Genetic Mutation Signature for Relapse Prediction in Normal Karyotype Acute Myeloid Leukemia

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Research Article

**Keywords:** acute myeloid leukemia, normal karyotype, genetic mutation, prognostic model, relapse

**Posted Date:** January 30th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-2262183/v1

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Abstract

**Background:** Risk stratification for normal karyotype acute myeloid leukemia remains unsatisfactory, which is reflected by the high incidence of leukemia relapse. This study aimed to evaluate the role of gene mutations and clinical characterization in predicting the relapse of patients with normal karyotype acute myeloid leukemia.

**Methods:** A prognostic system for normal karyotype acute myeloid leukemia was constructed based on gene mutations, measurable residual disease, and clinical characteristics. A panel of gene mutations was explored using next-generation sequencing. The least absolute shrinkage and selection operator, and nomogram algorithm were used to build a genomic mutation signature (GMS) nomogram (GMSN) model that combines GMS, measurable residual disease, and clinical factors to predict relapse in 347 patients with normal karyotype acute myeloid leukemia from four centers.

**Results:** Patients in the GMS-high group had a higher 5-year incidence of relapse than those in the GMS-low group ($P < 0.001$). The 5-year incidence of relapse was also higher in patients in the GMSN-high group than in those in the GMSN-intermediate and -low groups ($P < 0.001$). The 5-year disease-free survival and overall survival rates were lower in patients in the GMSN-high group than in those in the GMSN-intermediate and -low groups ($P < 0.001$) as confirmed by training and validation cohorts.

**Conclusions:** This study illustrates the potential of GMSN as a predictor of normal karyotype acute myeloid leukemia relapse.

Background

Acute myeloid leukemia (AML) is a highly heterogeneous disease with a poor prognosis, largely owing to its high incidence of relapse(1). Cytogenetic detection has been proven important for risk stratification of patients with AML(2, 3). However, AML with a normal karyotype (NK-AML) is observed in nearly half of AML cases(4). The identification of specific genetic mutations has remarkably augmented our understanding of AML molecular pathophysiology and revealed the prognostic significance of each mutation in NK-AML(5–7). The classification of AML and its prognostic profile have been improved owing to advances in molecular characterization and the application of high-throughput sequencing. NK-AML belongs to the largest cytogenetic AML subgroup, with a large proportion of patients experiencing relapse(8). However, the precise identification of patients at high risk of relapse remains unsatisfactory. It is, therefore, imperative to introduce novel prognostic biomarkers for determining high relapse risk.

In recent years, next-generation sequencing (NGS) has become a routine diagnostic method for hematological malignancies(7, 9). NGS outcomes, including single somatic mutation profiling, may improve diagnostic accuracy and support precise treatment strategies in clinical practice. However, the comprehensive use of genomic mutation databases and clinical factors to guide clinical decision-making remains at a relatively early stage and, therefore, difficult. In a previous study, we used a machine-learning algorithm based on 16s rRNA gene sequencing of intestinal microbiota to precisely predict the occurrence
of acute graft-versus-host disease during allogeneic hematopoietic stem cell transplantation (allo-HSCT) (10). Our results strongly suggested that NGS data and machine learning can be used for the identification of novel biomarkers for predicting NK-AML relapse.

In this study, we adopted the least absolute shrinkage and selection operator (LASSO) method and combined 22 gene mutations into a panel for NGS testing prior to induction therapy to establish a robust model (genomic mutation signature, GMS) for the prediction of relapse in NK-AML. Furthermore, we combined GMS, measurable residual disease (MRD) and clinical characteristics to generate a nomogram model for improved relapse and survival prognosis in 347 patients with NK-AML enrolled from four centers. Our model could provide novel insights into improving the precise evaluation of relapse risk in NK-AML.

