Integrated Genomic Profiling, Therapy Response, and Survival in Adult Acute Myelogenous Leukemia

Brian Parkin1, Peter Ouillette1, Mehmet Yildiz1, Kamliai Saiya-Cork1, Kerby Shedden2, and Sami N. Malek1

Abstract

Purpose: Recurrent gene mutations, chromosomal translocations, and acquired genomic copy number aberrations (aCNA) have been variously associated with acute myelogenous leukemia (AML) patient outcome. However, knowledge of the co-occurrence of such lesions and the relative influence of different types of genomic alterations on clinical outcomes in AML is still evolving.

Experimental Design: We performed SNP 6.0 array-based genomic profiling of aCNA/copy neutral loss-of-heterozygosity (cnLOH) along with sequence analysis of 13 commonly mutated genes on purified leukemic blast DNA from 156 prospectively enrolled non-FAB-M3 AML patients across the clinical spectrum of de novo, secondary, and therapy-related AML.

Results: TP53 and RUNX1 mutations are strongly associated with the presence of SNPs-based aCNA/cnLOH, while FLT3 and NPM1 mutations are strongly associated with the absence of aCNA/cnLOH. The presence of mutations in RUNX1, ASXL1, and TP53, elevated SNP-A-based genomic complexity, and specific recurrent aCNAs predicted failure to achieve a complete response to induction chemotherapy. The presence of ≥1 aCNA/cnLOH and higher thresholds predicted for poor long-term survival irrespective of TP53 status, and the presence of ≥1 aCNA/cnLOH added negative prognostic information to knowledge of mutations in TET2, IDH1, NPM1, DNMT3A, and RUNX1. Results of multivariate analyses support a dominant role for TP53 mutations and a role for elevated genomic complexity as predictors of short survival in AML.


Introduction

Substantial progress has been made in the characterization of recurrent genomic aberrations in acute myelogenous leukemia (AML) and their association with clinical outcomes. In particular, risk groups of AML based on karyotype-based cytogenetics remain important in daily clinical decision-making (1–3). Over the last few years, several groups have extended the discovery of multiple recurrently mutated protein-coding genes in AML by reporting the prognostic and therapeutically predictive significance of individual mutations [including NPM1 (4–6), FLT3 (7–9), CEBPA (10, 11), DNMT3A (12–14), RUNX1 (15–17), TET2 (18, 19), ASXL1 (20, 21), IDH1 (22, 23), IDH2 (24, 25), NRAS/KRAS (26, 27), TP53 (28–30), and others (31–34)] as well as combinations of these mutations (35–38) in AML patient cohorts. In addition, several groups have published studies demonstrating the correlation of specific SNP array-detected aCNA or the total number of aCNA/cnLOH (genomic complexity) with clinical outcomes (29, 39–43). Importantly, however, the relative influence on clinical AML outcomes of different types of genomic alterations, including gene mutations, structural genomic changes, or aCNA/cnLOH, remains unclear as lesion types rarely occur in isolation. While a few integrated analyses of the patterns of co-occurrence of various genomic alterations have been published (44), these studies have not extended findings to the study of chemoresistance and survival.

A majority of studies assessing the prognostic value of mutated genes in AML have been restricted to AML with normal karyotype (NK-AML), as this category is clinically heterogeneous, lacks a prognostic structural genomic marker, and because some mutated genes are enriched in NK-AML. The published cohorts studied are subject to selection based on age and other inclusion and exclusion criteria, which adds further restrictions on the population analyzed.

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Translational Relevance

Several gene mutations, structural chromosomal aberrations, specific acquired copy number aberrations (aCNA), and the total number of aCNAs as detected through SNP 6.0 array profiling have been associated with clinical outcome in acute myelogenous leukemia (AML). However, it remains incompletely understood how their interrelatedness affects their predictive and prognostic usefulness. In an AML cohort, failure to achieve a complete remission after one or two intensive induction regimens predicted for very poor long-term survival. The partial or complete resistance to induction chemotherapy significantly associated with mutations in RUNXI, ASXL1, and TP53, various recurrent aCNAs, and elevated SNP–A–based genomic complexity. Prognostically, the presence of ≥1 SNP–A–based aCNA/cnLOH and all higher thresholds predicted for poor long-term survival irrespective of TP53 status. When combining these data with AML-TCGA results in de novo AML, the presence of ≥1 aCNA/cnLOH added negative prognostic information to knowledge of mutations in TET2, IDH1, NPM1, DNMT3A, and RUNXI.

To further refine basic biologic knowledge of patterns of occurrence of genomic lesion types in AML and of the effects of various genomic aberration types on AML outcome, we profiled a consecutively enrolled prospective AML cohort for aCNA/cnLOH using SNP 6.0 array profiling and determined the mutation status of 13 recurrently mutated genes using Sanger sequencing. This approach has allowed us to integrate multiple genomic features across all major clinical subgroups of non-M3 AML and across the full age range of adult patients. Here, we identify genomic traits that associate with clinical AML subtypes, gene mutations that associate with genomic complexity or stability, and genomic traits that associate with sensitivity or resistance to standard induction therapy and ultimately survival.

