

# Preliminary evidence for the integration of CNAs, CN-LOH and mutational profiles into the prognostic stratification of elderly patients with *NPM1*-mutated acute myeloid leukemia

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**Abstract.** Acute myeloid leukemia (AML) exhibits substantial genetic heterogeneity in elderly patients. Although validated genomic alterations have improved precision prognostication, incomplete integration of genetic profiles, particularly the underutilization of copy number alterations (CNAs) and copy-neutral loss of heterozygosity (CN-LOH), contributes to prognostic uncertainty in elderly patients with nucleophosmin 1 (*NPM1*)-mutated AML. To address this gap, the present exploratory single-center study evaluated 61 elderly patients with *NPM1*-mutated AML. Shallow whole-genome sequencing was used to detect CNAs and CN-LOH, and targeted next-generation sequencing was performed to assess mutations in myeloid-associated genes. Genomic data were integrated with clinical and laboratory parameters to evaluate their prognostic significance. *NPM1* subtype A was the predominant variant, and most patients harbored 4 or 5 concurrent somatic mutations, frequently involving fms-related receptor tyrosine kinase 3 (*FLT3*), DNA methyltransferase 3 (*DNMT3A*), tet methylcytosine dioxygenase 2 (*TET2*), and isocitrate dehydrogenase 2 (*IDH2*). Overall, 65.57% of patients harbored CNAs and/or CN-LOH events,

with recurrent genomic aberrations including dup(4)(q11q35) in 4 cases. No significant differences in clinical parameters, complete remission rates or overall survival (OS) were observed between CNA/CN-LOH-positive and -negative groups. However, univariate survival analysis demonstrated that the OS of patients with  $\geq 5$  total mutations or  $\geq 3$  CNA/CN-LOH events was significantly shorter compared with that of other patients. Integrative analysis revealed a trend toward shorter OS in patients with *FLT3* internal tandem duplication-positive AML and  $\geq 2$  CNA/CN-LOH events, and longer OS in patients with *IDH2*-mutated AML without CNA/CN-LOH. Multivariate Cox regression analysis tentatively identified  $\geq 3$  CNA/CN-LOH events and  $\geq 5$  total mutations as potential independent predictors of a poor prognosis. These findings provide preliminary evidence that integrating CNA/CN-LOH burden with mutational profiles may improve risk stratification in elderly patients with *NPM1*-mutated AML. However, given the small sample size, single-center design and lack of external validation, larger multi-center studies are warranted to assess the robustness of these findings.

## Introduction

Rapid advancements in cytogenetics and molecular biology, together with their clinical translation, have advanced the mechanistic understanding of acute myeloid leukemia (AML), including its pathogenesis, risk stratification and prognosis. These advancements have provided critical evidence supporting risk assessment, minimal residual disease (MRD) monitoring and the development of precision therapeutic strategies (1). Notably, key genomic alterations such as chromosomal translocations, copy number alterations (CNAs), mutations in pan-tumor genes such as *TP53*, *NRAS* and *BRAF*, and lineage-specific driver mutations such as nucleophosmin 1 (*NPM1*) and fms-related receptor tyrosine kinase 3 (*FLT3*), are highly prevalent in hematopoietic malignancies, and have increasingly assumed a central role in laboratory diagnostics (2,3). Furthermore, technological innovations integrating conventional chromosome banding, fluorescence *in situ* hybridization (FISH) and high-throughput sequencing have improved the accuracy of risk stratification, supported the establishment of prognostic models, and facilitated the implementation of personalized treatment protocols for AML (4,5). However, this progress has not been translated into clinical

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**Abbreviations:** AML, acute myeloid leukemia; BM, bone marrow; CNA, copy number alteration; CN-LOH, copy-neutral loss of heterozygosity; ELN, European Leukemia Net; FAB, French-American-British; FISH, fluorescence *in situ* hybridization; *FLT3*-ITD, *FLT3* internal tandem duplication; HR, hazard ratio; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; MRD, minimal residual disease; NGS, next-generation sequencing; OS, overall survival; sWGS, shallow whole-genome sequencing

**Key words:** acute myeloid leukemia, *NPM1*, copy number alterations, copy-neutral loss of heterozygosity, survival

benefits for certain elderly patients with AML, such as those harboring *NPM1* mutations, who continue to face issues with ambiguous prognostic assessment and suboptimal risk stratification due to the incomplete integration of genomic profiling.

*NPM1* represents one of the most prevalent driver mutations in AML and is ubiquitously expressed across diverse cell types (6). Accumulating evidence indicates that approximately one-third of adult patients with AML harbor *NPM1* variants distributed across all French-American-British (FAB) subtypes (7). Although several studies have suggested that the prognosis of patients with *NPM1*-mutated AML, particularly isolated *NPM1*-mutated AML, is generally more favorable compared with that of patients with *TP53*-mutated AML, the current diagnostic and therapeutic framework continues to face substantial challenges in elderly patients, defined as patients  $\geq 60$  years old herein. Elderly patients often present with multiple comorbidities and age-related organ dysfunction, resulting in markedly lower treatment tolerance compared with that of their younger counterparts. More importantly, this population exhibits substantial prognostic heterogeneity: Even within the *NPM1*-mutated subtype, overall survival (OS) may range from several months to several years (8). A key contributor to this heterogeneity is suboptimal risk stratification, such as 'pseudo-isolated' *NPM1* mutations accompanied by undetected CNAs or copy-neutral loss of heterozygosity (CN-LOH). The latter is a loss of heterozygosity without changes in copy number, typically involving duplication of the retained allele. Such risk misclassification leads to biased risk stratification, ultimately resulting in the overtreatment of low-risk patients and the undertreatment of high-risk individuals (9). The core underlying issue is that current risk stratification systems inadequately account for genomic integrity in elderly patients: They focus primarily on single-gene mutations, such as *FLT3*-internal tandem duplication (ITD), or basic cytogenetic abnormalities, while failing to fully integrate key genomic alterations such as CNAs and CN-LOH.

Clinically actionable CNAs have emerged as pivotal molecular markers for prognosis and therapeutic decision-making in AML, and the characterization of their structural variants, including deletions, duplications and amplifications, has become central to basic and translational research (10). However, conventional cytogenetic techniques, including G-banding and FISH, are limited by low resolution and incomplete genome-wide coverage, leading to missed detection of relevant genetic information in a subset of AML patients and hindering accurate diagnosis and risk stratification (4,11). As a complementary tool, shallow whole-genome sequencing (sWGS) enables the comprehensive, high-sensitivity capture of genome-wide CNAs and CN-LOH, with higher sensitivity for large genomic segments compared with conventional cytogenetic testing such as G-banding. The sWGS approach also holds significant promise for the identification of critical biomarkers to support risk-adapted stratification and the refinement of prognostic models in AML (12).

Against this background, the present study aims to address the challenges of prognostic heterogeneity, diagnostic misclassification and suboptimal treatment decision-making in elderly patients with *NPM1*-mutated AML by systematically analyzing CNAs and CN-LOH in bone marrow specimens from 61 such patients using sWGS. By integrating these genomic data with

hematological parameters, myeloid gene mutation profiles, such as those of *FLT3*, isocitrate dehydrogenase 2 (*IDH2*) and *NRAS/KRAS*, and relevant clinical variables, including age and FAB subtype, the study seeks to identify potential integrative genetic and molecular biomarkers. Ultimately, the present study aims to provide preliminary evidence to optimize risk stratification and prognostic assessment in this vulnerable patient population, thereby contributing to improved stratification and treatment decision-making in elderly patients with *NPM1*-mutated AML.

