detectable NPM1 after consolidation, and both of these patients relapsed within 10 months. However, 13/15 relapsed patients did not have detectable NPM1 after consolidation. Thus MRD with our test provided poor negative predictive value, and is likely related to the limit of detection of our assay (0.5% VAF). There was a trend towards increased risk of relapse with persistent IDH1/2 mutations: 75% (6/8)

FIGURE 3 The combination of NPM1, DNMT3A, and IDH1/2 mutations predicts for inferior OS. A, Survival of patients was graphed using Kaplan-Meier method for patients with NPM1 mutations without DNMT3A and IDH1/2 (n = 12), NPM1 + DNMT3A (n = 11), NPM1 + IDH1/2 (n = 8) and NPM1 + DNMT3A + IDH1/2 (n = 9) mutations. B, The survival curves for just the triple combination, NPM1 + DNMT3A + IDH1/2, relative to the remainder of the cohort is shown. C and D, The same analysis as in A and B was done with publically available data from a larger cohort of patients (Gerstung) and plotted over 5 years for comparison; NPM1 mutations without DNMT3A and IDH1/2 (n = 205), NPM1 + DNMT3A (n = 131), NPM1 + IDH1/2 (n = 51) and NPM1 + DNMT3A + IDH1/2 (n = 30). P values calculated using log rank test [Color figure can be viewed at wileyonlinelibrary.com]
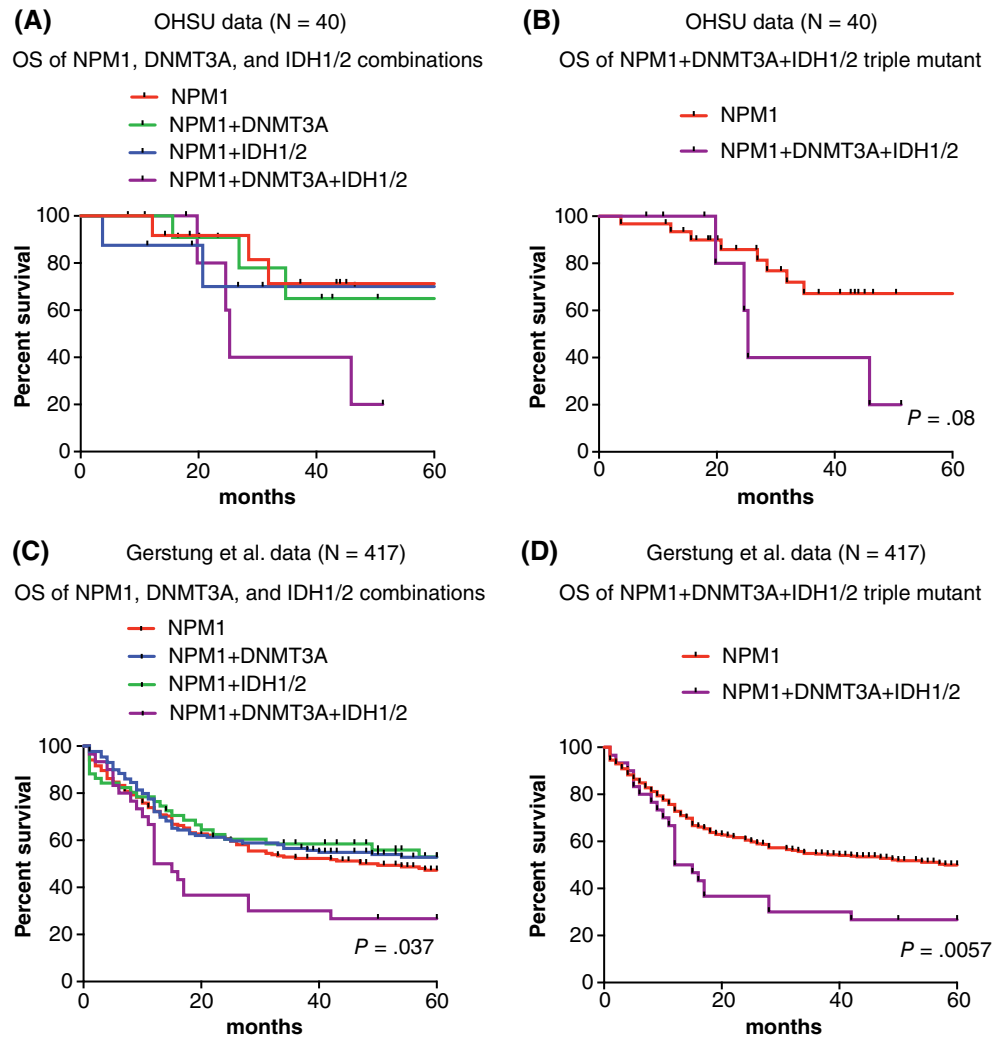
vs 37.5% (3/8), *P* = .08, further suggesting that early IDH1/2 mutations predispose to relapse, although larger numbers of patients are needed for analysis, in particular to analyze both DNMT3A and IDH1/2 mutations in remission.