Materials and Methods

Patients

Between March 2005 and June 2011, 173 patients with previously untreated non-M3 AML were enrolled into this study at the University of Michigan Comprehensive Cancer Center (Ann Arbor, MI). The study was approved by the University of Michigan Institutional Review Board (IRBMED #2004-1022) and written informed consent was obtained from all patients before enrollment. Of the samples from 173 patients, 156 resulted in written informed consent was obtained from all patients before enrollment. Of the samples from 173 patients, 156 resulted in

Array data analysis

Affymetrix CEL files for each blast and buccal sample underwent initial quality control screening with the Genotyping Console program after which Affymetrix "Birdseed" algorithm generated SNP call files with call rates for the entire sample group between 93.64% and 99.45%, with a mean call rate of 98.17%; none gave out-of-bounds results. Genomic copy number heatmaps were generated as previously described (45). Two independent observers visually inspected parallel heatmap copy number images of AML blast and paired normal DNA samples generated through dChip. Only those copy number changes detected in blast DNA but not in paired normal DNA were called somatic, and lesions had to be ≥30 SNP positions in length to be scored positive. All visually determined aCNA were corroborated with an algorithmic lesion calling method as previously described (47). LOH analysis was performed using LOH tool v2 as previously described (47). All resulting aCNA/cnLOH are listed in Supplementary Table S3 and SNP 6.0 array data files for all 156 patient samples analyzed were deposited in the GEO public database (accession number GSE61323).

TCGA data analysis

The recently published AML cohort of The Cancer Genome Atlas (TCGA) provided additional clinically annotated gene mutation and SNP–A–patient data (44). To facilitate a meaningful comparison of aCNA/cnLOH from TCGA with the Michigan cohort, SNP 6.0 Affymetrix CEL files from paired normal and followed by cryopreservation as previously described (45). Briefly, AML blast DNA used for SNP 6.0 profiling and DNA sequencing was extracted from thawed AML cell samples that were negatively column enriched for blasts using anti-human CD3 (Miltenyi Biotec 130-050-101), anti-human CD14 (if blasts were negative for CD14 expression; Miltenyi Biotec 130-050-201), anti-human CD19 (if blasts were negative for CD19 expression; Miltenyi Biotec 130-050-301), and anti-human CD235a (Miltenyi Biotec 130-050-301) microbeads to remove lymphocytes, mature monocytes, and nucleated red blood cells. The enriched samples were washed and stained with fluorescein isothiocyanate-conjugated-anti-CD33, phycoerythrin-conjugated anti-CD13, and allophycocyanin-conjugated anti-CD45 (all antibodies: eBioscience). Live cells (propidium iodide–negative) were gated for blasts by identifying cells with intermediate-intensity staining for CD45 and low- to moderate-intensity side scatter. CD33 and CD13 were used to further delineate myeloid and erythroid and mature myeloid lineage cells. This strategy resulted in a ≥90% pure blast population (45) that was confirmed with morphologic inspection of cytospins. DNA was extracted as previously described (46).

Exon resequencing of 13 recurrently mutated genes in AML

All coding exons and adjacent intronic sequences of CEBPA, ASXL1, TET2, DNMT3A, RUNXI, and BCORL1 along with exon 12 of NPM1, exons 13–15 and 20 of FLT3, exons 2–10 of TP53, exons 2–3 of KRAS and NRAS, exon 2 of IDH1, and exon 4 of IDH2 with adjacent intronic sequences were resequenced using PCR and sequencing primers designed with the primer3 tool; sequence information was generated as previously described (45). All mutations were confirmed using unamplified tumor DNA and paired patient buccal DNA as templates.

Cell isolation and preparation of sample DNA

Peripheral blood or bone marrow mononuclear cells from patients with AML were isolated using Ficoll gradient separation and determined the mutation status. When negative column enrichment for blasts using anti-human CD3 (Miltenyi Biotec 130-050-101), anti-human CD14 (if blasts were negative for CD14 expression; Miltenyi Biotec 130-050-201), anti-human CD19 (if blasts were negative for CD19 expression; Miltenyi Biotec 130-050-301), and anti-human CD235a (Miltenyi Biotec 130-050-301) microbeads to remove lymphocytes, mature monocytes, and nucleated red blood cells. The enriched samples were washed and stained with fluorescein isothiocyanate-conjugated-anti-CD33, phycoerythrin-conjugated anti-CD13, and allophycocyanin-conjugated anti-CD45 (all antibodies: eBioscience). Live cells (propidium iodide–negative) were gated for blasts by identifying cells with intermediate-intensity staining for CD45 and low- to moderate-intensity side scatter. CD33 and CD13 were used to further delineate myeloid and erythroid and mature myeloid lineage cells. This strategy resulted in a ≥90% pure blast population (45) that was confirmed with morphologic inspection of cytospins. DNA was extracted as previously described (46).
tumor TCGA AML specimens were downloaded from dbGaP after obtaining data access approval (dbGaP approval #29753-3). These files were analyzed with the identical informatics preprocesing pipeline and visual and algorithmic lesion calling methods used for the Michigan SNP 6.0 arrays above.

