ORIGINAL ARTICLE

Molecular Minimal Residual Disease in Acute Myeloid Leukemia

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ABSTRACT

BACKGROUND

Patients with acute myeloid leukemia (AML) often reach complete remission, but relapse rates remain high. Next-generation sequencing enables the detection of molecular minimal residual disease in virtually every patient, but its clinical value for the prediction of relapse has yet to be established.

METHODS

We conducted a study involving patients 18 to 65 years of age who had newly diagnosed AML. Targeted next-generation sequencing was carried out at diagnosis and after induction therapy (during complete remission). End points were 4-year rates of relapse, relapse-free survival, and overall survival.

RESULTS

At least one mutation was detected in 430 out of 482 patients (89.2%). Mutations persisted in 51.4% of those patients during complete remission and were present at various allele frequencies (range, 0.02 to 47%). The detection of persistent *DTA* mutations (i.e., mutations in *DNMT3A*, *TET2*, and *ASXL1*), which are often present in persons with age-related clonal hematopoiesis, was not correlated with an increased relapse rate. After the exclusion of persistent *DTA* mutations, the detection of molecular minimal residual disease was associated with a significantly higher relapse rate than no detection (55.4% vs. 31.9%; hazard ratio, 2.14; P<0.001), as well as with lower rates of relapse-free survival (36.6% vs. 58.1%; hazard ratio for relapse or death, 1.92; P<0.001) and overall survival (41.9% vs. 66.1%; hazard ratio for death, 2.06; P<0.001). Multivariate analysis confirmed that the persistence of non-*DTA* mutations during complete remission conferred significant independent prognostic value with respect to the rates of relapse (hazard ratio, 1.89; P<0.001), relapse-free survival (hazard ratio for relapse or death, 1.64; P=0.001), and overall survival (hazard ratio for death, 1.64; P=0.003). A comparison of sequencing with flow cytometry for the detection of residual disease showed that sequencing had significant additive prognostic value.

CONCLUSIONS

Among patients with AML, the detection of molecular minimal residual disease during complete remission had significant independent prognostic value with respect to relapse and survival rates, but the detection of persistent mutations that are associated with clonal hematopoiesis did not have such prognostic value within a 4-year time frame. (Funded by the Queen Wilhelmina Fund Foundation of the Dutch Cancer Society and others.)

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Acute myeloid leukemia (AML) is a heterogeneous group of clonal hematopoietic stem-cell disorders with a variable response to therapy.[1-3] Although the majority of patients with newly diagnosed AML have morphologic complete remission after they are treated with intensive induction chemotherapy, relapse rates remain high.[2] Decisions about the choice of postremission therapy in patients with AML currently depend on the identification of a selected set of genetic markers at diagnosis and the detection of residual disease with multiparameter flow cytometry.[2,4] Quantitative molecular evaluation during complete remission could further improve prognostication of outcomes in patients with AML.

The potential of the detection of molecular minimal residual disease after treatment to predict disease relapse in patients with AML has been explored, but assessment of molecular minimal residual disease is not widely established in clinical practice. Previous studies have dealt with only a few leukemia-specific genetic aberrations.[5-11] Next-generation sequencing enables comprehensive, simultaneous detection of somatic mutations that are often patient-specific, both at diagnosis and during treatment.[5,12] Initial studies showed the complex dynamics of residual mutations after induction therapy and the possible association between the persistence of certain somatic mutations and risk of relapse.[12,13]

In determining whether molecular monitoring may be applicable in patients with AML, the phenomenon of age-related clonal hematopoiesis (also known as clonal hematopoiesis of indeterminate potential),[14-17] a condition characterized by the recurrence of gene mutations (allele frequency, >2%) in healthy persons with no evidence of hematologic disease, has added an extra layer of complexity. Persons with age-related clonal hematopoiesis have a slightly increased risk of developing hematologic cancers over time.[14,15,18] Mutations in the epigenetic regulators *DNMT3A*, *TET2*, and *ASXL1* (i.e., *DTA* mutations) are most common in persons with age-related clonal hematopoiesis.[14-19] Residual leukemia-specific mutations that are present in the bone marrow during complete remission may represent either residual leukemic cells or age-related clonal hematopoiesis.[14,15,17] Whether posttreatment persistence of genetic mutations associated with age-related clonal hematopoiesis in the bone marrow from patients with AML has an effect on the disease course remains unclear.