4 | DISCUSSION

AML with mutated NPM1 is the largest genetic subgroup of AML, although it contains considerable genetic heterogeneity. The vast majority of NPM1 mutated AML patients harbor multiple additional mutations,[6] some of which, such as FLT3 ITD, increase the risk of relapse. For this reason, patients with NPM1 and FLT3 ITD are usually treated with allogeneic HSCT in CR1. However, recent studies have begun to refine prognostication further. The impact of the FLT3 ITD allelic ratio,[18,19] as well as FLT3 ITD co-mutation with DNMT3A,[6,20] were recently reported to have significant impacts on risk of relapse, and reduced OS. Improving risk stratification is important to identify patients who may benefit from allogeneic HSCT in first remission, or enrollment in clinical trials with targeted therapies such as IDH inhibitors. We sought to identify mutational profiles that confer poor

outcome in a well-curated, uniformly treated discovery cohort of favorable risk NPM1+ AML.

We evaluated mutations gained and lost at relapse to stratify the importance of different mutations on risk of relapse. Mutations in NRAS, KRAS, and PTPN11 were frequently lost at relapse, indicating that these mutations are more chemo-sensitive clones. The FLT3 TKD mutations were equally lost and gained, suggesting that they are also less important for driving relapse. The most commonly gained mutations at relapse were in tumor suppressor genes, including five WT1 mutations and one mutation in p53. The WT-1 mutations have been associated with worse overall survival in most, but not all, studies.[21] We also noted many newly acquired cytogenetic abnormalities at relapse, further implicating loss of tumor suppressor function. These findings suggest that loss of a tumor suppressor increases risk of relapse, although this might arise from a small, undetectable subclone at diagnosis, similar to what has been described for TP53.[22] Although WT1 was only detectable in three patients at diagnosis, our results suggest that deeper sequencing of tumor suppressors at diagnosis may be helpful to better predict relapse.

Mutations in DNMT3A and IDH1/2 were the most frequent additional mutations in the discovery cohort at diagnosis. Favorable risk
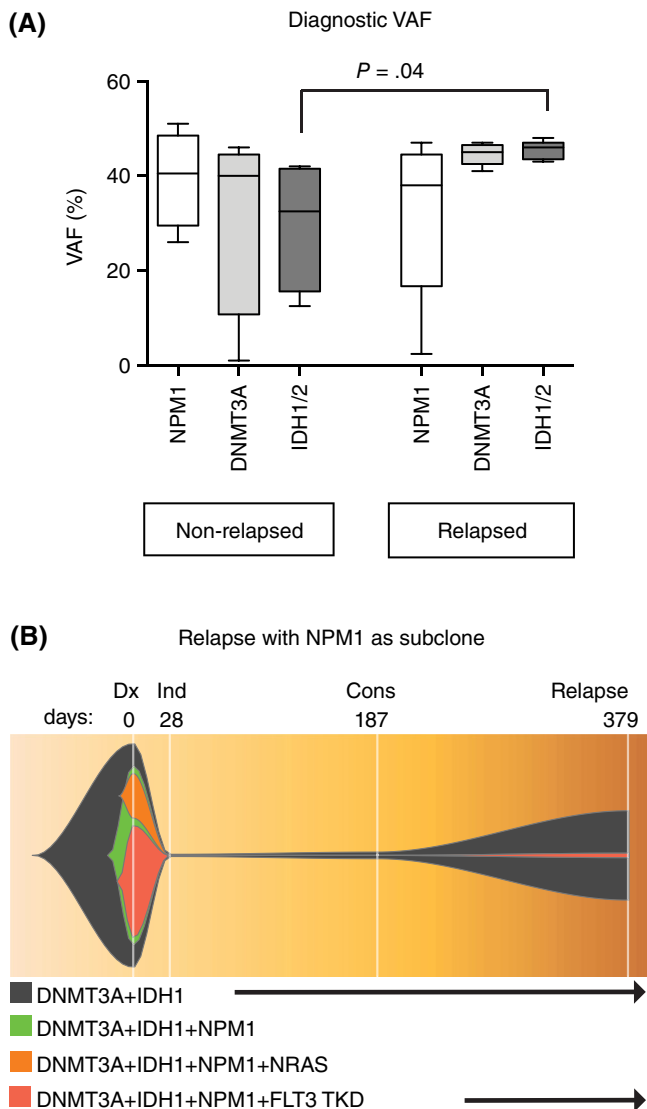
(A)

(B)

