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DIAGNOSTICS

original reports abstract

Persistent Molecular Disease in Adult Patients With AML Evaluated With Whole-Exome and Targeted Error-Corrected DNA Sequencing

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PURPOSE Persistent molecular disease (PMD) after induction chemotherapy predicts relapse in AML. In this study, we used whole-exome sequencing (WES) and targeted error-corrected sequencing to assess the frequency and mutational patterns of PMD in 30 patients with AML.

MATERIALS AND METHODS The study cohort included 30 patients with adult AML younger than 65 years who were uniformly treated with standard induction chemotherapy. Tumor/normal WES was performed for all patients at presentation. PMD analysis was evaluated in bone marrow samples obtained during clinicopathologic remission using repeat WES and analysis of patient-specific mutations and error-corrected sequencing of 40 recurrently mutated AML genes (MyeloSeq).

RESULTS WES for patient-specific mutations detected PMD in 63% of patients (19/30) using a minimum variant allele fraction (VAF) of 2.5%. In comparison, MyeloSeq identified persistent mutations above 0.1% VAF in 77% of patients (23/30). PMD was usually present at relatively high levels (>2.5% VAFs), such that WES and MyeloSeq agreed for 73% of patients despite differences in detection limits. Mutations in *DNMT3A*, *ASXL1*, and *TET2* (ie, DTA mutations) were persistent in 16 of 17 patients, but WES also detected non-DTA mutations in 14 of these patients, which for some patients distinguished residual AML cells from clonal hematopoiesis. Surprisingly, MyeloSeq detected additional variants not identified at presentation in 73% of patients that were consistent with new clonal cell populations after chemotherapy.

CONCLUSION PMD and clonal hematopoiesis are both common in patients with AML in first remission. These findings demonstrate the importance of baseline testing for accurate interpretation of mutation-based tumor monitoring assays for patients with AML and highlight the need for clinical trials to determine whether these complex mutation patterns correlate with clinical outcomes in AML.

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INTRODUCTION

Monitoring tumor burdens is a well-established clinical approach that informs prognostic assessment and guides treatment decisions for patients with cancer. In AML, monitoring relies on morphologic assessment of the bone marrow to identify immature AML blasts. This approach is now routinely supplemented with flow cytometry to identify cells with leukemia-associated immunophenotypes, or molecular analysis for leukemia-associated genomic alterations.[1] Genomic-based monitoring includes chromosomal abnormalities detected using metaphase karyotyping and/or fluorescence in situ hybridization, and AML gene mutations identified using molecular methods.[2-9] Although these approaches have demonstrated clinical utility, they are limited by the fact that not all patients with AML have mutations that can be monitored.[1] Advances in next-generation sequencing (NGS) technologies now allow for sequencing of multiple genes to identify patient-specific, leukemia-associated mutations for the purposes of tumor monitoring.[10-15] Indeed, these sequencing approaches have shown that the presence of persistent leukemia-associated mutations after induction chemotherapy predicts outcomes in AML.[13,16-18]

The compelling evidence for persistent molecular disease (PMD) as a risk assessment tool in AML has spurred the development of clinical NGS assays for molecular disease monitoring. However, the optimal parameters of such assays have not yet been clearly defined. Sequencing approaches vary in terms of breadth, depth, and sensitivity, and the mutation abundance thresholds (in terms of variant allele fraction [VAF]) that are most predictive remain unclear.[13,16,17] Furthermore, some studies suggest the number and

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

To compare different approaches to assessing persistent molecular disease after induction therapy for AML: deep assessment with a targeted, error-corrected next-generation sequencing panel (MyeloSeq) and broad assessment with whole-exome sequencing (WES).

Knowledge Generated

Persistent molecular disease after induction therapy is detectable in most patients by either MyeloSeq or WES. Deep targeted sequencing with MyeloSeq frequently finds previously undetected variants after induction, consistent with emerging clonal hematopoiesis. Broad WES may distinguish between persistent leukemia and residual clonal hematopoiesis through detection of passenger mutations.

Relevance

WES and MyeloSeq offer similar but distinct information regarding mutation persistence after induction. Baseline mutation determination is essential for interpretation of targeted next-generation sequencing panels given the frequent emergence of previously undetected variants after induction. Prospective trials coupled with outcome data will be needed to define the optimal target space and sequencing depth most relevant to clinical practice.

identity of the mutations that persist after therapy may be important, specifically regarding mutations prevalent in clonal hematopoiesis that may be less predictive of relapse, such as those in *DNMT3A*, *TET2*, or *ASXL1* (DTA mutations).[16-21] Finally, experience using ultrasensitive sequencing methods for testing of patient samples is limited, which makes it challenging to formalize interpretation guidelines for these assays. Although consensus guidelines for interpretation of NGS-based PMD have been proposed, additional data regarding interpretation of these assays are needed to clarify how they should be implemented.[1]

In this study, we used high-coverage (500×) exome sequencing (whole-exome sequencing [WES]) and a highly sensitive error-corrected targeted gene panel (MyeloSeq) to evaluate a cohort of 30 patients with adult de novo AML for PMD. All patients were in clinicopathologic remission after receiving standard intensive induction chemotherapy.[22] Postinduction bone marrow samples from all patients were then analyzed with WES and MyeloSeq to evaluate and compare broad versus deep methods for measuring PMD and assess clonal evolution that occurs after treatment. The analysis described here provides practical insights into the genetic architecture of AML persistence after induction chemotherapy and can inform the use of DNA sequencing for future studies that will define how to implement and interpret assessments of PMD in patients with AML. The clinical outcomes for the patients described here are being studied as part of an ongoing, prospective phase II clinical trial.

MATERIALS AND METHODS

Patients and Samples

The study population included a subset of patients enrolled in the Improving Risk Assessment of AML With a Precision Genomic Strategy to Assess Mutation Clearance trial (ClinicalTrials.gov identifier: NCT02756962; N = 28).[23] Enrollment criteria for this trial includes patients with newly diagnosed AML age 18 to 60 years who are either classified as intermediate risk by European Leukemia Net (ELN) 2010 criteria or have normal cytogenetics with mutated *NPM1* without *FLT3*-ITD.[22,24] All patients were treated with an induction regimen of cytarabine and either daunorubicin or idarubicin (either one or two cycles) and achieved remission with or without count recovery as defined by revised International Working Group criteria.[22] Presentation bone marrow samples were obtained before the initiation of treatment, and postinduction bone marrow samples were obtained approximately 30 days after induction. Normal skin biopsies or buccal swab samples were collected in remission to minimize leukemic contamination. An additional two patients met these criteria but were older than 60 years and were enrolled on an institutional banking protocol. Both the trial and banking protocol were approved by an institutional review board, and all patients provided written informed consent in accordance with the Declaration of Helsinki.