We evaluated a large cohort of patients with AML to investigate whether targeted molecular monitoring with next-generation sequencing could add clinical value for predicting the recurrence of leukemia.

Methods

Study Design

The study was designed by the first two and the last two authors, who wrote the manuscript with input from the other authors. The authors vouch for the completeness and accuracy of the data and analysis. No one who is not an author contributed to the manuscript. There was no commercial support for the study.

Patients and Cell Samples

Between 2001 and 2013, we obtained samples of bone marrow or peripheral blood from 482 patients, between the ages of 18 and 65, who had a confirmed diagnosis of previously untreated AML (428 patients) or had refractory anemia with excess of blasts, with a score on the Revised International Prognostic Scoring System of more than 4.5, indicating a high or very high risk of relapse (54 patients). To be included in the study, patients had to be in either complete remission or complete remission with incomplete hematologic recovery (defined according to the European Leukemia Net recommendation; hereafter collectively referred to as complete remission), with less than 5% blast cells in the bone marrow,[2,4] after receiving two cycles of induction chemotherapy (Fig. S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Among patients in whom at least one mutation was detected at diagnosis, samples were obtained during a defined period of remission, between 21 days and 4 months after the start of the second treatment cycle.

Patients were treated according to the clinical protocol of either the Dutch–Belgian Cooperative Trial Group for Hematology–Oncology (HOVON)[20] or the Swiss Group for Clinical Cancer Research (SAKK). The treatment protocols and patient eligibility criteria have been described previously.[21,22] All the patients provided written informed consent. Details about the

patients and cell samples are provided in the Supplementary Appendix.

TARGETED NEXT-GENERATION SEQUENCING AND MULTIPARAMETER FLOW CYTOMETRY

To detect the mutations in 54 genes that are often present in patients with hematologic cancers, we used targeted next-generation sequencing with the Illumina TruSight Myeloid Sequencing Panel (Illumina), following the manufacturer's protocol. Detection of residual disease with multiparameter flow cytometry was performed as described previously.[23] Details about these detection methods and data interpretation are provided in the Supplementary Appendix.

STATISTICAL ANALYSIS

The 430 patients in whom at least one mutation was detected at diagnosis were randomly assigned to either a training cohort (283 patients) or a validation cohort (147 patients); the two cohorts had similar clinical, cytogenetic, and molecular characteristics (Table 1, and Fig. S1 and Table S1 in the Supplementary Appendix). The primary end point was the 4-year cumulative incidence of relapse (defined according to the European LeukemiaNet recommendation[4]), and the secondary end points were the 4-year rates of overall survival and relapse-free survival. Within each cohort, the difference in the incidence of relapse between patients in whom residual disease was detected and those in whom residual disease was not detected was evaluated with the use of the method of Gray and the Fine and Gray model for competing risks. The log-rank test and the Cox proportional-hazards model were used for survival analyses. A two-sided P value of 0.05 or less was considered to indicate statistical significance. Details about the statistical analyses are provided in the Supplementary Appendix.

RESULTS

DETECTION OF MUTATIONS AT DIAGNOSIS

We performed targeted next-generation sequencing to detect gene mutations at diagnosis in samples obtained from 482 patients with AML (Fig. S1 in the Supplementary Appendix). We detected an average of 2.9 mutations per patient; at least 1 single mutation, which could potentially serve as a marker of residual disease, was present in 430

Table 1. Clinical, Cytogenetic, and Molecular Characteristics of the 430 Patients.*

Characteristic	Value
Age at diagnosis — yr	
Median	51
Range	18–66
Sex — no. (%)	
Male	216 (50)
Female	214 (50)
White-cell count per microliter at diagnosis — no. (%)	
≤100,000	387 (90)
>100,000	43 (10)
2017 European LeukemiaNet risk classification at diagnosis — no. (%)	
Favorable	204 (47)
Intermediate	113 (26)
Adverse	113 (26)
No. of chemotherapy cycles to attain complete remission — no. (%)	
1	360 (84)
2	70 (16)
Consolidation therapy — no. (%)	
None	46 (11)
Chemotherapy	117 (27)
Autologous hematopoietic stem-cell transplantation	78 (18)
Allogeneic hematopoietic stem-cell transplantation	189 (44)
Cytogenetic analysis at diagnosis — no. (%)†	
t(8;21)	27 (6)
inv(16)	24 (6)
Complex karyotype	38 (9)
Monosomal karyotype	30 (7)
Mutation at diagnosis — no. (%)	
ASXL1	31 (7)
CEBPA double mutation	19 (4)
DNMT3A	141 (33)
FLT3	
Tyrosine kinase domain	53 (12)
Internal tandem duplication, low ratio	40 (9)
Internal tandem duplication, high ratio	51 (12)
NPM1	168 (39)
RUNX1	50 (12)
TET2	48 (11)

* The percentages may not sum to 100 because of rounding.
† Karyotyping failed in 13 patients.