**Methods**

**Patients**

From July 2016 to December 2019, patients diagnosed with AML in our centers (the First Affiliated Hospital of Zhengzhou University, Henan Cancer Hospital, the First Affiliated Hospital of Xinxiang Medical University, and the Huaihe Hospital of Henan University) were enrolled based on the following inclusion criteria: (1) diagnosed with *de-novo* AML and normal karyotype; (2) between the ages of 14 and 60 years; (3) received ≥ 3 cycles of chemotherapy. The study was performed in accordance with the Helsinki Declaration and was approved by the ethical committees of the First Affiliated Hospital of Zhengzhou University, Henan Cancer Hospital, the First Affiliated Hospital of Xinxiang Medical University, and the Huaihe Hospital of Henan University.

**Diagnosis**

AML was diagnosed as previously described(2). Immunophenotyping was conducted on diagnostic bone marrow (BM) aspirate samples via eight-color CD45/SSC gated flow cytometry(11). Cytogenetic examinations were conducted as per standard techniques(12). Molecular screening for fusion genes and gene mutations was performed via RT-PCR, and sequencing analysis was performed for all patients(13). The diagnosis of AML was based on the European Leukemia Net 2017 recommendations version 3(2). Secondary AML (sAML) includes AML arising from myelodysplastic syndrome, myeloproliferative neoplasms, myelodysplastic/myeloproliferative neoplasm, and therapy-related AML(14).

**NGS**

BM samples containing at least 20% blasts were collected at diagnosis, and mononuclear cells were separated via density gradient centrifugation. Genomic DNA was extracted using the Tiangen DP318-02 blood genomic DNA Extraction Kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. The integrity and concentration of genomic DNA were determined using the Qubit 4.0 fluorometer dsDNA
HS Assay (Thermo Fisher Scientific, Waltham, MA, USA). Germline control DNA was obtained from matched BM during complete remission (CR). Approximately 500 ng to 1 µg of high-quality DNA was used for sequencing library construction.

The sequencing panel contained 22 frequently mutated genes related to AML diagnosis and prognosis. The panel kit was purchased from Shanghai Yuanqi Biomedical Technology Company Ltd. The threshold of the read depth was 1000×, resulting in a sensitivity of 1%. Raw reads were filtered using the Cutadapt software (version 2.10; https://cutadapt.readthedocs.io/en/stable), and clean reads were mapped to the human reference genome (GRCh37) using the BWA-mem algorithm (software version 0·7·17; http://bio-bwa.sourceforge.net/bwa.shtml). The Sambamba software (version 0·6·8; https://github.com/biod/sambamba) was used to mark duplication, and the GATK software (version 4·0·12·0; https://gatk.broadinstitute.org/hc/en-us) was used for the recalibration of the base quality score. Single-nucleotide variants and short insertions/ deletions were called using the GATK software. Finally, a variant allele frequency of 0.01 was used as the threshold to determine whether a mutation was positive or negative.

Sequencing libraries were prepared using the KAPA Hyper Prep Kit (Kapa Biosystems, Wilmington, MA, USA) according to the manufacturer's instructions. Genomic DNA was fragmented to 200 to 300 bp using the Covaris S220 Ultrasonicator (Covaris, Woburn, MA, USA). The overhanging ends were repaired into blunt ends. The 3′ ends of the fragments were additionally adenylated with a single adenine (A) nucleotide, allowing hybridization to the 3′ overhanging thymine (T) nucleotide of the sequencing adapters. The fragments were purified and ligated to adapters. The resulting DNA fragments were selected using AM Pure XP beads (Beckman Coulter, Krefeld, Germany) for the desired size of 420 bp. The fragments were then amplified via PCR. The purified libraries were sequenced and demultiplexed on a Nova Seq 6000 System (Illumina, San Diego, CA, USA), with 2 × 150 bp paired-end sequencing.

**Lasso-based Gene Selection And Establishment Of The Gms For Relapse Prediction**

In our previous studies, we employed LASSO for the regression analysis of high-dimensional variables (10, 15); herein, we used the LASSO algorithm to select the most important mutated genes in the training cohort. After repeated fine-tuning and 10-fold cross-validation, the standardized constraint parameters (minimum value of log λ) based on the 1-SE criteria were finally set to 0.036, and several non-zero coefficients were selected. We then used Cox regression to generate a predictive model of relapse and calculated the GMS for each patient. Using Kaplan–Meier survival analysis, we evaluated the predictive performance of the GMS model in both the training and independent validation cohorts. The best cut-off value estimation for the GMS model was 0.0069, as determined by repeated testing for relapse prediction.