## Materials and methods

**Patient characteristics.** The present single-center retrospective study was approved by the Clinical Ethics Committee of China-Japan Friendship Hospital (Beijing, China; approval no. 2023-KY-200). A total of 61 adult patients (27 men and 34 women) with *NPM1*-mutated AML were included. All patients in this study were diagnosed at the China-Japan Friendship Hospital between January 2012 and December 2023, based on the criteria outlined in the World Health Organization Classification of Tumours: Haematolymphoid Tumours (5th edition, 2022) (13). The median age of the cohort was 73 years (range, 60–88 years), with a median follow-up duration of 14 months from diagnosis to the last follow-up or death. Patients with incomplete medical records or concurrent active malignancies were excluded. Baseline demographic and clinical characteristics of the study cohort are summarized in Table I. The definitions of key characteristics are provided as follows: FAB subtype was determined by morphological review of Wright-Giemsa-stained bone marrow aspirate smears at diagnosis and was classified by two independent hematopathologists according to the FAB cooperative group criteria (7). Previous hematological disorder was defined as a documented history of myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), MDS/MPN overlap syndrome or therapy-related myeloid neoplasm prior to the current AML diagnosis. Complete remission (CR) and relapse were defined according to the 2022 ELN recommendations (14). CR required bone marrow blasts  $< 5\%$ , absence of circulating blasts and extramedullary disease, absolute neutrophil count  $> 1.0 \times 10^9/l$ , platelet count  $> 100 \times 10^9/l$ , and independence from red cell and platelet transfusions. Relapse was defined as the reappearance of  $> 5\%$  leukemic blasts in the bone marrow (not attributable to regeneration) or the development of extramedullary disease after achieving CR. The 1-year relapse rate was calculated as the proportion of patients who experienced relapse within 12 months from the date of CR attainment.

**DNA extraction, targeted next-generation sequencing (NGS) and sWGS.** Genomic DNA was extracted from bone marrow samples remaining from routine hematology laboratory tests performed at the initial diagnostic consultation using the Genomic DNA Extraction Kit (cat. no. 8.02.0014; Amoy Diagnostics Co., Ltd.) in strict accordance with the manufacturer's recommended protocol.

For targeted NGS, a custom-designed gene panel comprising 36 genes, including those recommended by the European Leukemia Net (ELN) and other frequently mutated genes in myeloid malignancies, was used (14). A list of all 36 target

Table I. Comparison of clinical manifestations and laboratory features in elderly patients with *NPM1*-mutated acute myeloid leukemia stratified by CNA/CN-LOH status.

Characteristic	Overall (n=61)	<3 CNA/CN-LOH (n=50)	≥3 CNA/CN-LOH (n=11)	P-value
Age, years	72.97 (60-88)	72.12 (60-88)	76.82 (65-87)	0.047
Sex, male/female	27/34	25/25	2/9	0.078
White cell counts at presentation, x10 <sup>9</sup> /l	61.23 (1.23-417.23)	58.74 (1.23-417.23)	72.32 (1.27-206.45)	0.611
Hemoglobin at presentation, g/l	74.47 (35-111)	73.59 (35-111)	78.36 (52-104)	0.415
Platelets at presentation, x10 <sup>9</sup> /l	65.65 (8-349)	69.43 (8-349)	48.82 (11-101)	0.315
Bone marrow blasts, %	63.42 (20.00-97.00)	62.33 (20.00-96.50)	68.37 (20.00-97.00)	0.507
<i>NPM1</i> mutation type, n				
Type A	47	39	8	1.000
Type B	3	2	1	0.455
Type D	3	3	0	1.000
Others	8	6	2	0.955
FAB subtype, n				
M2	13	12	1	0.492
M4	5	2	3	0.037
M5	31	26	5	0.694
Others	12	10	2	1.000
Previous hematological disorder, n	15	12	3	0.601
Induction chemotherapy regimens, n				
Containing cytarabine	16	13	3	1.000
Containing demethylation agent	35	30	5	0.585
Containing venetoclax	16	12	4	0.642
Others	15	11	4	0.539
Complete remission, n	23	19	4	1.000
1-year relapse, n	16	13	3	1.000
2-year overall survival, n	11	11	0	0.188

All values are presented as median (range), except where otherwise indicated. *NPM1*, nucleophosmin 1; CNA, copy number alteration; CN-LOH, copy-neutral loss of heterozygosity; FAB, French-American-British.

genes is provided in Table SI. Library preparation was carried out using the KAPA HyperPlus Kit (KAPA Biosystems; Roche Diagnostics), and sequencing was performed on an Illumina MiSeq platform (Illumina, Inc.) with a minimum sequencing depth of 2,000 reads per base. Raw sequencing data were processed using the Genome Analysis Toolkit (version 4.4.0.0; Broad Institute) with the following steps: Base calling for raw signal-to-base conversion, read alignment to the reference genome, and variant calling of single-nucleotide polymorphisms and small insertions/deletions. All identified variants were categorized following the joint guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Only mutations classified as pathogenic or likely pathogenic with a variant allele frequency ≥2% were retained for further analysis (15). *NPM1* mutations were categorized into types A, B, D, or others based on the characteristic 4-bp insertions in exon 12, as previously defined (6).

For sWGS, ~500 ng high-quality genomic DNA (OD260/280 ratio, 1.8-2.0) was used for library construction with the KAPA HyperPrep Kit (KAPA Biosystems; Roche Diagnostics), using

dual-indexed adapters. The final libraries were quantified using a Qubit fluorometer (Thermo Fisher Scientific, Inc.) and pooled at an equimolar concentration of 2 nM for sequencing. Sequencing was conducted on an Illumina NovaSeq platform (Illumina, Inc.) in a 2x150 bp paired-end mode, achieving a mean genomic coverage depth of 1 read per base. The sWGS data were processed using established bioinformatic pipelines to identify CNAs and CN-LOH events (11,16). Following quality control and data preprocessing, the CNA and CN-LOH datasets were formatted to meet the input requirements of the R package GenVisR (version 1.34.0; <https://bioconductor.org/packages/GenVisR>). To visualize the genomic alterations, the plotKaryotype function of GenVisR was used to create karyotype plots of the CNA/CN-LOH profiles. The CNA/CN-LOH burden was categorized as negative (0 events), low (1 or 2 events) or high (≥3 events) based on the total number of distinct events per patient.

*Chromosome karyotype analysis and FISH detection.* Bone marrow cells were harvested following culture for 24-48 h in

RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin, at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, without stimulation to preserve the native cytogenetic status of the cells. For conventional karyotype analysis, G-banded metaphase spreads were prepared from cultured bone marrow aspirates following unstimulated culture, and cytogenetic evaluation was carried out using standard laboratory techniques, which involved G-banding by trypsin digestion followed by Giemsa staining (GTG-banding) to produce characteristic light and dark bands for chromosome identification and analysis. A total of 20 metaphase spreads per sample were analyzed to ensure sufficient representation of the cell population, and the karyotypes were documented in strict accordance with the guidelines outlined in the International System for Human Cytogenetic Nomenclature (2020) (17).

In addition to karyotype analysis, FISH testing was performed in a subset of 36 patients to detect specific chromosomal aberrations. This testing used a FISH probe panel (MetaSystems) including the following probes: XL 5q31/5q33/5p15 (cat. no. D-5081-100-TC), XL del(7)(q22q31) (cat. no. D-5068-100-TC), XCE 8 (cat. no. D-0808-050-FI), XL TP53 (cat. no. D-5103-100-OG), XL ETV6 (cat. no. D-5139-100-OG), XL RB1/DLEU/LAMP (cat. no. D-5070-100-TC), XL 20q12/20qter (cat. no. D-5121-100-OG), XL RUNX1 (cat. no. D-5096-100-OG) and XCE X/Y (cat. no. D-0825-200-OG).