WES and Tumor/Normal Analysis

WES was performed in the Clinical Laboratory Improvement Amendments clinical sequencing laboratory at the McDonnell Genome Institute at Washington University School of Medicine (CLIA#26D2092546, CAP#9047655). Technical sequencing procedures have been described previously (see also the Data Supplement).[13] Variant identification was performed for single-nucleotide variants and small insertion-deletion variants (indels) that occurred in exons and any adjacent noncoding sequences with at least 20× coverage and were required to have a VAF >5% in the presentation bone marrow sample.[25,26] All variants were then queried in the WES data from the postinduction sample using *bam-readcount* to obtain variant allele counts for PMD assessment.[27]

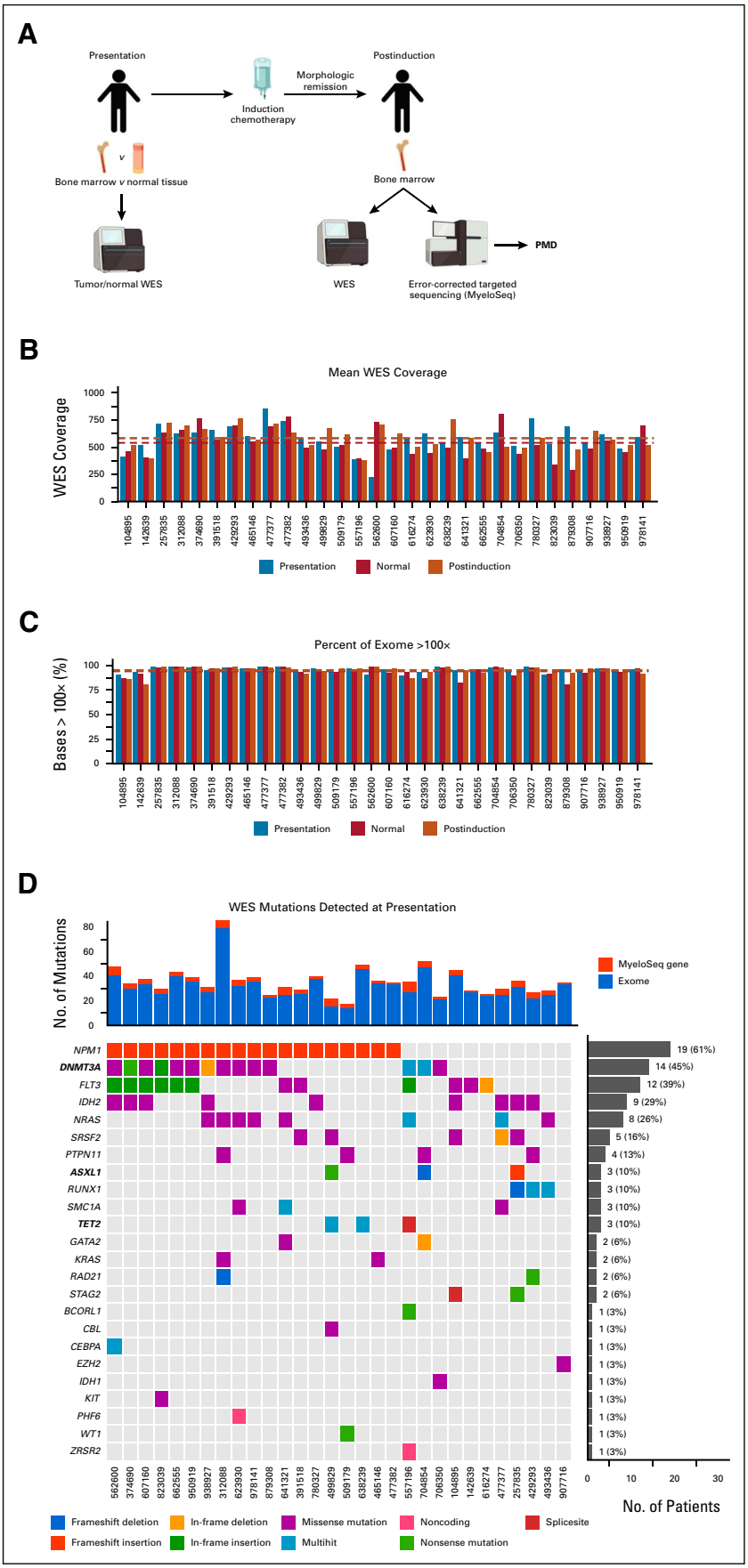

FIG 1. Study design and tumor/normal WES results for 30 patients with AML at presentation. (A) Study design, which included paired tumor/normal WES of 30 patients with de novo AML using presentation bone marrow aspirate samples and analysis of postinduction bone marrow samples (approximately 30 days after presentation) with both WES and MyeloSeq error-corrected targeted sequencing of 40 genes and mutation hotspots. (B) Mean coverage of the presentation bone marrow, normal (skin or buccal swab), and postinduction bone marrow samples using WES. (C) Percent of exome regions with >100× coverage from WES for presentation, normal, and postinduction samples. (D) Mutations identified at presentation from WES. Barplot at top shows the total number of mutations identified at presentation for each patient stratified by whether these mutations occurred in genes targeted by MyeloSeq (in orange) or elsewhere in the exome (in blue). The matrix shows the mutations and their consequences in a selected set of recurrently mutated AML genes (rows) for each patient (columns), with the total number of mutations in each gene indicated in the panel at the right. PMD, persistent molecular disease; WES, whole-exome sequencing.