**Monitoring And Definition Of Mrd**
BM samples were obtained to monitor MRD using eight-color multi-parameter flow cytometry after each cycle of chemotherapy (induction, six cycles of consolidation) (11). The identification of the leukemia-associated immunophenotype, as defined in AML diagnosis, was performed for MFC-MRD detection. The different-from-normal immunophenotype was applied to monitor MFC-MRD when leukemia-associated immunophenotype was not available at diagnosis. The sensitivity of MFC-MRD was defined as 0·1%. Any level of MRD $\geq 0.1\%$ was positive, and less than 0·1% was negative(16).

**Treatment**

Induction chemotherapy included anthracycline (10 mg/m$^2$ idarubicin or 45 mg/m$^2$ daunorubicin for 3 days) in combination with infusional cytarabine (Ara-C, 100 mg/m$^2$ for 7 days) or HAA (2 mg/m$^2$ homoharringtonine, 100 mg/m$^2$ cytarabine, and 20 mg aclacinorubicin for 7 days)(17, 18). Generally, induction chemotherapy was performed for two cycles if the patients achieved CR or partial remission (PR) in the first cycle; otherwise, those who experienced no remission (NR) after the first cycle received FLAG (30 mg/m$^2$ fludarabine on days 1–5, 2 g/m$^2$ Ara-C on days 1–5, and 300 µg of G-CSF on days 0–5) or CLAG (5 mg/m$^2$ cladribine on days 1–5, 2 g/m$^2$ Ara-C on days 1–5, and 300 µg of G-CSF on days 0–5)(19, 20). After two cycles of induction chemotherapy, patients with NR were given decitabine + CAG (Ara-C, aclacinorubicin) (21) or were enrolled in a clinical trial, and the patients of CR/CRi were administered consolidation chemotherapy, which comprised a cycle of cytarabine (2 g/m$^2$ q12h for 3 days).

Subsequently, the patients in CR/CRi received another three to four cycles of consolidation chemotherapy(17), allo-HSCT, or auto-HSCT based on MRD and donor availability. In auto-HSCT, peripheral blood stem cells were harvested following mobilization using intermediate-dose cytarabine and subsequent granulocyte colony-stimulating factor. In allo-HSCT, myeloablative conditioning regimens were administered to all patients, as previously described(10).

**Definitions Of Treatment Response**

CR was defined as follows: BM blasts less than 5%, absence of blasts with Auer rods, zero blasts in peripheral blood, no extramedullary disease, absolute neutrophil count $> 1.5 \times 10^9$/L, platelet count $> 100 \times 10^9$/L, and hemoglobin concentration $> 90$ g/dL. CRi was defined as CR with incomplete blood count recovery; PR was defined as 5% $<$ BM blasts $< 20\%$; NR was defined as BM blasts $\geq 20\%$; relapse was defined as the reappearance of BM blasts $> 5\%$, recurrence of blasts in the blood, or the appearance of extramedullary disease; non-CR was defined to include NR and PR as previously described(22).

**Development Of A Nomogram Combining Gms, Mrd, And Clinical Factors For Relapse Prediction**

Nomogram models have been extensively reported in previous studies on cancer prognosis (23, 24). We performed multivariate regression analysis to construct a GMS nomogram (GMSN) as a precise
quantitative model to predict relapse in patients with NK-AML. After multivariate analysis, candidate predictors of relapse were GMS, sAML, risk category, cycle3rd (remission status after the third cycle of chemotherapy), MRD3rd, and treatment choice. The predictive performance of the nomogram was estimated using an independent validation cohort. Relapse was then assessed considering the total points as a factor in the Cox regression analysis. Harrell's C-index was calculated to quantify the discriminating capability of the GMSN in the training cohort. The GMSN model was plotted using the “rms” package (https://cran.r-project.org/web/packages/rms/index.html).