**Statistical analysis.** Fisher's exact test was used to compare categorical variables. For continuous variables, normality was first assessed using the Shapiro-Wilk test. One-way analysis of variance was used for comparisons among different subgroups if variables were normally distributed, while the Kruskal-Wallis H test was used if normality was violated. OS was defined as the time from diagnosis to death (event) or last follow-up (censored). OS distributions were estimated by Kaplan-Meier analysis and differences between survival curves were assessed using the log-rank test. In addition, hazard ratios (HRs) with 95% confidence intervals (CI) were calculated to assess survival differences. Multivariate analyses were performed using binary logistic regression and Cox proportional hazards models for OS, with a limited backward elimination procedure used to exclude redundant variables. For analyses involving multiple comparisons, the Benjamini-Hochberg (BH) method was used to control the false discovery rate, with a corrected  $Q < 0.05$  considered to indicate statistical significance. All statistical analyses were performed using SPSS Statistics version 22.0 (IBM Corp.), and two-sided  $P < 0.05$  was considered to indicate a statistically significant result unless otherwise specified.

## Results

**Gene mutation landscape analysis.** A total of 61 bone marrow samples from elderly patients with AML were subjected to NGS using a 36-gene panel. Genomic profiling identified *NPM1* mutation subtypes A, B and D and other variants in 47 cases (77.05%), 3 cases (4.92%), 3 cases (4.92%) and 8 cases (13.11%), respectively (Fig. 1A). The majority of patients ( $n=32$ ; 52.46%) exhibited a total mutational burden of 4 or 5

concurrent somatic gene mutations, with the *NPM1* mutation consistently included in this mutational spectrum. Specifically, 14 cases (22.95% of the cohort) presented with a total of 4 mutations and 18 cases (29.51%) had a total of 5 mutations (Fig. 1B). The most prevalent co-mutations were detected in *FLT3* (45.90%), *DNMT3A* (39.34%), *TET2* (31.15%), *IDH2* (27.84%) and *NRAS/KRAS* (22.95%) (Fig. 1C). Notably, all samples exhibited wild-type status for *BRAF*, *CSF3R*, *ETV6*, *PHF6*, *PPMID*, *U2AF1*, and *ZRSR2*. By contrast, *ANKRD26*, *DDX41*, *EZH2*, *GATA2*, *JAK2*, *NF1*, *RUNX1*, *SETBP1* and *TP53* were each only mutated in a single case.

**Analysis of CNAs and CN-LOH.** Of the 61 patients with *NPM1*-mutated AML, 31 patients (50.82%) were found to harbor CNAs, while the remaining 30 patients (49.18%) had no detectable CNAs (Fig. 2A). The patients harboring CNAs included 13 patients (21.31%) with one CNA, 10 patients (16.39%) with 2 CNAs and 8 patients (13.11%) with  $\geq 3$  CNAs. In addition, 40 patients (65.57%) were identified to have CNA/CN-LOH events, whereas the remaining 21 patients (34.43%) had no detectable CNA/CN-LOH events (Fig. 2B). The cohort included 19 patients (31.15%) with 1 CNA/CN-LOH event, 10 patients (16.39%) with 2 such events and 11 patients (18.03%) with  $\geq 3$  CNA/CN-LOH events. The most frequently observed altered regions included dup(4)(q11q35) (4 cases), dup(18)(p11.31p11.23) (3 cases) and del(16)(q21q23) (3 cases); these represent putative recurrent alterations given the small sample size. Fig. 2C illustrates the genome-wide distribution of CNAs and CN-LOH across human chromosomes 1-22, X and Y.

The 61 patients were stratified into two groups based on the presence or absence of CNA/CN-LOH: The CNA/CN-LOH-positive group comprising 40 cases and the CNA/CN-LOH-negative group comprising 21 cases. Additionally, for further analysis, the patients were categorized by the burden of CNA/CN-LOH events: The  $< 2$  CNA/CN-LOH group, comprising all 21 CNA/CN-LOH-negative cases and the 19 CNA/CN-LOH-positive cases with 1 event ( $n=40$ ), and the  $\geq 2$  CNA/CN-LOH group ( $n=21$ ). Statistical analysis revealed no significant intergroup differences in the distribution of *NPM1* mutation subtypes, indicating genetic homogeneity in this aspect across the cohort. Subsequent comparative analyses demonstrated no significant differences between the groups in FAB subtype classification, sex distribution, age, baseline blood counts (including white blood cell count, platelet count and hemoglobin levels), CR rates or 2-year OS rates (Tables SII and SIII). However, these results were limited by the small single-center cohort and may not be generalizable. Gene mutation profiling identified recurrent alterations in *FLT3*, *DNMT3A*, *TET2*, *IDH2*, and *NRAS* in both the CNA/CN-LOH-positive and -negative groups, as well as in the  $< 2$  CNA/CN-LOH and  $\geq 2$  CNA/CN-LOH subgroups (Tables SIV and SV). However, no statistically significant differences in mutation frequencies were observed between these cohorts. To further explore the potential impact of a high CNA/CN-LOH burden on clinical and genetic characteristics, patients were stratified into two subgroups based on the number of CNA/CN-LOH variants: The  $\geq 3$  CNA/CN-LOH group ( $n=11$ ) and the  $< 3$  CNA/CN-LOH group ( $n=50$ ). Analysis demonstrated that patients harboring