(89.2%) of the patients. Mutations in *NPM1*, *DNMT3A*, *FLT3*, and *NRAS* were among the most common detectable mutations at diagnosis (Table 1 and Fig. 1A, and Table S1 in the Supplementary Appendix).

DETECTION OF MUTATIONS DURING COMPLETE REMISSION

We then performed targeted next-generation sequencing to detect persistent mutations after induction therapy in samples of bone marrow obtained from 430 patients who were in complete remission. Persistent mutations were detected in 51.4% of the patients (Fig. 1A, and Fig. S2A in the Supplementary Appendix). The rate at which mutations persisted was highly variable across genes. *DTA* mutations were most common, persisting at rates of 78.7% for *DNMT3A*, 54.2% for *TET2*, and 51.6% for *ASXL1* (Fig. 1A). In contrast, the majority of mutations in genes related to the RAS pathway were cleared after induction therapy, with mutations in *NRAS*, *PTPN11*, *KIT*, and *KRAS* persisting at rates of 4.2%, 7.0%, 13.5%, and 12.5%, respectively.

Of note, the allele frequencies of the mutations that persisted during complete remission ranged from 0.02 to 47% (Fig. 1B). This finding suggests that residual mutation-bearing cells could constitute a minor population of the cells or perhaps even a majority of the cells. An allele frequency of 50% is consistent with the presence of a heterozygous mutation in all cells. Thus, although the patients were in morphologic complete remission, which would typically imply that heterozygous mutations are present at allele frequencies lower than 2.5% (the equivalent of <5% blast cells in the bone marrow), the samples that were obtained during remission often contained mutations with much higher allele frequencies (Fig. 1B).

Mutations that persisted after induction therapy at allele frequencies higher than 2.5% were often *DTA* mutations (Fig. 1, and Fig. S2 and S3 in the Supplementary Appendix). In contrast, mutations in *IDH1*, *IDH2*, *STAG2*, *TP53*, and other genes only occasionally persisted after induction therapy at allele frequencies higher than 2.5%, and thus the allele frequencies of these mutations were typically consistent with the state of morphologic complete remission (<5% blast cells in the bone marrow).

Because *DTA* mutations have been established as the most common gene mutations in persons with age-related clonal hematopoiesis,[14-19] the persistent *DTA* mutations might have represented nonleukemic clones that repopulated the bone marrow after induction therapy. Among patients who had both *DTA* mutations and non-*DTA* mutations at diagnosis, non-*DTA* mutations were generally cleared after induction chemotherapy, whereas *DTA* mutations often remained detectable during complete remission and were the only persistent mutations in 90 of 133 (67.7%) of those patients (Fig. S2 in the Supplementary Appendix). These observations are consistent with the notion that residual cells bearing *DTA* mutations after induction therapy represent nonleukemic clones rather than persistent malignant disease.

RELAPSE AND SURVIVAL END POINTS

In the training cohort (283 patients), we found that the detection of any persistent mutation during complete remission was associated with an increased risk of relapse (4-year relapse rate, 48.2% with detection vs. 32.4% with no detection; P=0.03) (Fig. S4A in the Supplementary Appendix). We then imposed various thresholds for allele frequency to determine whether the prognostic value of the persistent mutations would improve after the exclusion of mutations with a high allele frequency, which could indicate a state of clonal hematopoiesis. The correlation of persistent mutations with an increased relapse risk appeared to be independent of allele frequency. A correlation with relapse risk generally remained present when we excluded persistent mutations with allele frequencies at or above the following thresholds: 30% (P=0.09), 20% (P=0.11), 10% (P=0.01), 5% (P=0.04), 2.5% (P=0.007), and 1% (P=0.07) (Fig. S4 in the Supplementary Appendix). The exclusion of persistent mutations with certain allele frequencies had no clear effect on the relationship between persistent mutations and an increased relapse risk, thus precluding the identification of a threshold for allele frequency that could be used to distinguish populations at higher or lower risk for relapse. As we mentioned previously, the patients with persistent mutations at high allele frequencies were enriched for *DTA* mutations (Fig. 1B).