Endpoints And Statistical Methods

The cumulative incidence of relapse was the primary endpoint of the study, while the secondary endpoints included disease-free survival (DFS) and overall survival (OS), determined via Kaplan-Meier analysis and compared using a log-rank test. In Cox regression analysis, variables associated with relapse or survival ($P<0.10$ in univariate analysis) or variables (e.g., age, high white blood cell count, cycles required to achieve CR $\geq 2$) known to influence outcomes were included in the final models. Statistical significance was established at $P<0.05$. The R software (http://cran.R-project.org) was used for all data analyses.

Results

Clinical characteristics

From July 2016 to December 2019, 1,066 adult patients were diagnosed consecutively with AML at our centers (Additional File Figure 1). Of the 1066 patients, 79 were not treated, 86 did not have complete cytogenetic or NGS data at diagnosis, and 464 had an abnormal karyotype. These patients were excluded from the study. Of the remaining 437 patients, 31 were excluded due to early death ($n = 31$), loss of follow-up ($n = 21$), or without MRD data during the two cycles of induction chemotherapy. Of the remaining 370 patients, patients lost to follow-up ($n = 13$) or without MRD ($n = 10$) were excluded after another cycle of chemotherapy consolidation. The remaining 347 patients (the First Affiliated Hospital of Zhengzhou University, $n = 197$; Henan Cancer Hospital, $n = 106$; the First Affiliated Hospital of Xinxiang Medical University, $n = 25$; and Huaihe Hospital of Henan University, $n = 19$) were enrolled and randomly divided into a training cohort ($n = 209$) and a validation cohort ($n = 138$).

The patient characteristics are shown in Table 1. The median age (range) of the patients was 40 (14–60) years, and the median follow-up time (range) was 17.0 (3.5–60.0) months. According to the risk categories of the European Leukemia Net 2017 guideline, 141, 178, and 28 cases were classified as unfavorable, intermediate, and favorable, respectively. Thirty-seven cases (10.7%) were sAML, and the other 310 cases were de novo AML. After the third cycle of chemotherapy, 317 (91.4%) patients achieved CR (including CRi), and 204 (58.8%) patients were MRD3 negative.

Table 1. Characteristics of patients in the training and validation cohorts.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Training cohort (n = 209)</th>
<th>Validation cohort (n = 138)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td>0.380</td>
</tr>
<tr>
<td>≤ 40</td>
<td>110 (52.63%)</td>
<td>66 (47.83%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 40</td>
<td>99 (47.37%)</td>
<td>72 (52.17%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.694</td>
</tr>
<tr>
<td>Female</td>
<td>109 (52.15%)</td>
<td>69 (50.00%)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>100 (47.85%)</td>
<td>69 (50.00%)</td>
<td></td>
</tr>
<tr>
<td>Risk category</td>
<td></td>
<td></td>
<td>0.803</td>
</tr>
<tr>
<td>favorable</td>
<td>17 (8.14%)</td>
<td>11 (7.98%)</td>
<td></td>
</tr>
<tr>
<td>intermediate</td>
<td>110 (52.63%)</td>
<td>68 (49.27%)</td>
<td></td>
</tr>
<tr>
<td>unfavorable</td>
<td>82 (39.23%)</td>
<td>59 (42.75%)</td>
<td></td>
</tr>
<tr>
<td>WBC</td>
<td></td>
<td></td>
<td>0.068</td>
</tr>
<tr>
<td>≤ 100×10^9</td>
<td>194 (92.82%)</td>
<td>120 (86.96%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 100×10^9</td>
<td>15 (7.18%)</td>
<td>18 (13.04%)</td>
<td></td>
</tr>
<tr>
<td>sAML</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27 (12.92%)</td>
<td>10 (7.25%)</td>
<td>0.094</td>
</tr>
<tr>
<td>No</td>
<td>182 (87.08%)</td>
<td>128 (92.75%)</td>
<td></td>
</tr>
<tr>
<td>Response cycle1st</td>
<td></td>
<td></td>
<td>0.264</td>
</tr>
<tr>
<td>non-CR</td>
<td>45 (21.53%)</td>
<td>23 (16.67%)</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>164 (78.47%)</td>
<td>115 (83.33%)</td>
<td></td>
</tr>
<tr>
<td>Response cycle2nd</td>
<td></td>
<td></td>
<td>0.405</td>
</tr>
<tr>
<td>non-CR</td>
<td>24 (11.48%)</td>
<td>12 (8.70%)</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>185 (88.52%)</td>
<td>126 (91.30%)</td>
<td></td>
</tr>
<tr>
<td>Response cycle3rd</td>
<td></td>
<td></td>
<td>0.978</td>
</tr>
<tr>
<td>non-CR</td>
<td>18 (8.61%)</td>
<td>12 (8.70%)</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>191 (91.39%)</td>
<td>126 (91.30)</td>
<td></td>
</tr>
<tr>
<td>MRD1st</td>
<td></td>
<td></td>
<td>0.647</td>
</tr>
<tr>
<td>negativity</td>
<td>50 (23.92%)</td>
<td>36 (26.09%)</td>
<td></td>
</tr>
</tbody>
</table>
Gene mutation landscape of *de novo* NK-AML