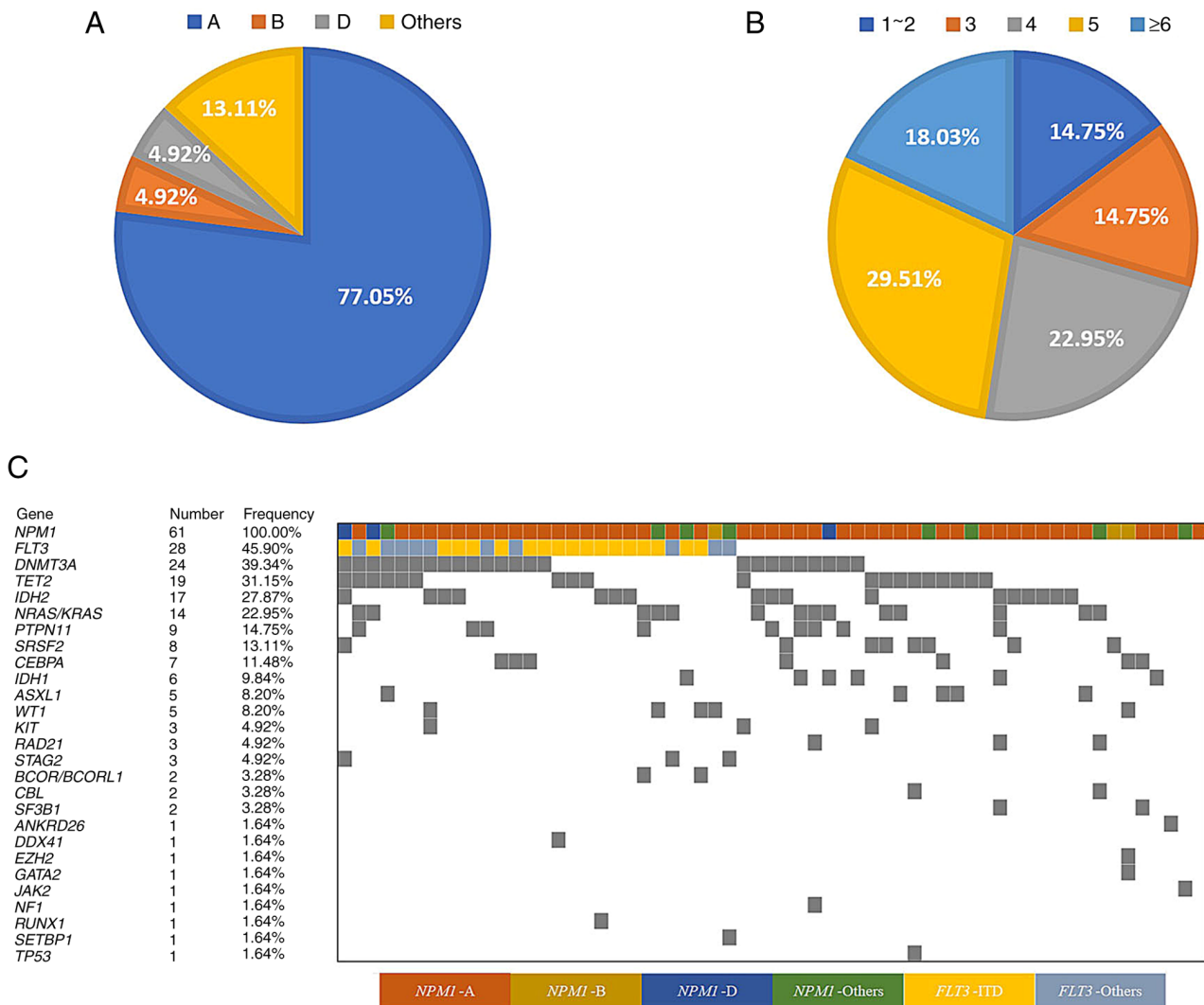


Figure 1. Genomic profiling of mutations in 61 patients with *NPM1*-mutated acute myeloid leukemia. Pie charts showing (A) the distribution of *NPM1* mutation subtypes A, B, D and other variants and (B) the frequency of co-mutation burden. (C) OncoPrint showing the mutational landscape of the patients. Genes and mutation frequency are presented on the y-axis; the colored bars represent samples classified by *NPM1* subtype, *FLT3*-ITD and other *FLT3* variants; each column represents 1 patient; and the colored or gray squares represent the presence of mutations in the corresponding genes. *NPM1*, nucleophosmin 1; *FLT3*, fms-related receptor tyrosine kinase; ITD, internal tandem duplication.

≥3 CNA/CN-LOH events were older ( $P=0.047$ ) and more frequently assigned to the M4 subtype according to the FAB classification ( $P=0.037$ ; Table I). However, after adjusting P-values via the BH method, these differences failed to reach the statistical significance threshold of  $Q<0.05$  ( $Q=0.447$  and  $0.703$ , respectively), indicating the absence of a robust association. In addition, no significant discrepancies were observed between the two subgroups regarding other hematological parameters, mutation frequencies, clinical indices or other relevant metrics (Tables I and II).

**G-banding and FISH analysis.** Chromosomal analysis was performed on all 61 elderly patients with *NPM1*-mutated AML using G-banding. Cytogenetic abnormalities were detected in 8 patients (13.11%), while the remaining 53 cases exhibited normal karyotypes. Among the 36 cases who additionally underwent FISH testing, 7 cases (19.44%) showed abnormalities in the targeted genomic regions, including 6 cases with CNAs and 1 case with a chromosomal

translocation. Integrative analysis of G-banding and FISH results identified chromosomal aberrations in 11 patients, while the remaining 50 cases had no detectable abnormalities. In a subset analysis of the 6 FISH-positive patients with CNA-type abnormalities, the comparison of FISH findings with sWGS-detected CNAs revealed the following cytogenetic abnormalities: Loss of chromosome X (-X), loss of chromosome Y (-Y), gain of chromosome X (+X), trisomy 8 (+8), 17p deletion (17p-) and concurrent gains of 21q/13q/20q, each occurring one in 1 patient. Representative FISH images are presented in Fig. S1. Quantitative evaluation using Cohen's  $\kappa$  coefficient demonstrated perfect inter-method agreement for CNA detection ( $\kappa=1.0$ ; 95% CI, 1.0-1.0; 100% concordance).

**Survival analysis.** To elucidate the impact of mutational burden on survival outcomes, the 61 elderly patients with *NPM1*-mutated AML were stratified into mutational burden subgroups ( $\geq 3$  vs.  $< 3$ ,  $\geq 4$  vs.  $< 4$ , and  $\geq 5$  vs.  $< 5$  mutations)

Table II. Comparison of genetic alterations in elderly patients with *NPM1*-mutated acute myeloid leukemia stratified by CNA/CN-LOH status.

Variant	Whole cohort (n=61)	<3 CNA/CN-LOH (n=50)	≥3 CNA/CN-LOH (n=11)	P-value
<i>FLT3</i>	28	22	6	0.525
<i>FLT3</i> -ITD	23	19	4	1.000
<i>DNMT3A</i>	24	20	4	1.000
<i>IDH2</i>	17	12	5	0.287
<i>TET2</i>	19	16	3	1.000
<i>NRAS/KRAS</i>	14	12	2	0.984
<i>PTPN11</i>	9	8	1	0.908
<i>CEBPA</i>	7	7	0	0.426
<i>IDH1</i>	6	4	2	0.294
<i>SRSF2</i>	8	7	1	1.000
<i>WT1</i>	5	4	1	1.000
<i>ASXL1</i>	6	5	1	1.000

*NPM1*, nucleophosmin 1; CNA, copy number alteration; CN-LOH, copy-neutral loss of heterozygosity; *FLT3*-ITD, fms-related receptor tyrosine kinase-internal tandem duplication; *DNMT3A*, DNA methyltransferase 3; *IDH1/2*, isocitrate dehydrogenase 1/2; *TET2*, tet methylcytosine dioxygenase 2; *PTPN11*, protein tyrosine phosphatase non-receptor type 11; *SRSF2*, serine and arginine-rich splicing factor 2; *WT1*, Wilms tumor 1; *ASXL1*, additional sex combs-like 1.