Of the 200 published matched pairs, twenty were excluded due to a diagnosis of APL (AML-M3), two for out-of-bounds results (TCGA-2855 and -2883), two for failure to pass the "Birdseed" algorithm (TCGA-2848 and -2891), and six for high visual and algorithmic background noise that precluded accurate lesion calling (TCGA-2802, -2833, -2843, -2847, -2876, and -2891). This resulted in 170 matched pairs with a complete catalog of aCNA/cnLOH, which is included in Supplementary Table S3. Gene mutation status for the thirteen genes of interest in this study and clinical characteristics including survival data based on the May 13, 2013, update were obtained from published Supplementary Data (44). Notable differences in the genomic profiling of TCGA specimens compared with the Michigan cohort include the use of whole exome or genome sequencing followed by non-Sanger based confirmation of gene mutation status and the use of tumor samples that were not enriched for leukemic cells.

Statistical analysis

Overall survival was defined as time (in months) between AML diagnosis and the patient's death. For patients still alive, the censoring date was September 1, 2013. Univariate and bivariate analyses were based on Kaplan–Meier estimates of survivor functions. Median survival times were estimated directly from the survivor function estimates. Significance levels for group-wise comparisons in the univariate analyses assess whether the HR between groups differs from 1 in a Cox proportional hazards model. For bivariate analyses, Cox models were fit to subsamples defined by the levels of one of the two factors being analyzed. The reported P value assesses whether the univariate HR for the other factor differs from 1 within the subsample. Multivariate analyses denoted using $q$ values. The SEM was calculated for the mean difference for the factors as reported in the results. The reported significance levels assess whether the hazard for a given factor differs from 1 when the other factors in the model are held fixed.

Comparison of clinical features of the various groups studied was performed using two-sided $\chi^2$, Mann–Whitney, and Kruskal–Wallis tests. Pairwise associations between gene mutations, recurrent aCNA/cnLOH, etiological AML subtypes, and type of response to induction chemotherapy were calculated using a two-sided Fisher exact test corrected for multiple hypotheses testing with the Benjamini–Hochberg FDR algorithm and denoted using $q$ values. The SEM was calculated for the mean number of aCNA/cnLOH associated with each mutated gene.

Results

Patient characteristics

Characteristics of the 156 prospectively enrolled, previously untreated, non-M3 FAB subtype AML patients (referred to hereafter as the "MI-All-AML" group) in this study are summarized in Supplementary Tables S1 and S2. Of the 156 patients, 114 underwent SNP-A analysis and limited gene sequencing in a previously published study (29). Sixty-six percent, 22%, and 12% of cases were dnAML, sAML, or tAML, respectively, and 35% (54/156) of cases had normal cytogenetics. Within the dnAML group, 17% had a recurrent genomic abnormality, 19% had myelodysplasia-related changes without a history of an antecedent myeloid neoplasm, and 64% were diagnosed as AML-nototherwise-specified. Forty-two percent (43/103) of dnAML cases had normal cytogenetics. Regarding therapy, 93%, 66%, and 78% of dnAML, sAML, and tAML cases, respectively, received high-intensity induction chemotherapy, and 30% (47/156) underwent allogeneic stem cell transplantation while in complete remission, 45% (21/47) of whom were alive >3 years after diagnosis. Seventy-six percent (119/156) of patients had died at the time of data analysis, and median follow-up for surviving patients was 57.4 months.

Clinical characteristics of the AML TCGA cohort are published elsewhere (44). Of note, the TCGA dataset comprises only dnAML cases and is compared in this study with the dnAML subgroup of Michigan cases (hereafter referred to as the "TCGA-dnAML" and "MI-dnAML" groups, respectively). The clinical features of the TCGA-dnAML and MI-dnAML groups are well matched with the exception of the fractions receiving high-intensity treatment (79% vs. 93%, respectively; $P < 0.01$). The comparison of these two groups is summarized in Supplementary Table S1.

Mutational spectrum of thirteen genes across clinical AML subsets

Mutation frequency of 13 recurrently mutated genes is summarized in Fig. 1A. Eighty percent (125/156) of cases had a mutation in at least one of these genes. IDH2 (19% vs. 3%), NPM1 (24% vs. 3%), and FLT3 (21% vs. 11%) mutations were enriched in dnAML compared with sAML ($P \leq 0.05$ for all), while IDH1 (17% vs. 8%), ASXL1 (26% vs. 13%), and TP53 (26% vs. 8%) mutations were overrepresented in sAML compared with dnAML ($P \leq 0.05$ for all). For tAML, 50% (9/18) of cases harbored mutant TP53 (Fig. 1B). ASXL1 occurred more frequently in patients ≥60 years old ($P = 0.05$) due to the increased proportion of sAML in that population; however, comparing age >60 to age <60 in the dnAML group alone showed enrichment of RUNX1 and TET2 in older patients (both 22% vs. 7%, respectively; $P < 0.05$), while the remaining eleven genes showed no significant difference in mutation frequency. Co-occurrence analysis of mutations in all AML cases demonstrated mutual exclusivity of TP53 and RUNX1, NPM1 and RUNX1, and IDH1 and IDH2 (Fig. 1A). A detailed catalog of gene mutations for each case is included in Supplementary Table S4. Mutation frequency in these subgroups was consistent with published data with the exception of ASXL1 in dnAML (13% vs. 3%) (36, 44) and TP53 in tAML (50% vs. 18%–21%; refs. 48, 49).