We next determined whether persistent *DTA* mutations, which are associated with age-related clonal hematopoiesis, might be correlated with

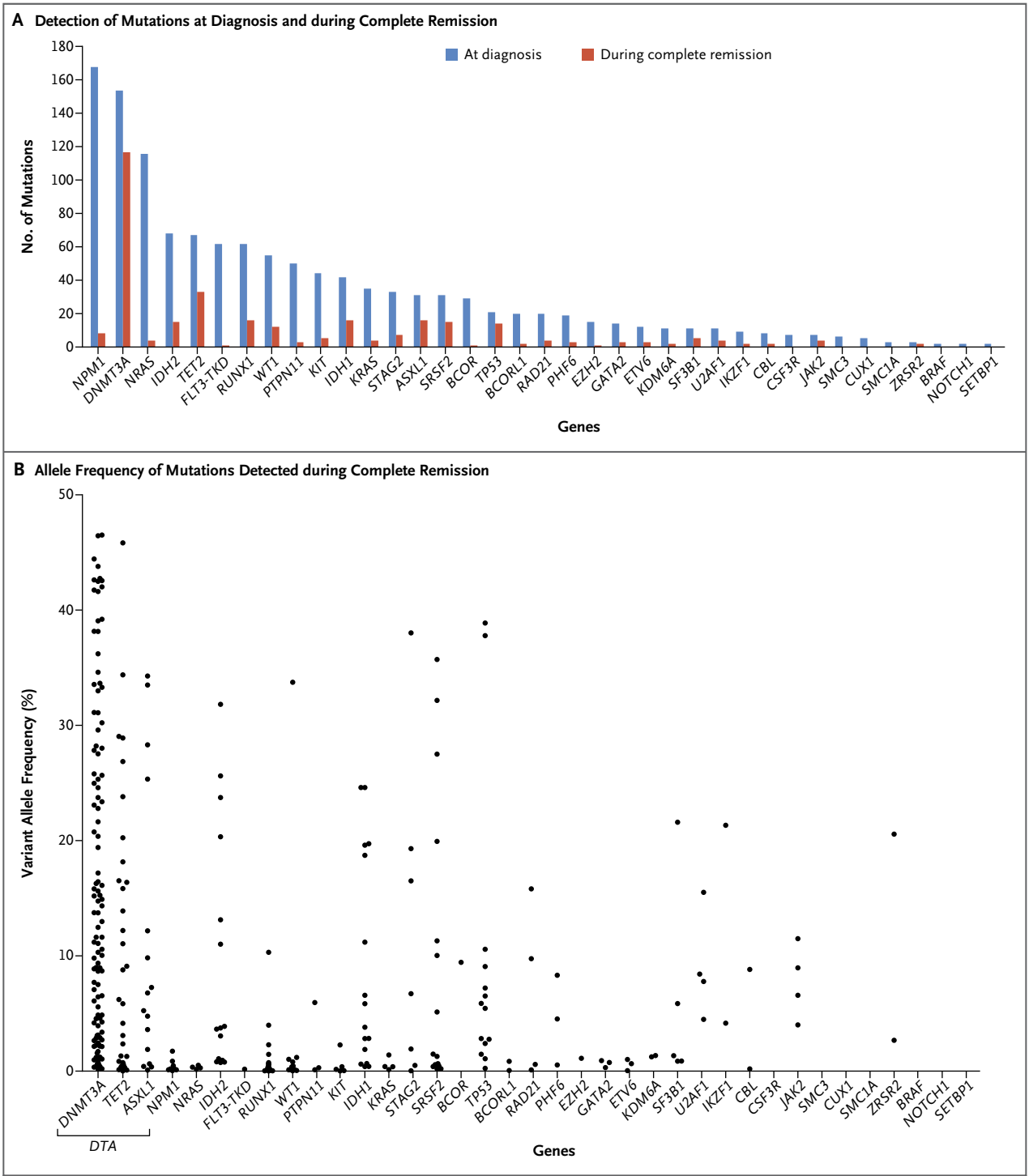


Figure 1. Detection of Mutations at Diagnosis and during Complete Remission and Allele Frequency of Mutations Detected during Complete Remission.