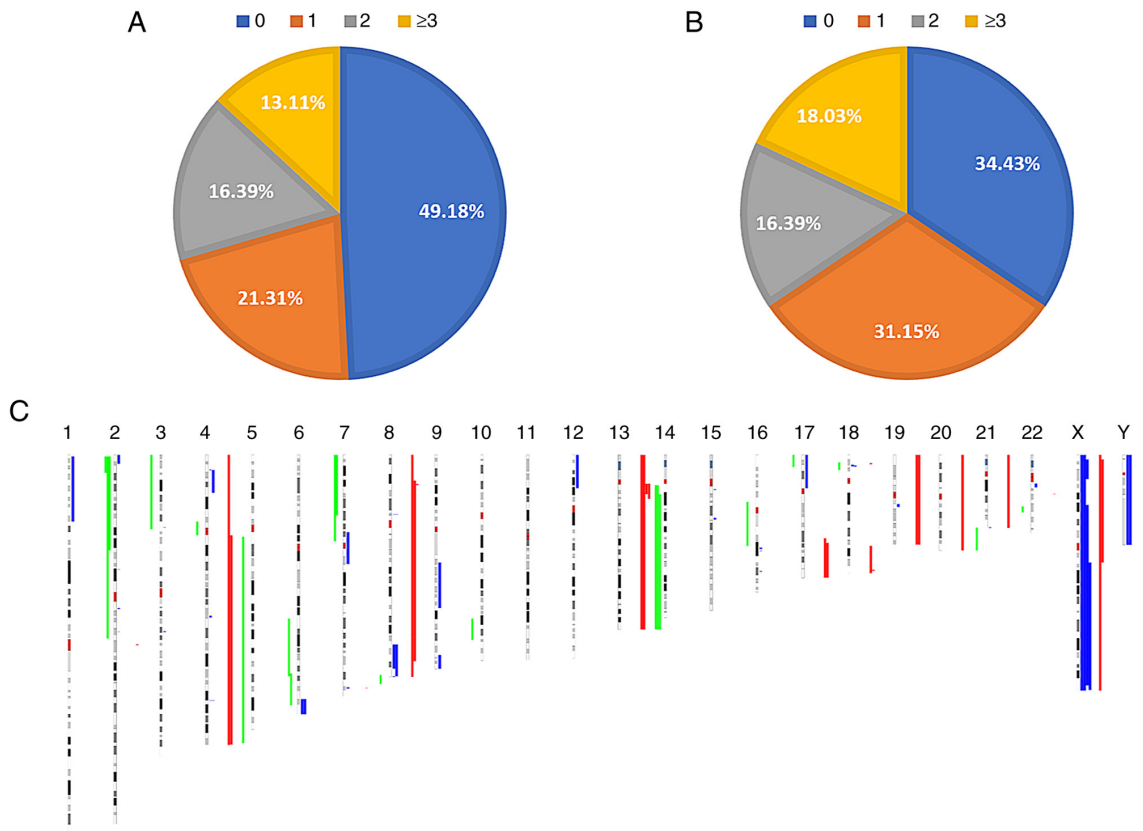


Figure 2. Distribution of CNAs and CN-LOH events in 61 patients with acute myeloid leukemia. Pie charts showing (A) CNA counts and (B) the number of combined CNA/CN-LOH events. (C) Genome-wide karyotype map of chromosomes 1-22, X and Y, generated by shallow whole-genome sequencing and visualized using the plotKaryotype function (GenVisR package, R). Red represents duplications; blue represents deletions; green represents CN-LOH; and gray banding represents canonical chromosomal G-banding (positional annotation). CNAs, copy number alterations; CN-LOH, copy-neutral loss of heterozygosity.

and subjected to univariate survival analysis. No significant differences in survival were observed between the ≥3 vs. <3 mutation groups ( $P=0.166$ ; Fig. 3A) or ≥4 vs. <4

mutation groups ( $P=0.055$ ; Fig. 3B) subgroups. However, patients with ≥5 mutations demonstrated a statistically significant reduction in OS compared with that of patients

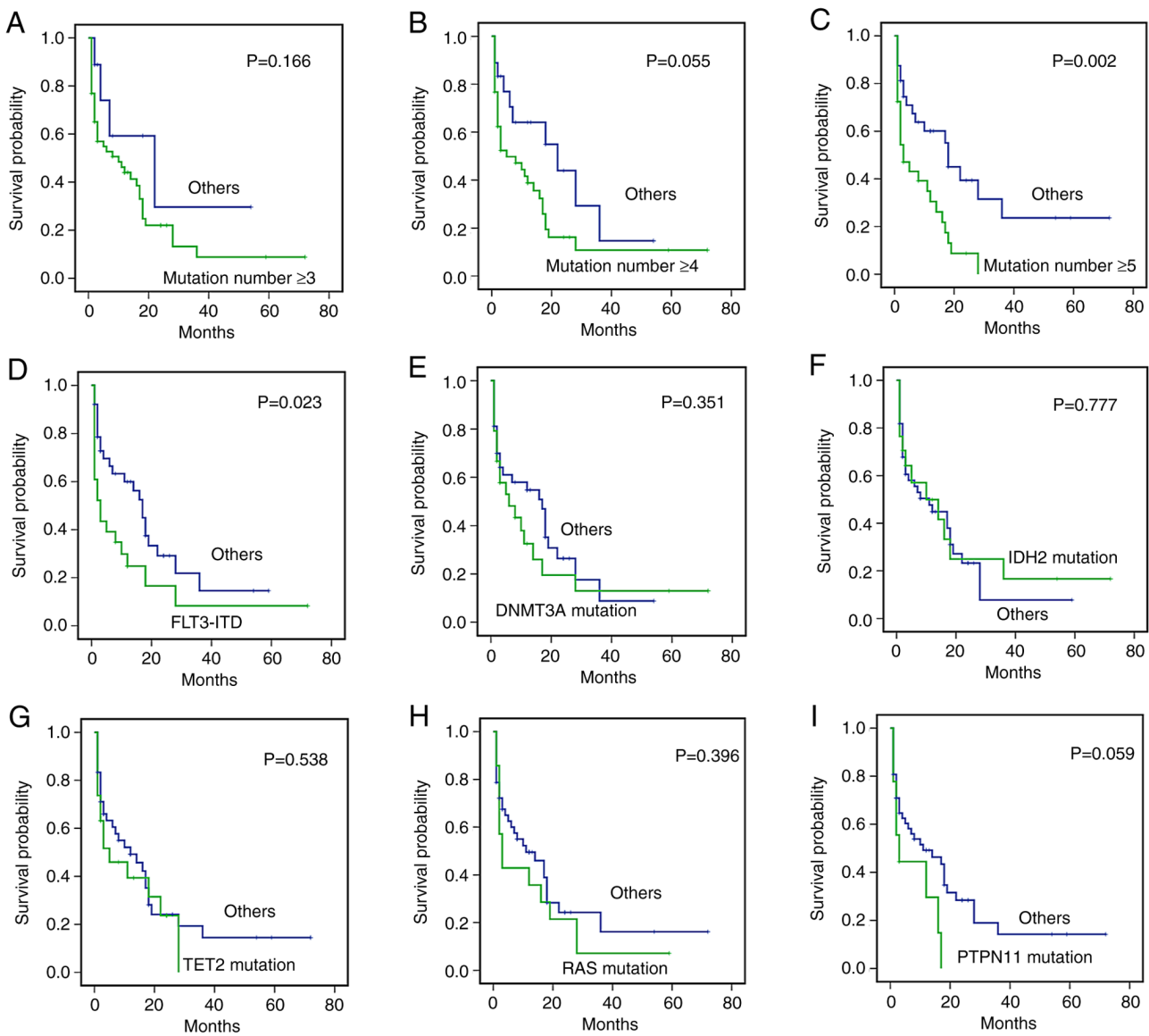


Figure 3. Kaplan-Meier analysis of overall survival in 61 patients with acute myeloid leukemia, stratified by genetic features. (A-C) Survival curves by cumulative mutation number: (A)  $\geq 3$  vs. others, (B)  $\geq 4$  vs. others and (C)  $\geq 5$  vs. others. (D-I) Survival by individual genetic mutations: (D) *FLT3*-ITD vs. others, (E) *DNMT3A* mutation vs. others, (F) *IDH2* mutation vs. others, (G) *TET2* mutation vs. others, (H) *RAS* (*NRAS/KRAS*) mutation vs. others and (I) *PTPN11* mutation vs. others. *FLT3*-ITD, fms-related receptor tyrosine kinase-internal tandem duplication; *DNMT3A*, DNA methyltransferase 3; *IDH2*, isocitrate dehydrogenase 2; *TET2*, tet methylcytosine dioxygenase 2; *PTPN11*, protein tyrosine phosphatase non-receptor type 11.

with  $<5$  mutations (HR, 2.446; 95% CI, 1.312-4.561;  $P=0.002$ ; Fig. 3C). Subgroup analyses of the frequently mutated genes *FLT3*, *DNMT3A*, *IDH2*, *TET2*, *NRAS/KRAS* and protein tyrosine phosphatase non-receptor type 11 (*PTPN11*) revealed that patients harboring *FLT3*-ITD mutations exhibited a significantly shorter OS compared with that of patients without such mutations (HR, 2.194; 95% CI, 1.192-4.036;  $P=0.023$ , Fig. 3D). However, mutations in *DNMT3A*, *IDH2*, *TET2*, *NRAS/KRAS* and *PTPN11* were not found to exert a significant impact on survival outcomes (Figs. 3E-I).