TABLE 1. Clinical Features of AML Cohort

Variable	Value
No.	30
Age, years, median (range)	52.5 (34-63)
Female sex, No. (%)	12 (40)
WBC >100,000, No. (%)	4 (13)
Bone marrow blast, %, median (range)	63 (15-96)
Normal cytogenetics, No. (%)	21 (70)
ELN 2010 risk group, No. (%)	
Favorable	13 (43)
Intermediate	17 (57)
ELN 2022 risk group, No. (%)	
Favorable	11 (37)
Intermediate	10 (33)
Adverse	9 (30)
CR/CRi at day 30, No. (%)	21 (70)/9 (30)
Second induction, No. (%)	2 (7)

Abbreviations: CR, complete remission; CRi, complete remission with incomplete blood count recovery; ELN, European Leukemia Net.

Error-Corrected Targeted Sequencing With MyeloSeq

The MyeloSeq error-corrected targeted sequencing assay was performed on the postinduction bone marrow sample as described previously (see the Data Supplement).[28-30] Error-corrected reads with ≥3 reads sharing the same alignment location and unique molecular identifier were generated using the DRAGEN software platform (version 3.9.3; Illumina, San Diego, CA) and variant calling was performed using FreeBayes followed by filtering for variants in ≥5 consensus reads.[31] Annotation was performed with Ensemble version 90 using VEP and filtered to exclude polymorphisms (ie, population allele frequency >0.1% in the gnomAD database).[32]

Analysis of Persistent Molecular Disease

Variant calls from the WES tumor/normal analysis were used to define patient-specific leukemia-associated mutations for analysis of PMD at the postinduction time point using both WES and MyeloSeq. Only variant alleles detected at presentation (using the above criteria) with ≥5 supporting reads (uncorrected reads for WES, error-corrected reads for MyeloSeq) were considered persistent. VAFs were calculated using high-quality reads with either the reference or variant alleles (minimum mapping quality of 1 and minimum base quality of 13). PMD was defined as having ≥1 persistent leukemia-associated mutation with a VAF of at least 2.5% using WES and any variant regardless of VAF for MyeloSeq (except where noted).

Confirmation of Previously Undetected Variants Identified by MyeloSeq

Confirmation of previously undetected variants in the MyeloSeq data used two approaches. First, new alleles were queried directly in the postinduction WES data using a custom script that obtained base counts for the variant positions. Selected previously undetected variants with adequate remaining DNA samples were also confirmed using polymerase chain reaction-NGS confirmation (see the Data Supplement).

Statistical Analysis

Differences in proportions were compared using the chi-square test and Fisher's exact test. Numeric variables were assessed using the Mann-Whitney U test, given the non-parametric distributions of variables observed in our data set. Given the hypothesis-generating nature of this study, no correction was made for multiple comparisons. All analyses were performed using R (v4.1), using standard statistical packages.[33]

RESULTS

Genetic Profiling of Patients With Adult De Novo AML Via Tumor/Normal WES

We studied the frequency and genetic features of PMD after induction chemotherapy in 30 uniformly treated patients with adult de novo AML using two approaches. These included tumor/normal WES at presentation with repeat WES post-induction to assess PMD via broad characterization of dozens of patient-specific mutations across the exome, versus targeted sequencing of postinduction samples with MyeloSeq, an error-corrected sequencing assay that performs deep analysis of mutations in 40 recurrently mutated genes to a limit of detection of approximately 0.1% VAF (Fig 1A; Data Supplement). The study cohort included patients younger than 65 years with either intermediate risk or favorable risk with normal cytogenetics and mutated *NPM1* without *FLT3*-ITD by ELN 2010 criteria. Per the updated ELN 2022 criteria, 11 (37%), 10 (33%), and nine (30%) were favorable, intermediate, and adverse risk, respectively (Table 1). All patients achieved remission after treatment with induction chemotherapy.[24] WES on presentation, post-induction, and a skin or buccal swab sample that was used for a matched normal control (N = 90 data sets) had a mean coverage of 567× (range, 227-859; see Fig 1B). At least 80% of targeted bases were covered >100× (Fig 1C; see also the Data Supplement). WES tumor/normal variant analysis using data from the presentation sample identified a median of 35 (range, 15-88) leukemia-associated variants above 5% VAF (Fig 1D; see Materials and Methods and the Data Supplement). The spectrum of recurrent mutations and patterns of co-occurrence in this cohort were consistent with previous studies of similar AML cohorts.[10,13,14]

Assessment of PMD by WES and MyeloSeq Error-Corrected Sequencing

We analyzed PMD in postinduction samples from all patients using WES and the MyeloSeq error-corrected sequencing assay. MyeloSeq of the postinduction samples achieved a mean error-corrected depth of 426×-4,909× and covered