Panel A shows the number of mutations in each leukemia-associated gene, both at diagnosis of acute myeloid leukemia and during complete remission, in 430 patients. Panel B shows the allele frequency of each mutation in each gene during complete remission in 430 patients. In male patients, the variant allele frequencies for *PHF6*, *KDM6A*, *ZRSR2*, *BCOR*, *BCORL1*, and *STAG2* (on the X chromosome) were divided by 2.

an increased relapse risk. We observed that the detection of persistent *DTA* mutations was not significantly associated with a higher 4-year relapse rate than no detection (P=0.29). The absence of a correlation was independent of allele frequency. No significant correlation of persistent *DTA* mutations with an increased relapse risk was apparent when we excluded persistent *DTA* mutations with allele frequencies at or above the following thresholds: 30% (P=0.91), 20% (P=0.66), 10% (P=0.89), 5% (P=0.82), 2.5% (P=0.53), and 1% (P=0.92) (Fig. S5 in the Supplementary Appendix). In contrast, among patients who had persistent *DTA* mutations during complete remission, coexisting persistent non-*DTA* mutations had high prognostic value with respect to relapse (4-year relapse rate, 66.7% with detection vs. 39.4% with no detection; P=0.002) (Fig. 2A). Thus, in patients with persistent *DTA* mutations, the presence of residual disease that specifically included coexisting non-*DTA* mutations represented a predictor of impending relapse.

We next assessed whether persistent non-*DTA* mutations might be correlated with an increased relapse risk. The detection of persistent non-*DTA* mutations at any allele frequency was strongly associated with an increased relapse risk (4-year relapse rate, 55.7% with detection vs. 34.6% with no detection; P=0.001) (Fig. 2B), as well as with reduced relapse-free survival (4-year rate of relapse-free survival, 56.7% with detection vs. 36.6% with no detection; P=0.006) and reduced overall survival (4-year rate of overall survival, 65.3% with detection vs. 43.7% with no detection; P=0.01) (Fig. 2C, and Fig. S6 in the Supplementary Appendix).

To assess the reproducibility of these results, we evaluated the effect of sequencing-based detection of persistent non-*DTA* mutations during complete remission on the rates of relapse, relapse-free survival, and overall survival in the validation cohort (147 patients). The rates with detection versus no detection were as follows: 4-year relapse rate, 55.1% versus 26.5% (P<0.001); 4-year rate of relapse-free survival, 60.6% versus 35.6% (P<0.001); and 4-year rate of overall survival, 67.6% versus 37.1% (P<0.001) (Fig. 2B and 2C, and Fig. S6 in the Supplementary Appendix). The results in the validation cohort confirmed the significant findings in the training cohort.

In the combined training and validation cohorts (a total of 430 patients), persistent non-*DTA* mutations were detected during complete remission in 28.4% of the patients. Detection of these mutations was associated with a significantly higher 4-year relapse rate than no detection (55.4% vs. 31.9%; hazard ratio, 2.14; 95% confidence interval [CI], 1.57 to 2.91; P<0.001), as well as with lower 4-year rates of relapse-free survival (36.6% vs. 58.1%; hazard ratio for relapse or death, 1.92; 95% CI, 1.46 to 2.54; P<0.001) and overall survival (41.9% vs. 66.1%; hazard ratio for death, 2.06; 95% CI, 1.52 to 2.79; P<0.001) (Fig. S6 in the Supplementary Appendix).

MULTIVARIATE AND SENSITIVITY ANALYSES

We performed multivariate analyses that accounted for the major established relevant prognostic factors, including age, white-cell count, 2017 European Leukemia Network risk classification, and the number of cycles of induction chemotherapy needed to attain complete remission. Sequencing-based detection of non-*DTA* mutations maintained significant independent prognostic value with respect to the rates of relapse (hazard ratio, 1.89; 95% CI, 1.34 to 2.65; P<0.001), relapse-free survival (hazard ratio for relapse or death, 1.64; 95% CI, 1.22 to 2.20; P=0.001), and overall survival (hazard ratio for death, 1.64; 95% CI, 1.18 to 2.27; P=0.003) (Table 2). No significant interactions were apparent between the detection of residual disease and the other prognostic factors in the multivariate model, type of consolidation therapy, or disease entity (AML vs. refractory anemia with excess of blasts) (data not shown).

In sensitivity analyses involving correction for variation in the time at which bone marrow specimens were obtained for sequencing analysis (within the remission period of 21 days to 4 months after the second treatment cycle), the prognostic value of sequencing-based detection of non-*DTA* mutations with respect to the rates of relapse, relapse-free survival, and overall survival remained unaffected (Table S2 in the Supplementary Appendix). In addition, an analysis that included postremission treatment with allogeneic stem-cell transplantation as a time-dependent variable conferred no effect on the prognostic value of the detection of residual disease (Table S3 in the Supplementary Appendix).