The prognostic impact of CNA/CN-LOH on OS was then investigated. Initial survival analyses focused on the genomic aberration dup (4)(q11q35), which was detected in 4 patients. Univariate analysis revealed no significant difference in OS between patients harboring dup (4)(q11q35) and those without

this aberration ( $P=0.397$ ; Fig. 4A). To determine whether CNA/CN-LOH status influenced clinical outcomes, patients were stratified into CNA/CN-LOH-positive and -negative groups, and no significant difference in OS was observed between these groups ( $P=0.148$ ; Fig. 4B). However, subgroup analysis based on the number of CNA/CN-LOH events revealed a trend toward shorter OS in patients with  $\geq 2$  events (HR, 1.375; 95% CI, 0.730-2.590;  $P=0.082$ ; Fig. 4C) and a statistically significant reduction in OS among those with  $\geq 3$  CNA/CN-LOH events (HR, 2.275; 95% CI, 1.101-4.698;  $P=0.024$ ; Fig. 4D).

The integration of gene mutation and CNA/CN-LOH profiles demonstrated that while an *IDH2* mutation alone had no distinct prognostic significance, patients with a concurrent *IDH2* mutation and no CNA/CN-LOH had a statistically significant improvement in OS compared with other patients (HR, 0.349; 95% CI, 0.103-1.185;  $P=0.043$ ; Fig. 4E).

Table III. Multivariate analysis of prognostic variables for overall survival in 61 elderly patients with *NPM1*-mutated acute myeloid leukemia.

Variables	P-value	Hazard ratio (95% confidence interval)
≥5 co-mutations	0.007	2.545 (1.458-4.441)
<i>FLT3</i> -ITD + ≥2 CNA/CN-LOH	0.136	2.399 (0.770-7.479)
<i>IDH2</i> mut + CNA/CN-LOH NEG	0.222	0.443 (0.201-0.977)
≥3 CNA/CN-LOH	0.018	1.978 (1.012-3.867)

*NPM1*, nucleophosmin 1; *FLT3*-ITD, fms-related receptor tyrosine kinase-internal tandem duplication; CNA, copy number alteration; CN-LOH, copy-neutral loss of heterozygosity; *IDH2*, isocitrate dehydrogenase 2; NEG, negative; mut, mutation.

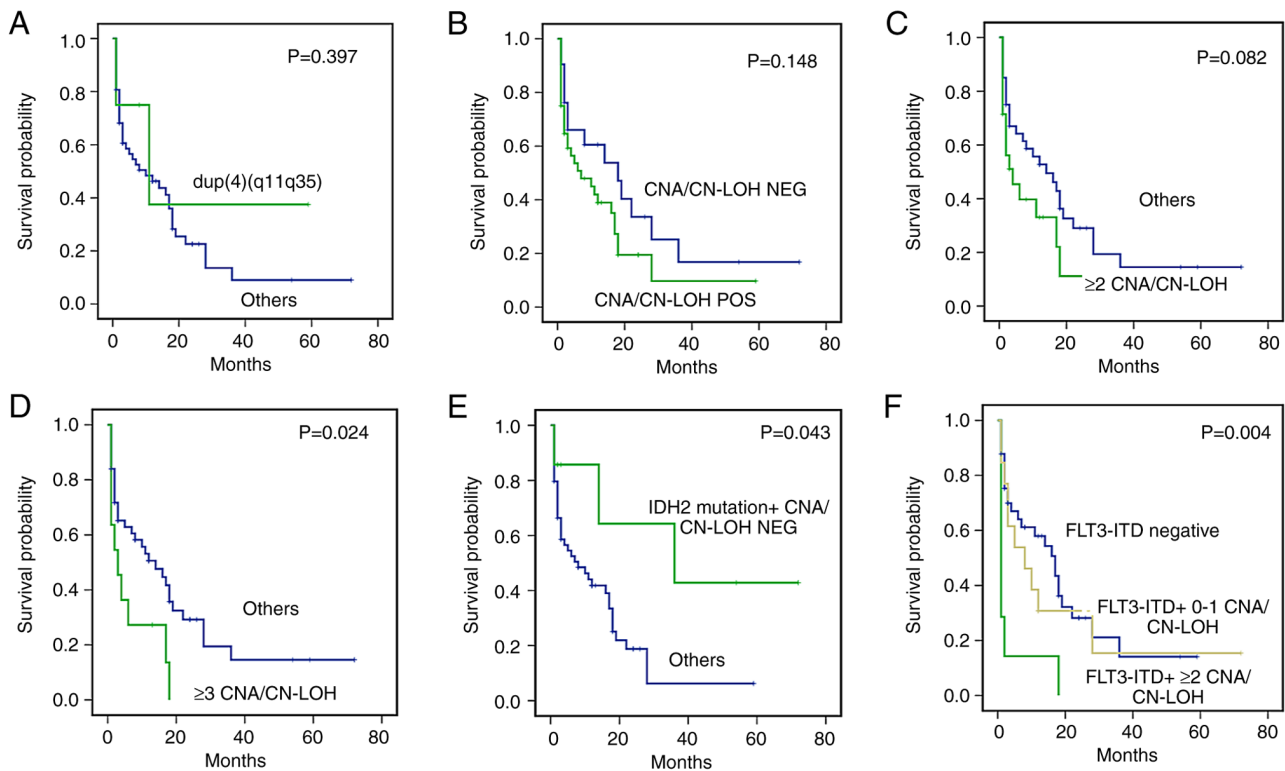


Figure 4. Kaplan-Meier analysis of overall survival in 61 patients with acute myeloid leukemia, stratified by distinct genetic and genomic alteration patterns. Survival analyses of (A) *dup(4)(q11q35)* vs. others, (B) CNA/CN-LOH NEG vs. CNA/CN-LOH POS, (C) ≥2 CNA/CN-LOH events vs. others, (D) ≥3 CNA/CN-LOH events vs. others, (E) *IDH2* mutation + CNA/CN-LOH NEG vs. others and (F) *FLT3*-ITD/CNA/CN-LOH combinations, namely *FLT3*-ITD negative, *FLT3*-ITD + 0-1 CNA/CN-LOH events and *FLT3*-ITD + ≥2 CNA/CN-LOH events. CNAs, copy number alteration; CN-LOH, copy-neutral loss of heterozygosity; NEG, negative; POS, positive; *IDH2*, isocitrate dehydrogenase 2; *FLT3*-ITD, fms-related receptor tyrosine kinase-internal tandem duplication.

Furthermore, patients with concurrent *FLT3*-ITD mutations and ≥2 CNA/CN-LOH events displayed poorer OS outcomes than those who were *FLT3*-ITD negative or had *FLT3*-ITD mutations and 0-1 CNA/CN-LOH events (HR, 4.528; 95% CI, 1.956-10.478;  $P=0.004$ ; Fig. 4F).