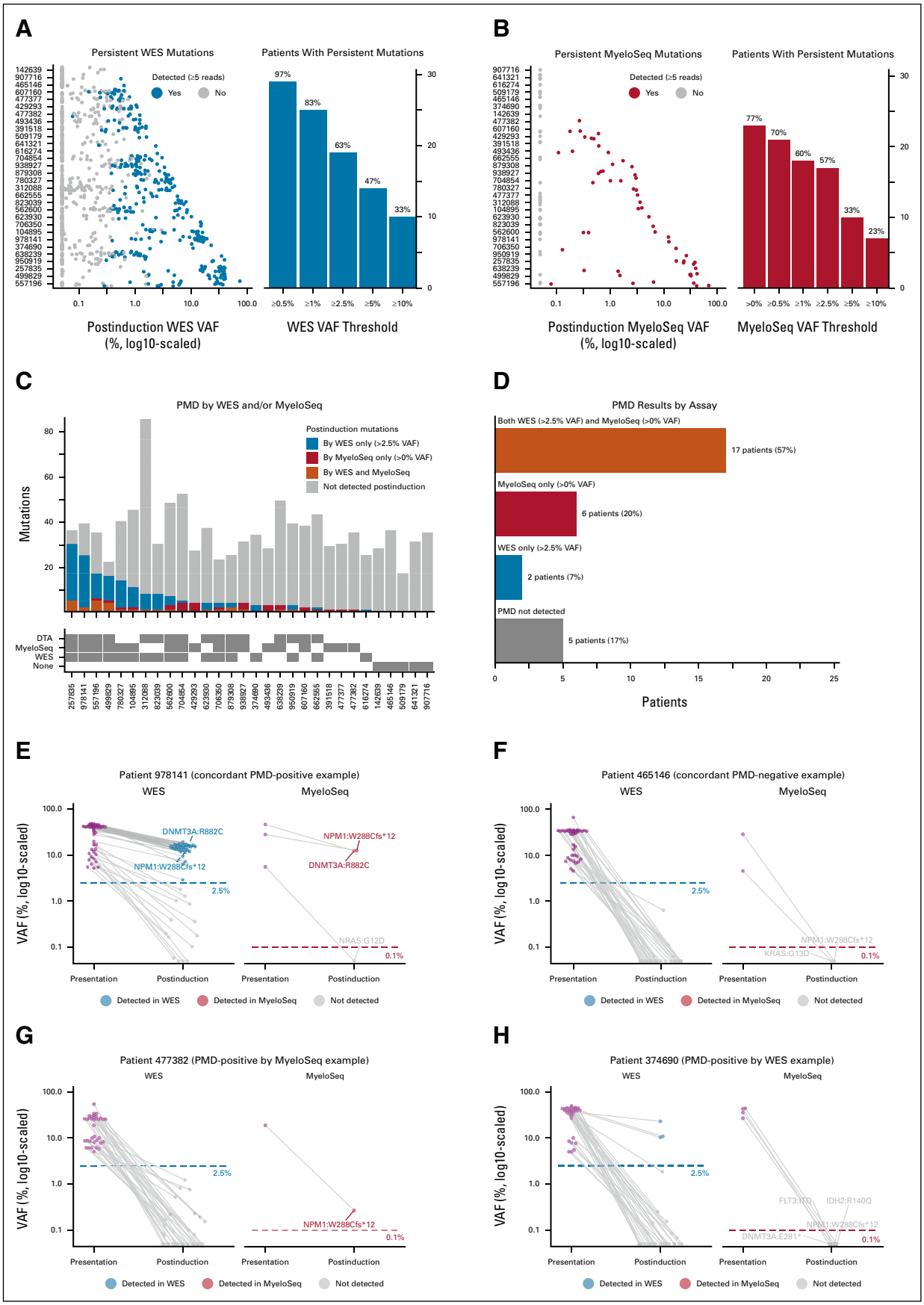

FIG 2. Comparison of PMD postinduction chemotherapy by WES and MyeloSeq. (A) Assessment of PMD in postinduction bone marrow samples by WES. The left panel shows VAFs of variants in postinduction bone marrow samples that were (continued on following page)

FIG 2. (Continued). detected at presentation from 30 patients. Variants with at least five reads of support (non–error-corrected) in the postinduction sample are shown in blue versus those that do not, which are shown in gray. The barplot at the right shows the percent of all patients with PMD using the VAF threshold indicated (from 0.5% to 10% minimum VAF). Patients were called PMD-positive if any mutation was present in the postinduction sample above the indicated VAF threshold. (B) Assessment of PMD in postinduction bone marrow samples by MyeloSeq shown in the same format as panel A with variants in red having a minimum of five consensus, error-corrected reads. Barplot shows the percent of patients who were PMD-positive if any mutation detected in the presentation sample was present in the postinduction MyeloSeq data above the indicated VAF threshold. (C) Comparison of PMD status by WES and MyeloSeq using a VAF cutoff of 2.5% for WES and five consensus (error-corrected) reads of support and any VAF for MyeloSeq. Top panel shows clearance versus persistence for each variant identified at presentation by WES in postinduction bone marrow samples for each patient. Variants not detected shown in gray, variants detected by both assays shown in orange, and variants detected by either WES or MyeloSeq shown in blue and red, respectively. (D) PMD status for all patients by WES and MyeloSeq. (E and F) Representative examples of patients with concordant PMD status between WES and MyeloSeq. Each point shows the VAF of variant identified by WES at presentation (in purple) in percent on a log base 10 scale. Variants detected in the postinduction sample by WES or MyeloSeq are shown in blue and red, respectively. Variants not detected (<2.5% VAF for WES and <5 reads for MyeloSeq) are shown in gray. Dashed lines show 2.5% and 0.1% VAFs for reference. (G and H) Representative examples of patients with discordant PMD status between WES and MyeloSeq, which occurred because of (G) recurrent mutations below 2.5% VAF that were missed by WES, or (H) variants in the exome that were not targeted by the MyeloSeq assay. PMD, persistent molecular disease; VAF, variant allele fraction; WES, whole-exome sequencing.

between 39.9% and 96.6% of the target space at >600× consensus coverage (Data Supplement). Analysis of these data for somatic variants with at least five supporting consensus reads[28] identified at least one variant in 29 of the 30 (97%) postinduction samples (median, 4 variants; range, 0-11; Data Supplement). Interestingly, only 57 of the total 119 variants (48%) were identified at presentation (median of 2 variants per patient; range, 1-6; mean VAF, 10.3%). The remaining 62 variants were not detected in the presentation sample and represent previously undetected new variants (see below).

PMD was defined as the presence of any variant identified at presentation in the postinduction sample. Different VAF thresholds were tested, given that prognostic VAF cutoffs for PMD have yet to be firmly established.[1] Using this approach, the frequency of PMD was 63% (19/30 patients) by WES and 57% (17/30 patients) by MyeloSeq using a 2.5% VAF cutoff for both assays (Figs 2A and 2B). PMD positivity at the 2.5% VAF level using WES by ELN 2022 risk group was 44% (4/9), 80% (8/10), and 64% (7/11) for adverse-, intermediate-, and favorable-risk patients, respectively. The frequency of PMD positivity increased when lower VAF cutoffs were used, with WES calling 83% (25/30) of patients PMD-positive at 1% VAF and 97% (29/30) PMD-positive using a 0.5% VAF cutoff (Fig 2A); we note that this assay did not use error-corrected sequencing, and so variant-supporting reads at this threshold may represent sequencing errors. MyeloSeq identified 60% (18/30) as PMD-positive at a 1% VAF cutoff, and 70% (21/30) at 0.5% VAF; 77% of patients (23/30) were PMD-positive if only five error-corrected consensus reads were required, with no minimum VAF threshold (Fig 2B; the lowest observed VAF was 0.08%).