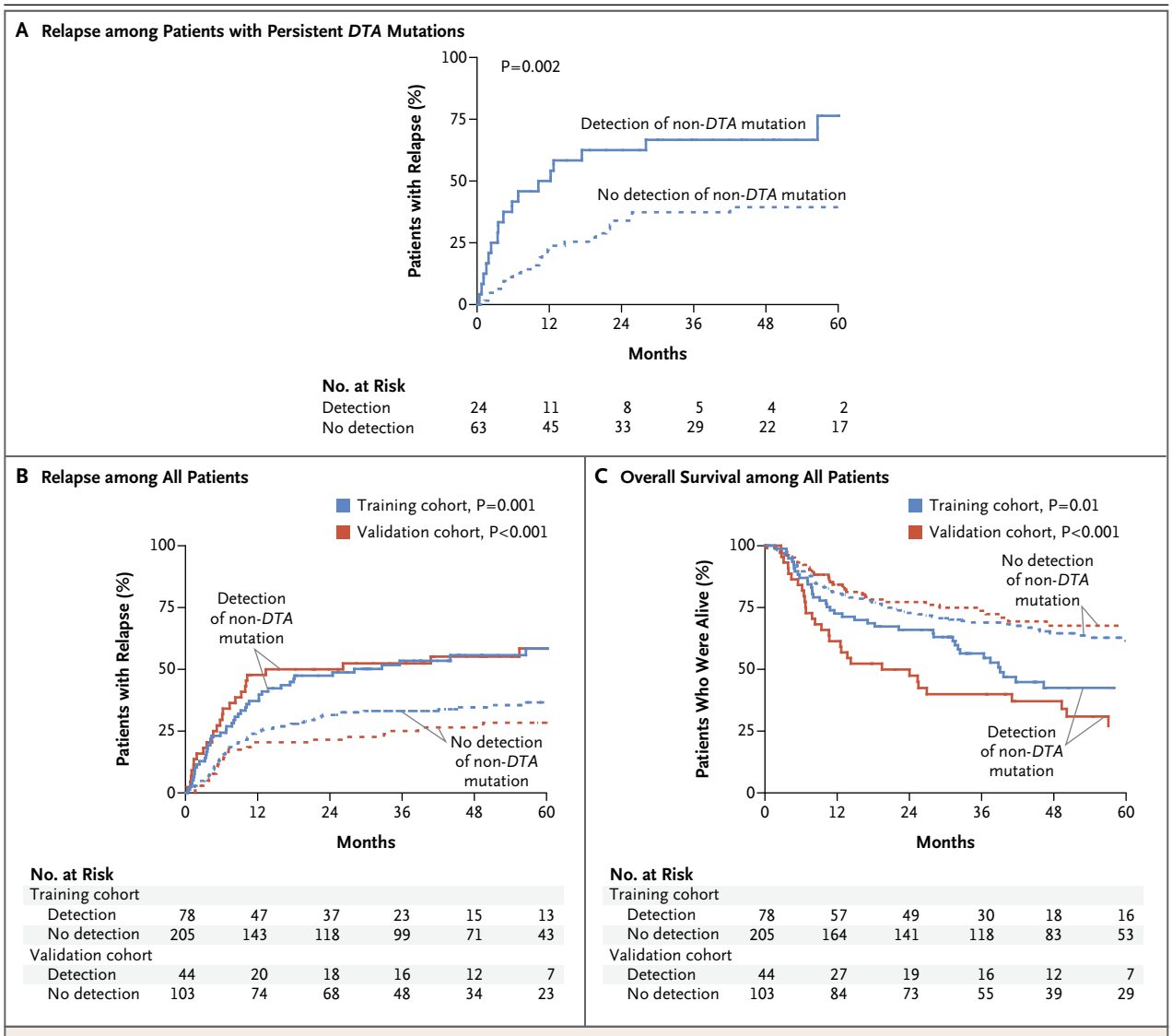


Figure 2. Rates of Relapse and Overall Survival.

Shown is the cumulative incidence of relapse among patients in the training cohort with persistent *DTA* mutations, according to the detection of coexisting persistent non-*DTA* mutations during complete remission (Panel A), and among all patients in the training and validation cohorts, according to the detection of any persistent non-*DTA* mutations during complete remission (Panel B). Panel C shows the rate of overall survival among all patients in the training and validation cohorts, according to the detection of any persistent non-*DTA* mutations during complete remission. *DTA* mutations are mutations in *DNMT3A*, *TET2*, and *ASXL1*.