A preliminary multivariate Cox regression analysis was performed incorporating the key molecular features that had been identified. The analysis revealed that high mutational burden (≥5 mutations; HR, 2.545,  $P=0.007$ ) was independently associated with an increased risk of unfavorable OS (95% CI, 1.458-4.441). Similarly, the presence of ≥3 CNA/CN-LOH (HR, 1.978,  $P=0.018$ ) conferred an elevated risk of adverse OS (95% CI: 1.012-3.867) in elderly patients with *NPM1*-mutated AML (Table III). Exploratory analyses further suggested

potential prognostic trends, albeit without reaching statistical significance. Specifically, *FLT3*-ITD combined with ≥2 CNA/CN-LOH events exhibited a trend toward an increased risk of adverse outcomes (HR, 2.399; 95% CI, 0.770-7.479;  $P=0.136$ ). By contrast, the co-occurrence of *IDH2* mutation in the absence of CNA/CN-LOH was associated with a trend toward a reduced risk of adverse outcomes (HR, 0.443; 95% CI, 0.200-0.977;  $P=0.222$ ). These associations did not achieve the statistical significance threshold.

## Discussion

With rapid advancements in genetic and molecular biology detection techniques, particularly high-throughput

sequencing, substantial progress has been achieved in the precise diagnostic classification, prognostic stratification and MRD monitoring of AML (5,18,19). Concurrently, the development and clinical application of novel therapeutic agents have significantly improved the survival outcomes of patients with AML (20,21). However, a substantial proportion of patients continue to be misclassified with respect to diagnostic subtype and prognostic risk. This prevents the matching of individuals with optimal treatment regimens, leading to suboptimal clinical outcomes, particularly among elderly patients. Although some patients with *NPM1* mutations can achieve a favorable prognosis, a subset of patients, particularly older individuals, experience poor outcomes. Therefore, the present study analyzed gene mutations, CNAs and CN-LOH in 61 elderly patients with *NPM1*-mutated AML, with the aim of preliminarily identifying genomic features that may further refine risk stratification and inform management strategies within existing precision medicine frameworks.

Targeted NGS was performed on 61 bone marrow samples obtained from elderly patients with *NPM1*-mutated AML using a 36-gene panel designed for myeloid malignancy profiling. Consistent with previous reports (22,23), *NPM1* mutation subtype A predominated, accounting for 77.05% of cases, affirming its status as the most common *NPM1* subtype in AML cohorts. Notably, 52.46% of patients exhibited a total of 4 or 5 gene mutations, including the pathogenic *NPM1* mutation, with *FLT3*, *DNMT3A*, *TET2*, *IDH2*, and *NRAS/KRAS* being the most frequently co-occurring genetic alterations. These findings are consistent with an independent study that has identified these genes as core components of the mutational landscape in *NPM1*-mutated AML (24). The high prevalence of such co-mutations underscores the genetic complexity of AML, where the accumulation of these mutations drives leukemogenesis and disease progression (25).

With respect to *TP53* mutations, a well-established high-risk genetic aberration in AML, only a single patient in the present study cohort carried a *TP53* mutation, which is consistent with previous studies reporting low *TP53* mutation frequencies in *NPM1*-mutated AML (26). Furthermore, within this elderly *NPM1*-mutated AML cohort, the mutation frequencies of genes such as *WT1*, *RUNX1* and *ASXL1* were markedly lower than those reported in patients with conventional (non-*NPM1*-mutated) AML, and the incidence of fusion genes was also relatively low (1,2,19).

A primary objective of the present study was to delineate the genomic landscape of elderly patients with *NPM1*-mutated AML, with particular emphasis on CNA, CN-LOH and their clinical implications, an area that remains relatively unexplored in elderly-specific cohorts (27). The data demonstrated a high CNA/CN-LOH rate (65.57%) in this population. Notably, recurrent amplifications, such as *dup(4q11q35)*, were identified and are hypothesized to activate oncogenes or disrupt tumor suppressor pathways in AML, although the specific driver genes within these loci remain to be characterized. By contrast, chromosomal translocations constitute a defining cytogenetic feature of conventional AML (5). In elderly patients with AML, while the overall incidence of chromosomal translocations is relatively low, specific chromosomal abnormalities, including 5q deletion (-5/5q-), 7q deletion (-7/7q-), as well as trisomy 8 (+8), are disproportionately represented (4,5). These findings

provide a preliminary rationale for the systematic exploration of these recurrent amplification hotspots, with the aim of identifying uncharacterized specific drivers as druggable targets within these genomic regions.

In the present study, elderly patients with *NPM1*-mutated AML harboring  $\geq 5$  concomitant mutations exhibited a significantly shorter OS, supporting the hypothesis that high mutational burden reflects underlying genomic instability and clonal heterogeneity (28). This observation provides preliminary evidence for the clinical relevance of mutation burden in elderly patients with *NPM1* mutations, a population that is often underrepresented in large-scale genomic studies of AML. Importantly, in routine clinical practice, a subset of elderly patients with *NPM1*-mutated AML, even those lacking ELN 2022-defined adverse-risk features (29), display distinct genomic profiles and experience suboptimal outcomes, underscoring the necessity for further investigation.

Using sWGS for the detection of CNAs and CN-LOH, it was further identified that the presence of  $\geq 3$  CNA/CN-LOH events may serve as an independent predictor of poor survival outcomes. This is a tentative but potentially novel finding, as the present study is among the first to use sWGS to quantify CNA/CN-LOH burden as a prognostic marker in elderly patients with *NPM1*-mutated AML. By contrast, previous studies in this field have primarily focused on individual chromosomal aberrations, such as *del(5q)* and monosomy 7, rather than using CNA/CN-LOH burden as a composite prognostic indicator (27,30).

In subtype-specific analyses, consistent with previous studies, isolated *IDH2* mutations did not demonstrate intrinsic prognostic value (22,31). However, by integrating mutational profiles with CNA/CN-LOH data, the present study observed that the co-occurrence of *IDH2* mutations and absence of CNA/CN-LOH events was associated with significantly improved clinical outcomes. Although multivariate Cox regression analysis did not confirm independent prognostic value, likely due to the small sample size and limited statistical power, these preliminary findings suggest that the combined assessment of *IDH2* mutation and CNA/CN-LOH status may help to tentatively identify subgroups with favorable prognosis among patients with *NPM1*-mutated AML. Future large-scale, multicenter studies are warranted to validate the generalizability of this combined molecular marker, including its prognostic implications in conventional AML subtypes.

With respect to *FLT3*-ITD mutations, the 2022 ELN guidelines classify patients with *NPM1*-mutated AML harboring *FLT3*-ITD as intermediate risk (29); however, some such patients still experience very poor outcomes. In the present study, it was found that among *FLT3*-ITD-positive, *NPM1*-mutated cases, those with  $\geq 2$  CNA/CN-LOH events exhibited the worst survival outcomes, underscoring the interplay between mutational status and genomic instability as a potential prognostic axis. Although this association did not reach statistical significance in the multivariate analysis, these observations tentatively suggest a mechanistic basis for the pronounced prognostic heterogeneity within this vulnerable subgroup and highlight the need to refine risk stratification beyond conventional AML classification systems.

Notably, in the exploratory analysis of associations between high CNA/CN-LOH burden ( $\geq 3$  events) and clinical

characteristics, the present study initially identified associations with advanced patient age and FAB subtype M4. However, these associations did not retain statistical significance following BH correction, underscoring that cautious interpretation is necessary, likely reflecting the impact of multiple testing adjustment and the limited sample size of the high-burden subgroup (n=11). These limitations highlight the necessity of validating these preliminary findings in larger, independent cohorts to clarify their potential clinical implications.