Topography of aCNA and cnLOH

A total of 455 copy number losses, 106 copy number gains, and 38 instances of cnLOH were identified, with 20% <1 Mb and 47% ≤5 Mb (Supplementary Table S3). aCNA/cnLOH were common in the MI-All-AML group with 62% (97/156) of cases harboring ≥1 lesion and 38% (59/156) of cases with ≥2 lesions (median 2, range 1–39); similar findings were noted in the dnAML subgroup with ≥1 lesion seen in 56% (58/103) of cases and ≥2 lesions in 29% (30/103) of cases (median 2, range 1–25). In addition, aCNA/cnLOH were detected in 31% (17/55) of cases with a normal karyotype. In cases with abnormal karyotype, additional SNP-A lesions not detected by karyotype were found in 51% of cases (53/102). The average number of copy number losses (1.6 vs. 4.4 vs. 7.7) as well as total aCNA/cnLOH (2.2 vs. 6.4 vs. 8.6)
increased substantially from dnAML to sAML and tAML ($P < 0.05$), while the average number of gains (0.3 vs. 1.8) increased of AML. Genomic boundaries of MADRs are listed in Supplementary Table S4.

**Figure 1.**
Landscape of gene mutations and aCNA/cnLOH in AML. A, mutational spectrum for all AML cases. Demonstration of mutual exclusivity between *TP53* and *RUNX1*: blue boxes indicate a mutation, gray boxes indicate wild-type. B, gene mutation frequency in clinical subtypes (dnAML, sAML, and tAML) of AML. C, average number of losses, gains, or cnLOH in clinical subtypes of AML. D, frequency of minimally amplified or deleted (MADR) regions in clinical subtypes of AML. Genomic boundaries of MADRs are listed in Supplementary Table S4.

**Associations of gene mutations with aCNA/cnLOH**
Mutation status and aCNA/cnLOH were analyzed for each case. Notably, co-occurrence of a gene mutation with an aCNA/cnLOH that produced a hemizygous or homozygous mutant state at that gene’s locus was uncommon. Occasional cases with cnLOH that produced a hemizygous or homozygous mutant state at that gene’s locus was uncommon. Occasional cases with co-occurring mutations and aCNA/cnLOH. The exception was the presence of 17p deletions and cnLOH involving the *TP53* locus [27% (7/26) of *TP53*-mutant cases with 17p deletions and 35% (9/26) of *TP53*-mutant cases with 17p- cnLOH].

Examining the association of gene mutations with each MADR revealed significant association between *KRAS* and trisomy 8 ($q = 0.04$), *BCORL1* and trisomy 21 ($q = 0.01$), and *TP53* with all recurrent aCNA/cnLOH except for 6p-, 11q-, 13q+, 21+, and 21q-.

**Associations of gene mutations with SNP-A–based genomic complexity**
Comparison of SNP-A–based genomic lesion loads in the MI-All-AML, MI-dnAML, or a combined dnAML (MI-dnAML and TCGA-dnAML) group further subdivided by individual gene mutations and organized by fraction of cases with 0, 1, 2, 3, 4, 5–10, and >10 aCNAs showed multiple interesting findings: (i) distinctly high genomic complexity in *TP53*-mutated cases (mean 15.5, SEM 2.1); (ii) low or no complexity in cases carrying *IDH1*, *FLT3*, *NPM1*, or *CEBPA* mutations; (iii) >50% of cases with mutations in *BCORL1*, *KRAS*, *RUNX1*, *NRAS*, *TET2*, *DNMT3A*, *ASXL1*, or *IDH2* harbored ≥1 aCNA/cnLOH (Fig. 2A), and, (iv) AML cases without mutations in the 13 genes analyzed here were quite complex. In the MI-All-AML group, *TP53* mutations were strongly associated with the presence of aCNA/cnLOH and *NPM1* mutations with their absence ($q < 0.01$ for both), while in the pooled dnAML group *TP53* and *RUNX1* mutations were strongly associated with the presence of aCNA/cnLOH and *NPM1* and *FLT3* mutations with their absence ($q < 0.01$ for all).

A parallel analysis was performed on *TP53* wild-type cases; in this group, a subset of cases within each mutation-carrying subgroup still carried high lesion loads (Fig. 2B) and substantial differences existed in the fraction of cases that carried no lesions. Specifically, in the pooled dnAML group, *RUNX1* mutations remained strongly associated with the presence of aCNA/cnLOH and *NPM1* and *FLT3* mutations remained strongly associated with their absence ($q < 0.01$ for all). In both the *TP53* any and
TP53 wild-type analyses, the majority of cases with BCORL1 and KRAS mutations also contained aCNA/cnLOH and almost all cases of CEBPA lacked aCNA/cnLOH; however, the lower number of cases with these mutations precluded statistically significant associations.

Association of gene mutations, aCNA/cnLOH and karyotype with refractory AML

Within the total cohort (MI-All-AML), 132 patients received full-intensity induction chemotherapy; of these, 13 patients died during cycle one of induction chemotherapy and one declined further treatment and these patients were excluded from subsequent analysis. Of the remaining 118 patients, 65% (76/118) achieved a morphologic complete remission (CR) after one cycle of therapy (referred to hereafter as the ‘sensitive’ group), 15% (18/118) achieved a CR after two cycles of therapy (the ‘intermediate’ group), and 20% (24/118) failed to achieve a CR after at least two cycles of therapy (the ‘refractory’ group).