We next compared PMD concordance using a 2.5% VAF cutoff for WES and a five read cutoff for MyeloSeq (and any VAF level), which is similar to criteria employed in previous studies using WES and targeted sequencing.[13,16,17] There were eight patients (27%) with discordant results, including six who were PMD-positive by MyeloSeq only, and two who were PMD-positive by WES only (Figs 2C and 2D). These occurred either because MyeloSeq detected persistent mutations below the 2.5% VAF cutoff used for WES, including variants in *NPM1*, *SRSF2*, *RAD21*, and *RUNX1* (N = 6; Fig 2G), or because patients had persistent mutations in the WES data that were not targeted by MyeloSeq (N = 2; Fig 2H). If either assay was considered, 25 of 30 patients (83%) had persistent mutations, and five patients (17%) were PMD-negative by both assays (Figs 2C-2F; see also the Data Supplement).

Gene and Mutation Spectrum of Persistent Mutations

Additional analysis of the persistent mutations was performed to characterize the genetic features associated with PMD in AML. The pretreatment VAF was correlated with mutation persistence after induction chemotherapy (Fig 3A), reflecting the hierarchy of clonal mutations in AML.[12,34] *DNMT3A* had the most persistent mutations (15 mutations in 13 patients), followed by *NPM1* (N = 9) and *IDH2* (N = 6), *RUNX1* (N = 5), *TET2* (N = 5), *SRSF2* (N = 4), and *ASXL1* (N = 3; Fig 3B). The frequency of mutation persistence in *DNMT3A*, *TET2*, or *ASXL1* was 96% (23/24), which was significantly higher than mutations in other genes (39%; 34/87; $P < 10^{-4}$, chi-squared test; Fig 3C). DTA mutations displayed uniformly high VAFs at presentation, but variable VAFs in the postinduction samples (Figs 3D-3F). This is consistent with previous studies showing that persistent DTA mutations can reflect residual AML cells or clonal hematopoiesis with a nontransformed ancestral clone.[35]

Because WES identifies a larger number of leukemia-associated mutations than targeted gene sequencing, we reasoned that these additional markers might change the interpretation of persistent DTA mutations for some patients. Comparison of WES and MyeloSeq for the 17 patients with DTA mutations at presentation showed that persistent non-DTA mutations were detected in 14 of these patients (82%) by WES, compared with 11 (65%) using MyeloSeq (Figs 4C

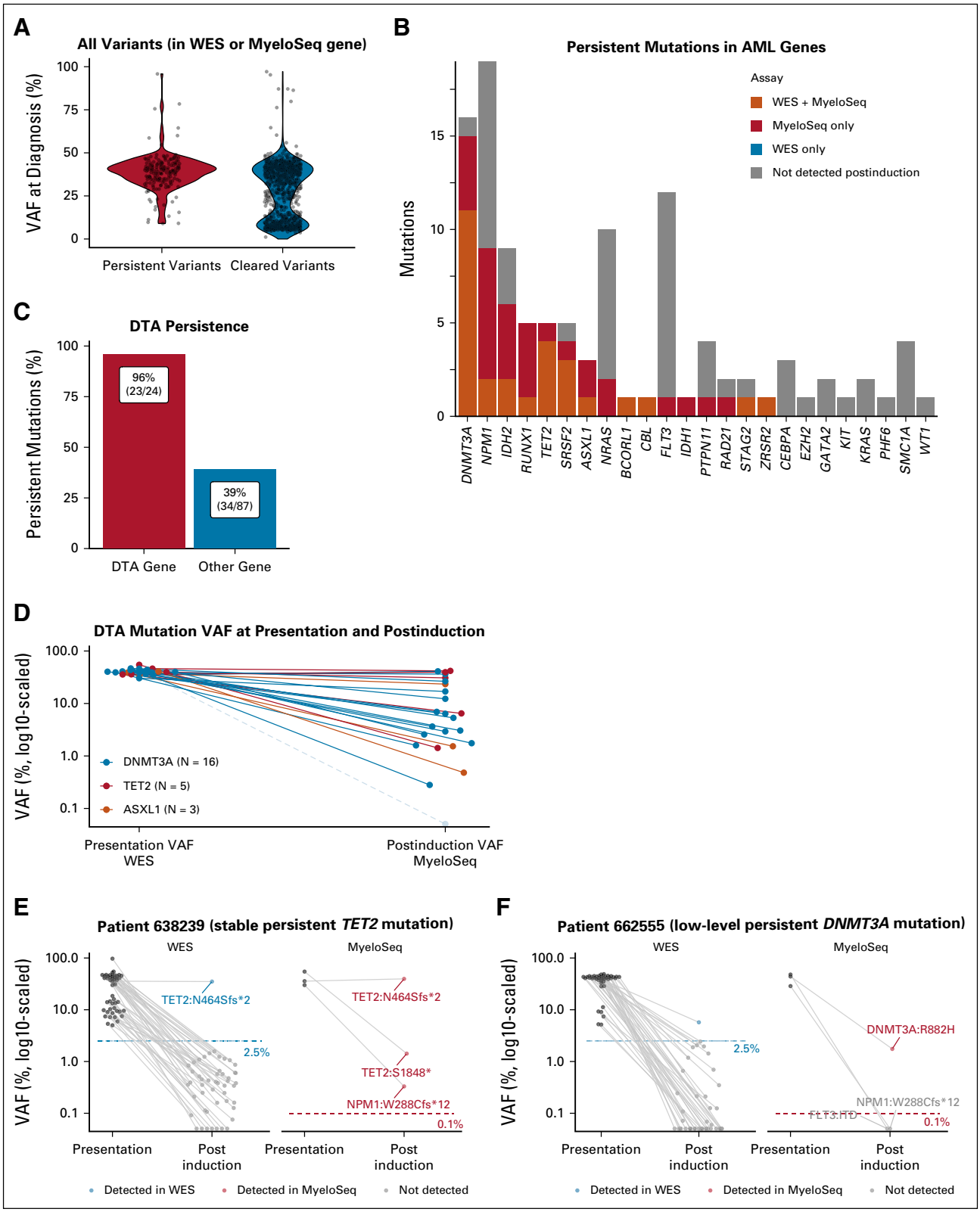

FIG 3. Features of persistent mutations detected in postinduction bone marrow samples. (A) VAF distribution from WES at presentation for variants that were detected postinduction (persistent mutations, in red), or that were not detected and therefore cleared (cleared variants, in blue). Note that cleared variants include many with VAFs below 25%, whereas persistent mutations rarely have VAFs below this level. (B) Persistent mutations detected by gene across all patients. The height of the bar shows the total number of variants with colors indicating the number of variants detected by both WES and MyeloSeq (in orange), by either WES or MyeloSeq alone (in blue and red, respectively), or that were (continued on following page)