To define high-risk thresholds for genomic burden, the CNA/CN-LOH cut-off was first determined. A threshold of  $\geq 3$  events was statistically derived by rounding the sum of the cohort mean (1.3770) plus 1 standard deviation (1.5723), yielding a value of 2.9494. This threshold included 11 patients (18.03%), which provides sufficient discriminatory power for survival analysis while preserving the ability to distinguish a clinically relevant high-risk subgroup. A higher threshold ( $\geq 4$  events) reduced the subgroup to 5 patients (8.20%), compromising statistical reliability. Kaplan-Meier analysis showed that the separation of OS was optimal with  $\geq 3$  events, whereas a lower threshold exhibited a weaker association. For mutation burden, the high-risk threshold of  $\geq 5$  mutations was selected based on the distribution within the 61-patient cohort (mean, 4.34; median, 4.0), to effectively identify patients with above-average mutational burden. This cut-off included 29 patients (47.54%), ensuring balanced group sizes and adequate analytical power. Importantly, the  $\geq 5$  mutation threshold was associated with shorter OS, supporting its utility for risk stratification, while lower thresholds lacked robust prognostic relevance.

Regarding the clinical implications of integrated genomic stratification, the insufficiently defined prognosis of *NPM1*-mutated AML in current clinical practice underscores the urgent requirement for precise risk stratification. Integrating CNA/CN-LOH burden ( $\geq 3$  events) with mutational load ( $\geq 5$  mutations) may help to refine existing risk stratification systems and offer translatable clinical value by providing a more personalized basis for tailoring therapeutic intensity and disease surveillance strategies. Specifically, patients with low genomic burden ( $< 3$  CNA/CN-LOH events and  $< 5$  mutations) may benefit from de-escalated therapy, potentially minimizing treatment-related toxicity while maintaining therapeutic efficacy. By contrast, patients with high genomic burden ( $\geq 3$  CNA/CN-LOH events and/or  $\geq 5$  mutations), which is associated with shorter OS, may require more aggressive upfront treatment regimens. Regarding surveillance, patients with a high burden may benefit from more frequent monitoring to enable the early detection of relapse, whereas those with a low burden could be surveilled less frequently. Beyond overall genomic burden, subtype-specific analyses suggest the potential value of integrating CNA/CN-LOH with driver mutations. Among *FLT3*-ITD-positive patients, those with  $\geq 2$  CNA/CN-LOH events exhibited the poorest OS, indicating that combinatorial therapies including *FLT3* inhibitors may be explored, while accounting for the potential contribution of high CNA/CN-LOH burden to treatment resistance. Conversely, in patients with *IDH2* mutations, those without CNA/CN-LOH exhibited a longer OS, suggesting that CNA/CN-LOH status may serve as a stratification marker for the efficacy of *IDH2* inhibitors and provide guidance for the design of future clinical trials.

The present study has several inherent limitations that warrant careful consideration when interpreting the results. As a single-center retrospective analysis with a modest cohort size, the generalizability of the findings may be limited. The small sample size was particularly pronounced in clinically relevant subgroups, including patients with  $\geq 3$  CNA/CN-LOH events (n=11) and those harboring *FLT3*-ITD alongside  $\geq 2$  CNA/CN-LOH events (n=10). This may compromise the robustness of inter-subgroup comparisons and locus-specific CNA/CN-LOH analyses. In addition, the relatively low number of OS events may introduce variability in the prognostic parameters, including HRs and 95% CIs, generated by the preliminary Cox regression model. This also restricts the ability of the model to adjust for potential confounding variables or to detect subtle interactions among covariates. Therefore, the observed independent prognostic value of high mutational burden and elevated CNA/CN-LOH burden necessitates external validation in larger, multi-institutional cohorts. Furthermore, the clinical applicability of these findings is currently constrained by the lack of associative analyses linking genomic aberrations to responses to targeted therapies.

To address these limitations, subsequent studies should prioritize the following: i) Validation of prognostic thresholds for mutational and CNA/CN-LOH burdens in large, prospectively curated cohorts; and ii) integrative, multidimensional analyses of co-occurring gene mutations and CNA/CN-LOH events, and their association with therapeutic outcomes. Such efforts will be critical for refining risk stratification models and informing personalized treatment strategies for older patients with *NPM1*-mutated AML. In alignment with these objectives, the current research team is actively conducting a multicenter study to systematically compare CNA/CN-LOH profiles across AML molecular subtypes, including *NPM1*-mutated AML and conventional subtypes, such as *TP53*-, *RAS*- and *ASXL1*-mutated AML. The study is structured around two pivotal analytical axes: i) Quantification of CNA/CN-LOH burden at the patient level to define subtype-specific distribution patterns, and ii) genome-wide mapping of recurrent events, such as *dup(4q11q35)* enrichment in *NPM1*-mutated AML. By constructing a provisional subtype-resolved CNA/CN-LOH landscape and associating these genomic features with clinical outcomes, the study aims to clarify inter-subtype heterogeneity in burden distributions, identify subtype-discriminatory recurrent events, and elucidate the mechanistic interplay between CNA/CN-LOH and driver mutations.

In conclusion, the present study preliminarily delineates the genomic landscape of *NPM1*-mutated AML in elderly patients, with a focus on co-mutational patterns, CNAs and CN-LOH, which are genomic features that have been relatively unexplored in previous studies. It identifies high mutational burden ( $\geq 5$  mutations) and  $\geq 3$  CNA/CN-LOH events as potential indicators of a poor prognosis, tentatively supporting the established concept that genomic instability and clonal complexity are associated with adverse outcomes in this subgroup. In addition, the present study suggests that the combination of *IDH2* mutation with the absence of CNA/CN-LOH may be associated with favorable outcomes, while *FLT3*-ITD positivity combined with  $\geq 2$  CNA/CN-LOH events is associated with poor survival. These findings may partially explain the prognostic heterogeneity observed within

intermediate- and favorable-risk categories defined by the ELN 2022 guidelines, highlighting the potential value of integrating multidimensional genomic profiles, rather than single-gene mutations alone, to optimize risk stratification beyond existing guidelines. However, it should be emphasized that the generalizability of the genomic thresholds proposed in the present study requires validation in large-scale multicenter studies, and may ultimately provide a preliminary framework for advancing the personalized diagnosis, treatment selection and precise management of elderly patients with *NPM1*-mutated AML.

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#### Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The raw sequencing data have been deposited in the China National Genomics Data Center (NGDC) under the BioProject accession number PRJCA053981 (<https://ngdc.cnbc.ac.cn/bioproject/browse/PRJCA053981>). The sample-level data can be accessed via GSA accession number HRA015549 (<https://ngdc.cnbc.ac.cn/gsa-human/browse/HRA015549>).

#### Authors' contributions

ZL, LG and CZ conceived and designed the study, and drafted the manuscript. ZL, CZ and MG contributed to the collection of clinical data and patient management. LG, ZC, CL and SW performed the laboratory work. ZL, LG and CZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

#### Ethics approval and consent to participate

This study was approved by the Ethics Committee of China-Japan Friendship Hospital (approval no. 2023-KY-200) and conducted in strict adherence to the principles outlined in the Declaration of Helsinki. Prior to the initiation of the study, written informed consent was obtained from all enrolled patients or their legal representatives in accordance with ethical requirements.

#### Patient consent for publication

Not applicable

#### Competing interests

The authors declare that they have no competing interests.

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