From the patients mentioned above, the NGS analysis for each BM sample was performed under strict quality control. The NGS analysis of 347 patients identified 715 mutations, 57 non-sense mutations, and 658 other mutations, namely non-synonymous point mutations (missense \( n = 298 \)), insertions/deletions (in-frame \( n = 174 \) or causing a frameshift \( n = 162 \)), and splice site mutations \( n = 24 \). The number of molecular variations per patient ranged from 0 to 6, with a median of 2. For 38 patients, no mutations were detected using the applied gene panel. The mutation frequency and clinical characteristics are shown in Figure 1. The most common NK-AML-related mutations were found in CEBPA (27.66%), including CEBPA biallelic mutations (18.15%) and a CEBPA single mutation (9.51%), followed by NPM1 (22.48%), DNMT3A (20.46%), TET2 (21.61%), FLT3-ITD (17.58%), NRAS/KRAS (15.56%), IDH1 (8.36%), and IDH2 mutations (6.92%). We also explored the correlation between different gene mutations. DNMT3A and FLT3-ITD mutations occurred more commonly in patients with mutant NPM1 \( r = 0.45 \) and 0.22, respectively; all \( P < 0.001 \); Figure 2a). IDH1 mutations also often co-occurred with alterations in NPM1 \( r = 0.19, P < 0.001 \). RUNX1 was associated with mutations in EZH2, PHF6, and SF3B1 \( r = 0.31, 0.26, \) and 0.23, respectively; all \( P < 0.001 \). CEBPA double mutations and the NPM1 mutation were mutually exclusive \( r = -0.24, P < 0.001 \).

Potential of GMS for determining NK-AML relapse
To determine suitable predictors for NK-AML relapse, the above gene mutations were assessed at diagnosis. Based on the 10-fold cross-validation via minimum criteria, nine coefficients were selected as the vertical lines shown in Figure 2b. The final optimal selection genes included NPM1, KIT, CEBPA double, FLT3-ITD, RUNX1, TP53, ETV6, ZRSR2, and JAK2. The GMS was determined, and the correlation weights are shown in Table 2. NPM1 and CEBPA double mutations were protective variables for NK-AML relapse (\(P = 0.026\) and 0.002; hazard ratio [HR] = 0.477 [0.248–0.918] and 0.266 [0.111–0.632], respectively), whereas KIT, FLT3-ITD, RUNX1, ETV6, and JAK2 mutations were risk variables for relapse (\(P < 0.001, < 0.001, 0.018, 0.004,\) and \(< 0.001; \text{HR} = 3.478 [1.672–7.234], 2.768 [1.611–4.757], 2.567 [1.170–5.628], 5.886 [1.744–19.857], \)and \(8.200 [2.896–23.213], \)respectively); TP53 and ZRSR2 mutations were not significant variables for relapse (\(P = 0.145\) and 0.106, respectively).