FIGURE 4 Variant allele frequencies (VAF) of DNMT3A, IDH1/2 and NPM1 in relapsed vs non relapsed patients. A, The VAF of NPM1, DNMT3A and IDH1/2 at diagnosis in patients who either did not relapse or relapsed is graphed using box and whiskers plot. IDH1/2 VAF was compared using students *t* test. B, Fishmouth plot of the inferred clonal architecture showing an example where NPM1 is a subclone at diagnosis and relapse [Color figure can be viewed at wileyonlinelibrary.com]

AML patients with concomitant NPM1, DNMT3A and IDH1/2 mutations showed a trend towards decreased overall survival. This result was independently confirmed in a large validation cohort of 417 NPM1-mutated AML patients (Figure 3A-D). In the validation cohort, there was a significant difference in OS for patients with all three mutations, with a 5-year OS of only about 30% (Figure 3C,D). The survival curves were quite similar between our cohort and this much larger dataset. It is notable that the curves separate 1 to 2 years after initiation of treatment, consistent with relapse after completion of consolidation therapy.

Of note, the combination of NPM1 with IDH1/2 mutations, but *without* DNMT3A mutations, did not have inferior OS This suggests that co-mutation patterns are important for determining prognosis

with IDH1/2 mutations. This may explain some of the previous conflicting reports of prognostic impact of IDH1/2 mutations in NPM1 mutated AML. Some groups report that NPM1 and IDH1/2 mutations confer a worse prognosis,[23] while others have reported favorable impact of NPM1 and IDH2 mutations.[24] The additional impact of DNMT3A was not accounted for in these reports. Our results are similar to the recent revelation that FLT3 ITD mutations are much worse in combination with DNMT3A, and FLT3 ITD has a less negative impact without additional DNMT3A.[6] These results may also help explain some of the conflicting data on DNMT3A as a prognostic marker, since the context of additional mutations is important to take into consideration.

We considered two potential explanations for inferior overall survival with the triple combination: 1) the combination of these three mutations is intrinsically more resistant to chemotherapy or 2) the order of acquisition of mutations alters the underlying biology. This is not to say that these explanations are mutually exclusive. Early acquisition of IDH1/2 and DNMT3A in an indolent founder clone may lead to intrinsic chemoresistance (akin to myelodysplastic syndrome that transforms into AML). Indeed, closer analysis of the relative VAF of each of the mutations, suggests that early expansion of a premalignant clone, containing both DNMT3A and IDH1/2 during leukemogenesis is at least part of the explanation.[25,26] In patients that relapsed we noted relatively higher IDH1/2 and DNMT3A VAF compared to NPM1, indicating growth and expansion of a DNMT3A + IDH1/2 clone, with subsequent acquisition of NPM1 mutation later in leukemogenesis (Figure 4A). The VAF of IDH1/2 was significantly higher at diagnosis in patients that relapsed, vs those that did not (P = .04). The NPM1 mutation was also frequently present as a subclone at relapse in these patients, with the DNMT3A and IDH1/2 clone present as the dominant clone. This is illustrated in Figure 4B; in this example, DNMT3A and IDH1 are both mutated at a higher VAF than NPM1. After NPM1 mutation, two additional mutations emerge in separate subclones: one with NRAS and one with FLT3 TKD. After consolidation, the DNMT3A and IDH1 clones were still detected at low levels, but then expanded with relapse. Although NPM1 is detectable at relapse, it is present at very low levels (1% VAF), indicating that it is a subclone. For comparison, please see Figure S4.

In non-relapsed patients with the triple combination of NPM1 + DNMT3A + IDH1/2 mutations, we noticed the reverse pattern was true, with NPM1 more commonly having the highest relative VAF, consistent with earlier acquisition of NPM1. This result initially appears to run counter to a recent publication that higher NPM1 VAF was associated with an increased risk of relapse.[27] However, this NPM1 cohort included FLT3 ITD mutations, and NPM1 VAF was also correlated with elevated WBC. In contrast, Abbas et al. showed that NPM1 allele frequency correlated with leukemia burden (and FLT3 ITD) but found no significant correlation with overall survival.[28] Once again, context is important in NPM1 mutated AML, and associated mutations impact OS.

In summary, we demonstrate in independent discovery and validation cohorts, that the triplet combination of NPM1, IDH1/2 and DNMT3A mutations is associated with significantly inferior OS. These patients may benefit from allogeneic HSCT in first remission, or from

clinical trials with IDH1/2 inhibitors: either concurrently with chemotherapy, and/or as maintenance after chemotherapy. Our data further refines the genetic subclassification of AML, which is crucial to better understand the biology of this heterogeneous disease, and to select patients who will benefit from additional therapy.

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

E.T., J.D, and J.L. developed the project; collected and analyzed data, and wrote the manuscript. M.R analyzed the European dataset and survival data. R.C., G.F., P.R., and R.P. collected data. All authors reviewed the data and the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.