DETECTION OF RESIDUAL DISEASE WITH MULTIPARAMETER FLOW CYTOMETRY

Multiparameter flow cytometry is an increasingly used method for predicting relapse in patients with AML who are in complete remission.[7,24] We compared next-generation sequencing for the detection of persistent non-*DTA* mutations with flow cytometry for the detection of residual disease in a representative subgroup of 340 patients, from whom sufficient samples were obtained for both analyses. Concordant results (either detection or no detection on both assays) were found in 69.1% of the patients (30 patients with detection and 205 with no detection), whereas persistent non-*DTA* mutations were detected only on sequencing in 64 patients and only on flow cytometry in 41

Table 2. Multivariate Analysis of Prognostic Factors for Relapse, Relapse-free Survival, and Overall Survival.

Prognostic Factor	Relapse		Relapse-free Survival		Overall Survival	
	Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)	P Value	Hazard Ratio (95% CI)	P Value
Molecular minimal residual disease: detection vs. no detection	1.89 (1.34–2.65)	<0.001	1.64 (1.22–2.20)	0.001	1.64 (1.18–2.27)	0.003
Age: per year	1.01 (0.99–1.03)	0.21	1.02 (1.00–1.03)	0.009	1.03 (1.01–1.04)	0.001
White-cell count per microliter at diagnosis: >100,000 vs. ≤100,000	2.16 (1.31–3.56)	0.003	2.03 (1.34–3.08)	0.001	2.02 (1.27–3.21)	0.003
2017 European Leukemia Network risk classification						
Intermediate vs. favorable	1.67 (1.12–2.49)	0.01	2.01 (1.42–2.83)	<0.001	2.53 (1.72–3.72)	<0.001
Adverse vs. favorable	1.83 (1.26–2.66)	0.002	2.21 (1.58–3.10)	<0.001	2.67 (1.83–3.92)	<0.001
Number of chemotherapy cycles to attain complete remission: 2 cycles vs. 1 cycle	2.17 (1.50–3.15)	<0.001	2.43 (1.74–3.39)	<0.001	2.96 (2.09–4.21)	<0.001

patients. The 4-year relapse rate was 73.3% among patients in whom both assays were positive, 52.3% among those who had residual disease on sequencing but not on flow cytometry, 49.8% among those who had residual disease on flow cytometry but not on sequencing, and 26.7% among those in whom both assays were negative (Fig. 3). In a multivariate analysis that combined the results of sequencing and flow cytometry, the combined use of the two assays for the detection of residual disease conferred independent prognostic value with respect to the rates of relapse (P<0.001), relapse-free (P<0.001), and overall survival (P=0.003) (Table S4 in the Supplementary Appendix).

DISCUSSION

In addition to the presence of genetic abnormalities at diagnosis, the continued presence of particular gene mutations during or after treatment carries prognostic information for certain genetically defined AML subtypes.[5-11] This applies, for example, to AML associated with a mutation in *NPM1*, for which the detection of a residual mutation in *NPM1* transcripts during complete remission is indicative of an increased probability of relapse.[8,9] However, this example is only representative of a single-gene approach. We report the results of a systematic study that involved a large number of patients with AML, in which we used a genomewide approach to evaluate the persistence of multiple gene mutations for the effect on treatment outcomes. Patients were treated with intensive chemotherapy regimens and attained morphologic complete remission, with a median follow-up exceeding 3 years.

Of note, age-related clonal hematopoiesis,[14-17] which is characterized by recurrent somatic mutations in leukemia-associated genes in persons with no apparent hematologic disease, adds a challenge in the detection of residual disease. Our study showed that the persistence of mutations that are most commonly associated with age-related clonal hematopoiesis (i.e., *DTA* mutations [mutations in *DNMT3A*, *TET2*, and *ASXL1*]) during complete remission did not contribute to a measurably increased risk of relapse within a follow-up period of 4 years in adults with AML who were younger than 65 years of age. This appeared to be true for mutations that were present at various allele frequencies, which suggests that the

clone size in age-related clonal hematopoiesis yields no prognostic value with respect to the end points defined in this study.