FIG 3. (Continued). not detected in the postinduction by either assay (in gray). (C) Frequency of persistence by either assay for variants in *DNMT3A*, *TET2*, or *ASXL1* (ie, DTA genes) versus variants in all other recurrently mutated genes targeted by the MyeloSeq assay (N = 40 total genes). (D) VAFs of DTA mutations at presentation (by WES) and in the postinduction sample (by MyeloSeq). (E and F) Examples of patients with persistent DTA mutations that either (E) remained stable postinduction or (F) were only detected at a low level via MyeloSeq error-corrected sequencing. VAF, variant allele fraction; WES, whole-exome sequencing.

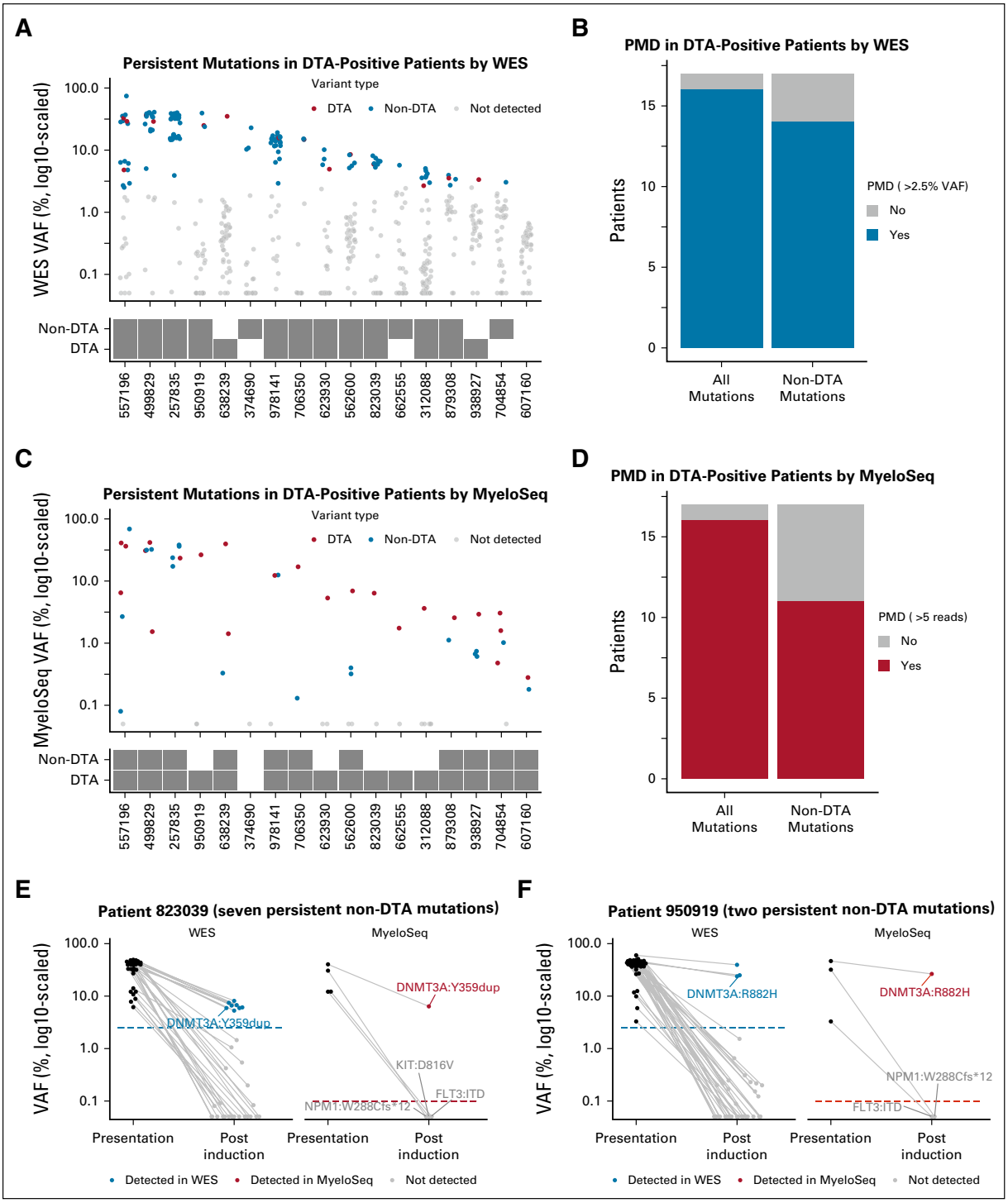

FIG 4. Spectrum of persistent mutations detected by WES and MyeloSeq in patients with mutations in *DNMT3A*, *TET2*, or *AXSL1*. (A) Persistent mutations by WES in patients with mutations in DTA genes (N = 17 patients). Points show (continued on following page)

FIG 4. (Continued). the postinduction VAFs (in percent, log10-scaled) from WES. Mutations in DTA genes are shown in red and other genes in blue. Mutations that were not detected postinduction (2.5% VAF or <5 reads) are shown in gray. The matrix shows whether DTA and non-DTA mutations were detected. (B) PMD by WES in 17 DTA-positive patients when all mutations are considered (including those in DTA genes) and when DTA mutations are excluded. There were 16 of 17 patients with PMD when all mutations were used versus 14 of 17 considering only non-DTA mutations. (C) Persistent mutations by MyeloSeq in DTA-positive patients. Panel is displayed as in B. (D) PMD by MyeloSeq in 17 patients with mutations in DTA genes. Excluding DTA mutations resulted in fewer PMD-positive patients, with 11 of 17 patients having persistent non-DTA mutations. (E and F) Example patients with DTA mutations and additional exome mutations identified by WES. (E) A patient with a *DNMT3A* mutation, and seven additional persistent exome mutations detected in the postinduction sample that could indicate the presence of a preleukemic cell population. (F) A patient with more total mutations (42 *v* 30) but only two persistent mutations, which could suggest the persistent DTA mutation is more consistent with clonal hematopoiesis than persistent AML. PMD, persistent molecular disease; VAF, variant allele fraction; WES, whole-exome sequencing.

and 4D). As expected, the greater number of patient-specific mutations in WES resulted in more persistent non-DTA mutations per patient (median of 3.5 mutations for WES, *v* median of 1 for MyeloSeq). However, the range was quite broad (1-29 additional mutations; Figs 4E and 4F). This variability indicates that persistent DTA mutations can reside in clonal cell populations with varying degrees of relatedness to the founding AML clone and suggests that WES may provide additional information about the potential for these mutations to predict relapse.