Univariate analysis of the 118 patients examining the effect of response to induction therapy on median OS demonstrated a significant and profound difference in outcome between the sensitive, intermediate, and refractory groups (median OS of 25.8, 12.5, and 4.2 months, respectively; \( P < 0.03 \) for all; Fig. 3A).

This profound survival difference prompted evaluation of genomic features associated with these distinct responses to therapy. All cases with CEBPA biallelic mutations, t(8;21), inv(16), and any 11q23 abnormality (\( N = 3, 5, 6, \) and 4, respectively) were sensitive to treatment. NPM1 mutations and the absence of any aCNA/cnLOH were highly associated with the sensitive group (\( q < 0.001 \) for both). RUNX1 mutations, on the other hand, were the only genomic feature highly associated with the intermediate group (\( q < 0.001 \)). TP53 mutations (\( q < 0.001 \)); SNP-A detected deletions of 3p, 5q, 7q, 12q, 16q, 17p, and 17q (\( q < 0.05 \) for all); the presence of \( \geq 2 \) or \( \geq 3 \) aCNA/cnLOH (\( q = 0.01 \) and \( q < 0.001 \), respectively); and monosomal (MK) or complex karyotype (CK, \( q < 0.001 \) for both) were all associated with the refractory group. The frequency of all measured genomic features in each response group is displayed in Fig. 3B.

Given the dominant correlation of TP53 mutations with refractory disease, MK, CK, and aCNA/cnLOH lesion load, the TP53 wild-type fraction (\( N = 105 \)) was evaluated in a similar fashion. NPM1 mutations and the absence of aCNA/cnLOH remained highly associated with the sensitive group (\( q < 0.02 \)) and RUNX1 mutations with the intermediate group (\( q < 0.001 \)). SNP-A-based deletions of 5q, 7q, 12p, and 16q still correlated with a decreased response to therapy but were associated with the intermediate group (\( q < 0.05 \) for all) rather than the refractory group. ASXL1 mutations emerged as significantly associated with the refractory group (\( q = 0.05 \)) as well as cases with \( \geq 2 \) aCNA/cnLOH (\( q = 0.02 \)). The frequency of all measured genomic features in the TP53 wild-type group is shown in Fig. 3C. Additional representations of genomic lesion frequency within specific types of therapy response are shown in Supplementary Fig. S1.

Age, cytogenetics, and gene mutations and survival in the AML cohorts studied

In the MI-All-AML group, median overall survival (OS) for age \( \geq 60 \) (6.5 months) versus age <60 (24.0 months) and for favorable (not reached) versus intermediate (14.0 months) versus unfavorable (4.3 months) cytogenetics were significantly different (\( P < 0.01 \) for all). Likewise, in the MI-dnAML-only subgroup, the median OS for age \( \geq 60 \) (8.4 months) versus age <60 (26.3
Figure 3.
Genomic lesion associations with response to induction chemotherapy. Univariate analysis of median overall survival in the MI-All-AML (N = 156) and MI-dnAML (N = 103) subgroups based on type of response to therapy (A). Frequency of gene mutations and structural genomic aberrations, minimally amplified or deleted regions, and aCNA/cnLOH load stratified by response to treatment in (B) all cases or in (C) TP53 wild-type cases only.
DNMT3A and TP53 in survival was again seen in (11.5 months) versus (6.8 months) versus decrease in median survival was seen with (difference in median survival. Kaplan were significantly different (P < 0.04 for all).

In the MI-All-AML cohort, a statistically significant increase in median survival was observed for NPM1 mutated (26.9 months) versus NPM1 wild-type (9.0 months) cases (P = 0.01), and a significant and profound decrease in median survival was seen with TP53-mutated (2.9 months) versus TP53 wild-type (13.4 months) cases (P < 0.001). The remaining eleven genes showed no significant difference in median survival.

In the MI-dnAML subgroup alone (N = 103), a significant decrease in median survival was seen with TET2-mutated (6.8 months) versus TET2 wild-type (17.8 months) cases (P = 0.03) and TP53-mutated (2.9 months) versus TP53 wild-type (18.0 months) cases (P < 0.01). A trend toward decreased median survival was noted with RUNX1-mutated (11.5 months) versus RUNX1 wild-type (18.1 months) cases (P = 0.06). The remaining ten genes showed no significant difference in median survival.

In the TCGA-dnAML group (N = 170), a significant decrease in survival was again seen in TP53-mutated (7.0 months) versus TP53 wild-type (14.6 months) cases (P = 0.01), RUNX1-mutated (12.5 months) and RUNX1 wild-type (14.6 months) cases (P = 0.04), and DNMT3A-mutated (9.9 months) versus DNMT3A wild-type (15.5 months) cases (P = 0.05). The remaining ten genes showed no significant difference in median survival. Kaplan–Meier curves of univariate analyses based on gene mutation status for the MI-All-AML, MI-dnAML, and TCGA-dnAML cohorts are shown in Supplementary Fig. S2.

Kaplan–Meier analysis of SNP 6.0 array-based genomic complexity and effects on survival in AML

Univariate analysis of the MI-All-AML group demonstrated a significant decrease in median survival for ≥1 lesions (7.9 months) versus 0 lesions (23.9 months; P < 0.001), ≥2 lesions (6.6 months) versus <2 lesions (15.9 months; P < 0.001), ≥3 lesions (4.2 months) versus <3 lesions (13.6 months; P < 0.001), and ≥4 lesions (4.0 months) versus <4 lesions (13.5 months; P < 0.001; Fig. 4A).