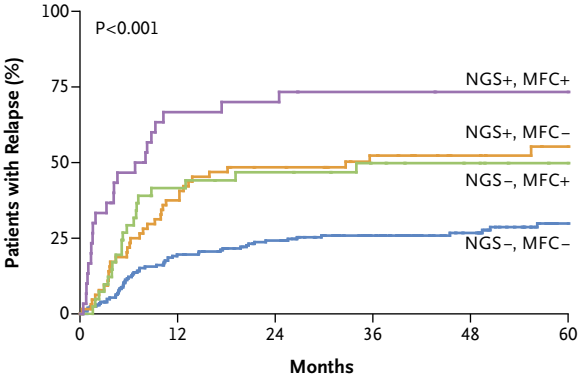
The cells bearing *DTA* mutations appeared to persist and possess a selective clonal advantage over normal stem cells when they repopulated the bone marrow after induction therapy. This finding is consistent with the competitive clonal advantage of hematopoietic stem cells with deficiencies and mutations in *DNMT3A* and *TET2*, an advantage that has been reported previously.[25-27] The proliferative advantage of hematopoietic stem cells with *DTA* mutations and their capacity to withstand chemotherapy because of inherent resistance may explain why persistent premalignant *DTA* mutations were not correlated with an increased probability of relapse and thereby did not constitute a reliable molecular biomarker for the assessment of relapse risk.

It is possible that gene mutations other than *DTA* mutations also partially reflect clonal hematopoiesis. However, at this time, we cannot rigorously verify the possibility that gene mutations associated with age-related clonal hematopoiesis also reside as subfractions among the other gene abnormalities in leukemia cells. In addition, mutations in *TP53*, *IDH1*, and *IDH2*, along with genes related to the RAS pathway and spliceosome genes, have been shown to have distinct biologic features in the context of AML pathogenesis.[28-31] Therefore, in this study, we collectively considered non-*DTA* mutations to be abnormalities that are unrelated to clonal hematopoiesis.

Our study had a median follow-up of almost 40 months. Among patients with AML who have complete remission, most relapses generally occur within the first 4 years. We found that the continued persistence of *DTA* mutations was not associated with an increased relapse risk, and thus these residual cells may not need to be eliminated to prevent relapse. However, the limited follow-up of 40 months does not rule out the possibility that persistent *DTA* mutations represent an increased risk of relapse at a later time point.

Although sequencing-based detection enables assessment for residual disease in virtually all patients with AML, it is imperfect in two ways. First, not all patients with residual mutation-bearing cells have a relapse. Second, some patients with no measurable residual disease have a relapse. It is conceivable that relapse estimation can be improved with the development of technological variations of sequencing-based approaches that have greater sensitivity or a broader scope (e.g., those with molecular barcoding, exome sequencing, or whole-genome sequencing) or with the identification of additional molecular and phenotypic markers so that quantitative minor clones or subclones associated with the leukemia are captured by the assay. In this respect, it is of particular interest that the use of multiparameter flow cytometry[7,24] — which identifies patients with AML who have an increased risk of relapse according to an entirely different approach that is based on a residual leukemia-associated immunophenotype[23,32] — can increase the yield of identification of residual leukemia during complete remission.

In this study, gene sequencing and multiparameter flow cytometry each had independent and additive prognostic value with respect to rates of relapse and survival in patients with AML. The detection of residual leukemia with both methods is associated with an excessively high probability of relapse (approximately 75%), and the absence of detection of residual disease with both methods is correlated with a relatively low probability of relapse (approximately 25%). Thus, the combined use of sequencing and flow cytometry dur-



No. at Risk						
NGS+, MFC+	30	8	7	5	4	4
NGS–, MFC+	41	22	18	14	11	7
NGS+, MFC–	64	39	30	22	15	11
NGS–, MFC–	205	153	130	101	69	42

Figure 3. Rate of Relapse According to Results of Next-Generation Sequencing and Multiparameter Flow Cytometry.

Shown is the cumulative incidence of relapse, according to the presence of positive (+) or negative (–) results for the detection of persistent non-*DTA* mutations during complete remission on next-generation sequencing (NGS) and on multiparameter flow cytometry (MFC).

ing complete remission warrants further development and evaluation in clinical practice.

In conclusion, targeted sequencing-based detection of molecular minimal residual disease during complete remission was associated with an increased risk of relapse or death in patients with AML. However, over a 4-year follow-up period, the risk of relapse or death was not influenced by the persistence of genetic lesions that are associated with age-related clonal hematopoiesis.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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APPENDIX

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