Previously Undetected Variants Identified After Chemotherapy Are Consistent With Clonal Hematopoiesis

We next analyzed the 62 variants detected in 22 patients that were not identified at presentation and therefore represent new variants that emerged postinduction. We first analyzed the postinduction WES data for evidence of these variants (see the Data Supplement), which showed read support for 27 of 56 (48%) of the previously undetected variants (Fig 5A; six variants were not analyzed because the data were unavailable). The VAFs for these variants were too low to be detected by automated pipelines (mean VAF, 1.6%), but the read support was significantly above position-specific background noise (see the Data Supplement). The remaining 29 variants without WES support had low VAFs, making supporting reads unlikely, given the coverage levels obtained (approximately 500×). We further verified six variants in five genes (*CUX1*, *DNMT3A*, *NF1*, *RAD21*, and *RUNX1*) via direct polymerase chain reaction and deep sequencing of the postinduction DNA samples; selection of these variants was based solely on sample availability. All six variants were confirmed at VAFs similar to those present in MyeloSeq (Fig 5B; Data Supplement). The previously undetected variants included frameshifts and nonsense mutations and were most common in DTA genes, *PPM1D*, *CBL*, and *CUX1* (see Fig 5C), which are rarely mutated in AML and are more common in clonal hematopoiesis.[36-42] The VAFs of these variants ranged between 0.1% and 10% (Fig 5D) and were not significantly different in patients with or without co-existing persistent mutations ($P = .07$; T-test; Fig 5E). To determine whether any of these clonal hematopoiesis variants may have been present at low levels (and were therefore not detected) before induction chemotherapy, the presentation WES data sets were directly queried for these variants. This identified two patients with clonal hematopoiesis variants that were detectable at presentation. Patient 142639 had a *TET2* nonsense mutation at a VAF just below 5% in the postinduction sample that was present at nearly the same abundance in the presentation sample (Fig 5F). Patient 104895 had two *CUX1* variants that were also present before chemotherapy, including one at a VAF of approximately 2%; interestingly, this patient had two additional variants emerge after chemotherapy, including a *TET2* frameshift and a canonical *JAK2* V617F allele (Fig 5G). These observations are consistent with prior data indicating that maintenance and/or expansion of pre-existing clonal hematopoiesis is common after induction chemotherapy in patients with AML.[19,35,43-45]

DISCUSSION

We used two sequencing-based assays to characterize the frequency, abundance, and genetic features of PMD in patients with AML after induction chemotherapy. These included approximately 500× WES that detected patient-specific mutations across the exome to a limit of detection of approximately 2.5% VAF, and MyeloSeq, an error-corrected deep sequencing assay that detected mutations in selected AML genes at VAFs of approximately 0.1% in the same samples. These assays have been shown to predict relapse in patients with AML after induction chemotherapy, and the purpose of this study was to perform a head-to-head comparison of the two methods.

Both sequencing approaches identified PMD in most patients with AML in first remission, with 83% of patients in this cohort having persistent mutations by either one or both assays. Although this cohort was primarily composed of relatively young patients and is therefore not representative of all patients with AML, it contained nearly equal numbers of adverse-, intermediate-, and favorable-risk patients using the 2022 ELN criteria. PMD was detectable in most patients across these categories and was concordant for 73% of patients using WES (with a VAF threshold of 2.5%) versus MyeloSeq (with a limit of detection of approximately 0.1% VAF) despite the differences between these assays. DTA mutations were nearly always detected in the postinduction samples, with only a single patient having complete clearance of their *DNMT3A* mutation. Most patients with persistent DTA mutations also had detectable non-DTA