### Table 2. Genomic mutation signature (GMS) calculation formula.

<table>
<thead>
<tr>
<th>Mutated genes</th>
<th>Correlation coefficient</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPM1</td>
<td>-0.7390</td>
<td>0.026*</td>
</tr>
<tr>
<td>KIT</td>
<td>1.2467</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>CEBPA double</td>
<td>-1.3238</td>
<td>0.002*</td>
</tr>
<tr>
<td>FLT3-ITD</td>
<td>1.0184</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>RUNX1</td>
<td>0.9428</td>
<td>0.018*</td>
</tr>
<tr>
<td>TP53</td>
<td>0.7860</td>
<td>0.145</td>
</tr>
<tr>
<td>ETV6</td>
<td>1.7726</td>
<td>0.004*</td>
</tr>
<tr>
<td>ZRSR2</td>
<td>0.7214</td>
<td>0.106</td>
</tr>
<tr>
<td>JAK2</td>
<td>2.1042</td>
<td>&lt; 0.001*</td>
</tr>
</tbody>
</table>

*\(P < 0.05\)*.

We evaluated patient relapse and survival based on GMS. For the 209 patients in the training cohort, the 5-year cumulative incidence of relapse was higher in the GMS-high than in the GMS-low group (72.41% vs. 24.60%, \(\text{HR} = 4.093 [2.100–7.977], P < 0.001; \)Figure 3a). The 5-year DFS and OS were lower in the GMS-high group than in the GMS-low group (23.42% vs. 63.83%, and 25.27% vs. 71.06%; \(\text{HR} = 3.142 [1.771-5.576] \)and 4.093 [2.100–7.977], respectively; each \(P < 0.001; \)Figure 3b and c). The 5-year incidence of relapse was higher (71.97% vs. 16.93%, \(\text{HR} = 4.993 [2.293–10.870], P < 0.001; \)Figure 3d), whereas the 5-year DFS and OS were lower in the GMS-high-group than in the GMS-low-group of the validation cohort (24.76% vs. 78.41% and 21.84% vs. 82.30%; \(\text{HR} = 4.063 [2.049–8.058] \)and 4.761 [2.283–9.930]; \(P < 0.001 \)and 0.001, respectively; Figure 3e and f). Our results also indicated that FLT3-ITD-high status was associated with a higher 5-year relapse rate than FLT3-ITD-low and wild-type status according to the mutation allelic ratio in all patients (100% vs. 34.5% vs. 35.0%, \(P < 0.001; \)Additional File.
Furthermore, the FLT3-ITD-high subgroup included more patients with high GMS than the FLT3-ITD-low and wild-type subgroups (26/26 vs. 29/35 vs. 47/239, \( P < 0.001 \); Additional File Figure 2b).

Univariate analysis of the training cohort revealed that GMS-high, Cycle3rd in non-CR, MRD3rd-positive, chemotherapy consolidation, high white blood cell count, unfavorable and intermediate risk, and sAML were all risk factors for relapse \( (P < 0.001, < 0.001, < 0.001, < 0.001, 0.020, 0.046 \text{ and } 0.002, \text{ respectively}; \) Table 3). In multivariate Cox analysis, GMS-high, Cycle3rd in non-CR, MRD3rd positivity, and sAML were also independent risk factors for relapse \( (\text{HR} = 6.92 [3.35–14.30], 2.27 [1.35–3.84], 2.44 [1.26–4.73] \text{ and } 3.43 [1.65–7.11]; P < 0.001, 0.002, 0.008 \text{ and } < 0.001, \text{ respectively}) \). Allo-HSCT/auto-HSCT and favorable risk were independent protective factors for relapse \( (0.54 [0.37–0.78] \text{ and } 0.52 [0.31–0.89]; P<0.001 \text{ and } 0.017, \text{ respectively}) \). In the multivariate Cox analysis of DFS and OS, GMS was also a significant independent predictor \( (\text{HR} = 3.09 [1.85–5.17] \text{ and } 3.40 [1.90–6.06], \text{ respectively}; \text{ each } P < 0.001; \) Additional File Table 1).