Corresponding results for the MI-dnAML group are: SNP-A–derived aCNA/cnLOH lesions ≥1 lesion (11.0 months) versus 0 lesions (28.4 months; P = 0.01), ≥2 lesions (8.5 months) versus <2 lesions (22.4 months; P = 0.03), ≥3 lesions (3.8 months) versus <3 lesions (20.5 months; P < 0.001), and ≥4 lesions (2.9 months) versus <4 lesions (20.0 months; P < 0.001; Fig. 4A). This effect was also seen in the TCGA-dnAML group for ≥3 lesions (9.9 months) versus <3 lesions (16.4 months; P = 0.01) and ≥4 lesions (8.9 months) versus <4 lesions (16.4 months; P = 0.02; Fig. 4A).

In MI-dnAML cases with wild-type TP53 (N = 95), aCNA/cnLOH burden remained associated with a decrease in median survival, with a trend toward significance for ≥3 lesions (6.6 months) versus <3 lesions (20.5 months; P = 0.06) and a significant difference for ≥4 lesions (4.0 months) versus <4 lesions (20.6 months; P = 0.04; Fig. 4B).

A combined analysis of gene mutations and SNP-A–detected aCNA/cnLOH on survival in AML

As mutational profiling is increasingly employed for clinical risk stratification, we assessed the prognostic value of knowledge of aCNA/cnLOH counts in AML patients stratified by individual
Figure 5. Bivariate analyses of gene mutations and aCNA/cnLOH. Bivariate analyses of the effect of gene mutation status combined with the presence or absence of aCNA/cnLOH on median overall survival in the pooled Mi-dnAML and TCGA-dnAML groups (N = 273).
gene mutations. To increase power of this analysis, the MI-dnAML and the TCGA-dnAML cohorts were combined.

The presence of $\geq 1$ aCNA/cnLOH in patients with mutations in TET2, IDH1, NPM1, or DNMT3A significantly worsened survival as compared with lack of aCNA/cnLOH, with similar trends detected in patients carrying RUNX1 or FLT3 mutations (Fig. 5). For example, median survival was 6.6 months versus 29.4 months in patients with TET2 mutations and presence or absence of aCNA/cnLOH, respectively ($P < 0.01$), and 12.9 months versus 56.3 months for patients with IDH1 mutations and presence or absence of aCNA/cnLOH, respectively ($P = 0.01$).

Multivariate survival analyses of age, cytogenetics, SNP 6.0 array-based genomic complexity, and gene mutations in AML

Multivariate analysis was performed using a Cox proportional hazards model of single variables in combination with age and cytogenetic risk group. For the MI-All-AML group, increased genomic complexity defined as $\geq 4$ SNP-A lesions demonstrated a strong, independent negative impact on overall survival [HR = 1.98; 95% confidence interval (CI), 1.20–3.28; $P < 0.01$] with $\geq 3$ SNP-A lesions trending toward an independent negative effect as well [HR = 1.57; 95% CI, 0.95–2.57; $P = 0.07$]. In separate models, TP53 mutations showed a strong, independent negative prognostic effect [HR = 2.65; 95% CI, 1.47–4.81; $P = 0.001$; Table 1]. No other gene mutations showed an independent effect on overall survival in this model. Similarly, in multivariate analysis of the MI-dnAML subgroup, the presence of $\geq 3$ SNP-A lesions had an independent negative effect on overall survival (HR = 1.92; 95% CI, 1.01–3.68; $P = 0.04$), as did the presence of $\geq 4$ lesions (HR = 2.50; 95% CI, 1.29–4.84; $P < 0.01$). In separate models, TP53 mutations likewise showed a strong, independent negative impact on prognosis (HR 4.08; 95% CI, 1.02–16.38).