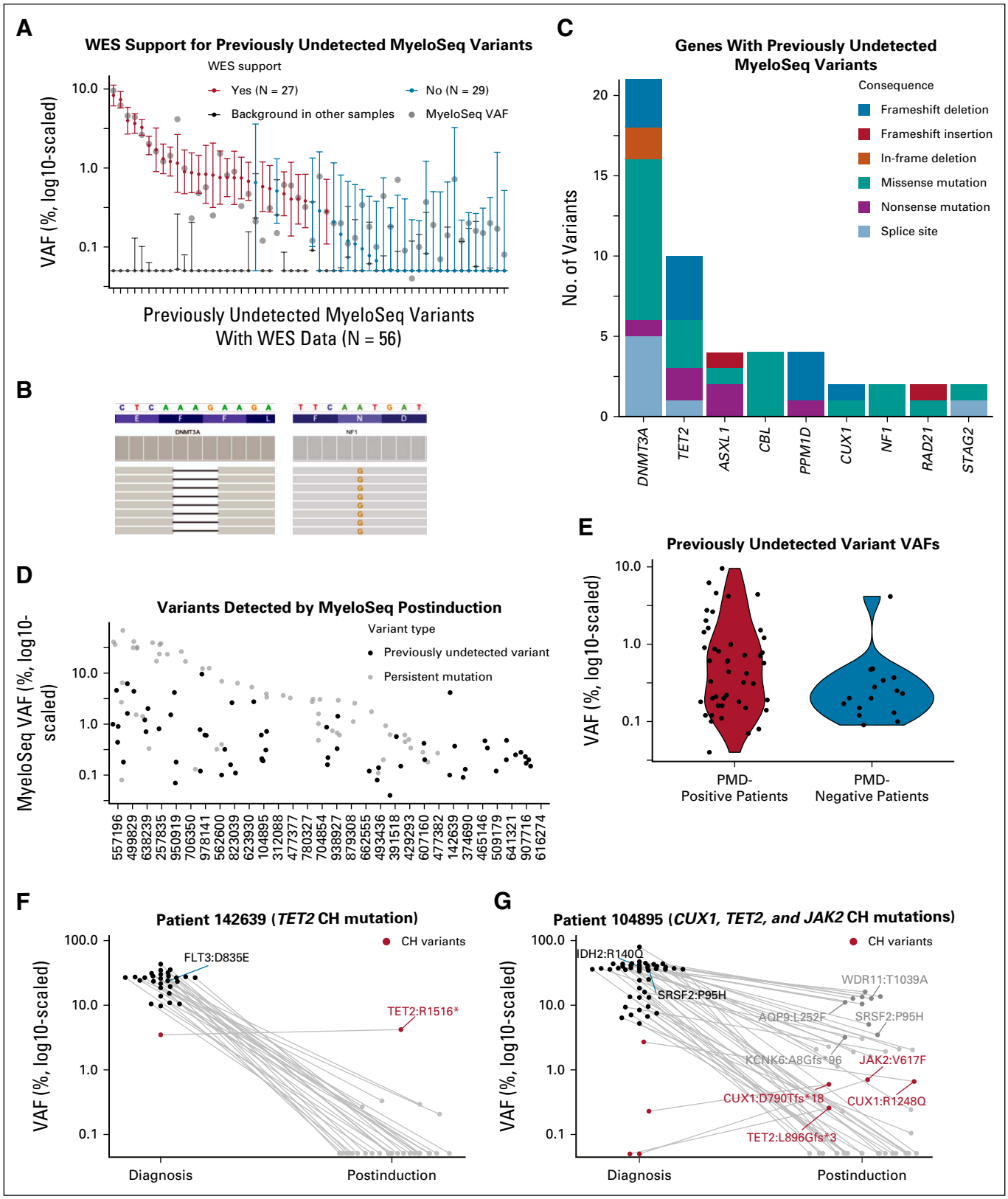

FIG 5. Features of previously undetected variants identified in postinduction samples by MyeloSeq error-corrected sequencing. (A) Support for new variants detected by MyeloSeq in WES from postinduction samples. Shown are data for 56 new variants detected in 22 patients by MyeloSeq in the postinduction sample. The MyeloSeq VAF for each previously undetected variant is shown in gray. The VAFs in WES reads from the postinduction time point are shown in red for variants with evidence for read support or blue if there was not. Evidence for support was based on whether the 95% (continued on following page)

FIG 5. (Continued). binomial CI for each variant (indicated by the error bars) did not overlap the 95% CI of the background VAF for that allele across WES data from all other patients in the cohort (in black; see Materials and Methods). (B) Examples of two variants confirmed by direct polymerase chain reaction and amplicon sequencing. See also the Data Supplement. (C) Distribution of genes with new variants detected by MyeloSeq postinduction. (D) MyeloSeq VAFs of previously undetected variants (in black) and persistent mutations (in gray). (E) VAF distributions of previously undetected variants in PMD-positive and PMD-negative patients, shown in red and blue, respectively. No significant difference in VAF was observed ($P = .07$, T-test). (F and G) Examples of patients with variants detected in the postinduction sample that were present at a low level at the presentation time point. (F) A patient with a *TET2* nonsense mutation that was present in the WES data at approximately 4% VAF at both time points. (G) A patient with two *CUX1* mutations that were present in both samples and new variants in *TET2* and *JAK2* that were not detected at presentation but emerged after therapy. CH, clonal hematopoiesis; PMD, persistent molecular disease; VAF, variant allele fraction; WES, whole-exome sequencing.

mutations, such that excluding DTA mutations changed PMD status in a minority of cases. Surprisingly, MyeloSeq found previously undetected variants in 73% of patients after induction, which was nearly as many patients with persistent mutations by this assay (77% of patients). These new variants were consistent with clonal hematopoiesis and some were detectable before treatment at nearly the same VAF, indicating they were unrelated to the leukemic clone and represent a separate clonal process that was either maintained or selected for by induction therapy.

This study further demonstrates that PMD in AML is common after induction chemotherapy, even in relatively young patients in morphologic remission. Previous studies have identified persistent mutations at this time point in approximately 50% of patients.[13,16,17] We detected at least one leukemia-associated mutation in 83% of patients using VAF cutoffs comparable with previous studies. This high prevalence suggests that additional parameters may be necessary for PMD to have clinical utility. Several studies have addressed this by excluding DTA mutations, with the assumption that these mutations could reflect clonal hematopoiesis and not persistent disease. However, at least one recent study suggests that persistent DTA mutations are not always clinically benign.[21] Other strategies could include testing later during the disease course (ie, after consolidation) or monitoring the mutation abundance dynamics at multiple time points. The presence of certain mutations at or above a specific VAF level, or their trajectory over time, could maximize the predictive power of PMD assessments. The target size of the sequencing assay could be an important variable, especially for patients with DTA mutations, where additional persistent mutations in non-DTA genes could inform predictions about relapse and survival.[13,16] Future clinical trials that include larger sequencing panels like WES will therefore be needed to determine whether measuring persistent ancestral clones at this resolution provides additional prognostic information that improves clinical management of patients with AML.

A striking finding in this data set was the number of previously undetected variants identified by MyeloSeq after induction chemotherapy. Nearly three quarters of patients had new variants that were not detected at presentation and that displayed a mutation spectrum consistent with clonal hematopoiesis. These variants likely represent non–leukemia-associated clonal cell populations that either emerged or persisted after chemotherapy, which has been reported by previous studies using single-cell approaches.[46-50] Because the spectrum of these variants overlaps that observed in AML, it would be extremely challenging to determine whether they represent persistent disease, or a separate clonal process on a per-patient basis without prior information. Knowledge of the mutations in the presentation sample will therefore be critical for determining whether variants detected after chemotherapy reflect persistent disease, or clonal hematopoiesis. Although several studies have now reported on the predictive value of persistent mutations in AML, the clinical significance of newly detected mutations is unknown. This uncertainty argues against reporting new variants detected at low levels in clinical reports for patients who are sequenced during remission, until more is known about the clinical outcomes of patients with AML who have detectable clonal hematopoiesis after induction chemotherapy.

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DATA SHARING STATEMENT

All variant calls are included in the Data Supplement.

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