### Table 1. Results of multivariate survival analyses

<table>
<thead>
<tr>
<th>Variables</th>
<th>MI-All-AML group ($N = 156$)</th>
<th>$P$</th>
<th>MI-dnAML group ($N = 103$)</th>
<th>$P$</th>
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<td>Age $\geq 60$ vs. $&lt;60$</td>
<td>2.74 (1.85–4.05)</td>
<td>&lt;0.001</td>
<td>2.16 (1.32–3.55)</td>
<td>&lt;0.01</td>
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<td>aCNA/cnLOH $\geq 3$ vs. $&lt;3$</td>
<td>2.50 (1.52–3.94)</td>
<td>&lt;0.001</td>
<td>2.61 (1.45–4.68)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TET2 mutated vs. wild-type</td>
<td>1.10 (0.64–1.89)</td>
<td>0.73</td>
<td>1.85 (0.97–3.51)</td>
<td>0.06</td>
</tr>
<tr>
<td>Age $\geq 60$ vs. $&lt;60$</td>
<td>2.62 (1.76–3.89)</td>
<td>&lt;0.001</td>
<td>2.29 (1.40–3.74)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>aCNA/cnLOH $\geq 3$ vs. $&lt;3$</td>
<td>2.42 (1.59–3.68)</td>
<td>&lt;0.001</td>
<td>2.45 (1.37–4.38)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ASXL1 mutated vs. wild-type</td>
<td>1.45 (0.87–2.36)</td>
<td>0.15</td>
<td>1.22 (0.62–2.39)</td>
<td>0.56</td>
</tr>
<tr>
<td>Age $\geq 60$ vs. $&lt;60$</td>
<td>2.73 (1.85–4.04)</td>
<td>&lt;0.001</td>
<td>2.35 (1.45–3.82)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aCNA/cnLOH $\geq 3$ vs. $&lt;3$</td>
<td>2.29 (1.52–3.43)</td>
<td>&lt;0.001</td>
<td>2.37 (1.34–4.39)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>DNMT3A mutated vs. wild-type</td>
<td>1.00 (0.65–1.55)</td>
<td>0.99</td>
<td>1.11 (0.64–1.95)</td>
<td>0.69</td>
</tr>
<tr>
<td>Age $\geq 60$ vs. $&lt;60$</td>
<td>2.72 (1.84–4.03)</td>
<td>&lt;0.001</td>
<td>2.35 (1.44–3.81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aCNA/cnLOH $\geq 3$ vs. $&lt;3$</td>
<td>2.27 (1.50–3.45)</td>
<td>&lt;0.001</td>
<td>2.40 (1.34–4.32)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FLT3-ITD mutated vs. wild-type</td>
<td>0.92 (0.52–1.64)</td>
<td>0.77</td>
<td>1.07 (0.55–2.11)</td>
<td>0.83</td>
</tr>
<tr>
<td>Age $\geq 60$ vs. $&lt;60$</td>
<td>2.72 (1.84–4.02)</td>
<td>&lt;0.001</td>
<td>2.34 (1.44–3.81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aCNA/cnLOH $\geq 3$ vs. $&lt;3$</td>
<td>2.08 (1.37–3.17)</td>
<td>&lt;0.001</td>
<td>2.23 (1.24–4.02)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>NPM1 mutated vs. wild-type</td>
<td>0.62 (0.36–1.09)</td>
<td>0.09</td>
<td>0.79 (0.43–1.43)</td>
<td>0.42</td>
</tr>
<tr>
<td>Age $\geq 60$ vs. $&lt;60$</td>
<td>2.82 (1.90–4.19)</td>
<td>&lt;0.001</td>
<td>2.46 (1.52–4.00)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aCNA/cnLOH $\geq 3$ vs. $&lt;3$</td>
<td>1.23 (0.69–2.34)</td>
<td>0.52</td>
<td>1.60 (0.77–3.31)</td>
<td>0.19</td>
</tr>
<tr>
<td>TP53 mutated vs. wild-type</td>
<td>3.23 (1.54–6.77)</td>
<td>&lt;0.001</td>
<td>3.65 (1.31–10.2)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

**NOTE:** Multivariate analysis of overall survival in the MI-All-AML cohort (middle column) and MI-dnAML cohort (right column). Models using age- and aCNA/cnLOH-based risk factors along with gene mutation status are shown in the top section; models using age- and cytogenetics-based risk factors along with number of aCNA/cnLOH or gene mutation status are shown in the bottom section.
HR of 1.60 (95% CI, 0.77–3.63; \( P = 0.04 \), respectively). No other gene mutations showed an independent effect on overall survival in multivariate analysis.

Models of MI-dnAML that included age, TP53 mutations, and the presence of \( \geq 3 \) or \( \geq 4 \) SNP-A lesions showed elevated HR of 1.60 (95% CI, 0.77–3.31) and 1.80 (95% CI, 0.74–4.37) for \( \geq 3 \) and \( \geq 4 \) SNP-A lesions, respectively, that were NS (\( P = 0.20 \) and 0.19); however, conclusions are limited by the small number of cases (\( N = 11 \)) that both had an aCNA/cnLOH count \( \geq 3 \) and were TP53 wild-type.

Multivariate analyses were also performed on the TCGA-dnAML group. In models that included age and cytogenetics, DNMT3A and FLT3-ITD mutations showed an independent adverse prognostic effect (HR 1.67; 95% CI, 1.12–2.48; \( P = 0.01 \)) and HR 1.60; 95% CI, 1.00–2.55; \( P = 0.04 \), respectively) with a trend toward an independent adverse effect of TP53 mutations (HR 1.81; 95% CI, 0.92–3.56; \( P = 0.08 \); Supplementary Table S5). An independent adverse effect of aCNA/cnLOH count \( \geq 3 \) in TP53 wild-type or mutant cases was not detected. Analysis of the clinical features of the TP53-mutant and wild-type subgroup within the TCGA and MI-AML groups shows that while the TP53-mutant groups are well matched (median age 67 vs. 68 and BMT as consolidation in 27% vs. 25%, respectively), the TCGA TP53 wild-type subgroup with aCNA/cnLOH count \( \geq 3 \) harbored substantial differences compared with MI-AML (median age 60 vs. 69 and BMT as consolidation in 67% vs. 18% \( P = 0.03 \)); which may explain the lack of observed effect of elevated genomic complexity on outcomes in the TCGA TP53 wild-type subgroup.

Discussion

Recent publications in AML have underscored the importance of simultaneous analysis of competing genomic variables when determining an individual mutated gene’s association with survival (35–37, 50). While these studies have focused on the relative influence of a given mutant gene compared with a pool of other mutant genes, our study integrates the majority of known recurrently mutated genes with SNP-A–based genomic complexity. We characterized the DNA of leukemic blasts from 156 consecutive prospectively enrolled AML patients using SNP 6.0 arrays and Sanger-based mutational analysis of TP53, RUNX1, DNMT3A, NPM1, FLT3, ASXL1, TET2, IDH1, IDH2, Nras, KRAS, BcorL1, and CEBPA to evaluate the relationship between chromosomal translocations, aCNA/cnLOH, and gene mutations and their association with response to induction chemotherapy and overall survival. The cohort studied comprised consecutively evaluated patients without a priori selection for clinically defined subgroups, such as dnanAML, sAML, and tAML, or normal karyotype versus aberrant karyotype AML. In addition, we analyzed 170 dnanAML patients published by TCGA to expand our findings.

Together, the genomic and clinical findings allow for the following principle conclusions: (i) RUNX1 mutations, TP53 mutations, elevated SNP 6.0 array-based genomic complexity, SNP 6.0 array-defined deletions/cnLOH of 3p, 5q, 7p, 7q, 12q, 16q, 17p, and 17q, and complex and monosomal karyotypes are strong predictors of partial or complete resistance to intensive induction therapy, resulting in dismal overall survival, (ii) in all AML subcohorts after exclusion of TP53-mutant cases, NPM1 mutations predicted for chemotherapy sensitivity (in addition, all AML cases carrying mutations of CEBPA, KRAS, and t(8;21), inv (16), and 11q23 abnormalities achieved a CR), while mutations in RUNX1 and ASXL1, elevated SNP 6.0 array-based genomic complexity and SNP 6.0 array-defined deletions/cnLOH of 3q, 7q, 12p, and 16q were often associated with resistance to induction chemotherapy, and (iii) presence of any aCNA/cnLOH in patients carrying mutations in TET2, IDH1, NPM1, DNMT3A significantly worsened prognosis when compared with patients carrying such mutations but no aCNA/cnLOH, with similar trends observed in patients carrying either RUNX1 or FLT3 mutations.

Additional novel conclusions center on (i) the finding that genomic losses or gains infrequently affect the loci of genes known to be recurrently mutated in AML, providing clear evidence that these lesion types serve largely independent biologic functions in AML, (ii) that genomic complexity markedly associates with TP53 mutation status, but that mutations in RUNX1 also associated with the presence of \( \geq 1 \) aCNA/cnLOH in TP53 wild-type dnAML and that previous reports that mutations in TP53 and RUNX1 were mutually exclusive in dnanAML (44) can be extended to all clinical subtypes, and (iii) that NPM1, CEBPA, and FLT3-ITD mutations are significantly associated with stable genomes (absence of SNP-A lesions) and that marked differences exists in the aCNA/cnLOH load of AML subgroups stratified by specific gene mutations.

While this is the first study to integrate the majority of recurrently mutated genes with SNP-A data in a survival analysis, there are limitations to this analysis. First, our methodology did not evaluate all known recurrent mutations, and the use of Sanger sequencing, while capable of identifying clonally represented mutations, likely did not detect subclonal mutations that are of unclear clinical significance. In addition, no uniform therapy was used in this cohort, and while most patients were treated with intensive induction regimens, differences in specific induction chemotherapies may have influenced outcomes. Furthermore, the smaller size of the cohort studied precluded the ability to comprehensively analyze all competing variables in multivariate analysis. The TCGA cohort provided additional patients for study, but the use of unpurified tumor DNA, a different sequencing methodology and lower rates of use of intensive therapies limit to some extent a direct comparison of genomic findings and may account for some of the differences observed.

Given that genomic complexity resulting from genomic instability is as measured by SNP-A–based aCNA/cnLOH counts is strongly associated with poor survival and chemoresistance in AML, and given that the effect of the presence of such genomic instability supersedes that of mutations in all commonly mutated genes in AML evaluated with the exception of TP53 and possibly TET2, multiple relevant questions arise. Is genomic complexity (and the underlying pathogenic mechanisms that lead to it) one of the defining characteristics upon which clinical and biologic AML subgroups should be defined? Are recurrent AML-associated gene mutations other than TP53 more associated with AML initiation or clonal outgrowth rather than with chemotherapy resistance? If so, are the observed relationships of these mutations with AML prognosis in part indirect and due to co-occurrence or conversely the lack of association with either TP53 mutations or genomic instability?

In summary, our data demonstrate that RUNX1 mutations, ASXL1 mutations, SNP 6.0 array-based deletions/cnLOH of 3q, 7q, 12p, and 16q and elevated SNP 6.0 array-based genomic complexity significantly predict partial or complete resistance to induction chemotherapy even in the absence of a co-occurring TP53 mutation. Furthermore, highly adverse prognostic information in AML can be derived from determinations of patient’s age,
karyotype, TP53 mutation status, and/or SNP 6.0 array-based genomic complexity, which should be measured routinely for all patients with AML at diagnosis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.N. Malek
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Parkin, P. Ouillette, M. Yildiz, K. Saiya-Cork, S.N. Malek
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Parkin, K. Shedden, S.N. Malek
Writing, review, and/or revision of the manuscript: B. Parkin, S.N. Malek
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Parkin, S.N. Malek
Study supervision: S.N. Malek

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Integrated Genomic Profiling, Therapy Response, and Survival in Adult Acute Myelogenous Leukemia

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