Table 3. Univariate and multivariate analysis of cumulative relapse in the training cohort
<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Age (&gt; 40 vs. ≤ 40)</td>
<td>1.71 (0.98-2.98)</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Gender (male vs. female)</td>
<td>1.22 (0.70-2.13)</td>
<td>0.457</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Risk category (favorable vs. unfavorable/intermediate)</td>
<td>0.17 (0.07-0.42)</td>
<td>0.046*</td>
</tr>
<tr>
<td>WBC (≥100 vs. &lt; 100) ×10^9/L</td>
<td>2.60 (0.72-9.34)</td>
<td>0.020*</td>
</tr>
<tr>
<td>sAML (Yes vs. No)</td>
<td>2.67 (1.04-6.86)</td>
<td>0.002*</td>
</tr>
<tr>
<td>Cycle1st (non-CR vs. CR)</td>
<td>1.87 (0.91-3.81)</td>
<td>0.035*</td>
</tr>
<tr>
<td>Cycle2nd (non-CR vs. CR)</td>
<td>1.49 (0.54-4.05)</td>
<td>0.351</td>
</tr>
<tr>
<td>Cycle3rd (non-CR vs. CR)</td>
<td>5.69 (1.53-21.07)</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>MRD1st (positivity vs. negativity)</td>
<td>1.63 (0.85-3.12)</td>
<td>0.194</td>
</tr>
<tr>
<td>MRD2nd (positivity vs. negativity)</td>
<td>1.40 (0.80-2.42)</td>
<td>0.237</td>
</tr>
<tr>
<td>MRD3rd (positivity vs. negativity)</td>
<td>2.75 (1.58-4.79)</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>Treatment (allo-HSCT/auto-HSCT vs. chemotherapy)</td>
<td>0.33 (0.19-0.58)</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>GMS (high vs. low)</td>
<td>4.09 (2.10-7.97)</td>
<td>&lt; 0.001*</td>
</tr>
</tbody>
</table>

Allo-HSCT, allogeneic hematopoietic stem cell transplantation; Auto-HSCT, autologous HSCT; CI, confidence interval; CR, complete remission; Cycle1st~3rd, Response after the 1st~3rd chemotherapy cycle, respectively; GMS, genomic mutation signature; HR, hazard ratio; MRD, measurable residual disease; MRD1st~3rd, MRD after the 1st~3rd chemotherapy cycle, respectively; NA, not available; non-CR, partial remission + no remission; sAML, secondary acute myeloid leukemia. *P < 0.05.

**Establishment of a prognostic model that combines GMS, MRD, and clinical characteristics**

To further improve predictive accuracy, we established GMSN, a comprehensive nomogram that combines GMS, MRD, and clinical characteristics. We quantified the degree of agreement between the actual and predicted relapse in the training cohort, divided into GMSN-low, -intermediate, and -high groups.
(Figure 4). The detailed formula for the GMSN calculation is shown in Additional File Table 2. The 5-year cumulative incidence of relapse was higher in the GMSN-high group than in the GMSN-intermediate and -low groups (100.00% vs. 48.63% vs. 8.70%, \( P < 0.001 \); Figure 5a). Furthermore, the 5-year DFS and OS were lower in the GMSN-high group than in the GMSN-intermediate and -low groups (00.00% vs. 43.20% vs. 79.25% and 00.00% vs. 50.30% vs. 86.15%, \( P < 0.001 \) and < 0.001, respectively; Figure 5b and c).

Likewise, in the validation cohort, the 5-year incidence of relapse was higher in the GMSN-high group than in the GMSN-intermediate and -low groups (91.11% vs. 49.90% vs. 7.46%, \( P < 0.001 \); Figure 5d), whereas the 5-year DFS and OS were lower (8.88% vs. 45.68% vs. 85.05% and 8.33% vs. 54.24% vs. 86.01%, \( P < 0.001 \) and < 0.001, respectively; Figure 5e and f). To evaluate the predictive power of the model, we presented the morphology and MRD (at the time of relapse) of two relapsed patients with AML-M2, with high GMS and GMSN scores (Additional File Figure 3a and 3b).

**Discussion**

Over the past 10 years, the application of NGS and gene expression profiling has fostered the identification of an increasing number of genetic alterations with prognostic value in AML(6, 7, 25). Although the prognostic impact of various single markers has been established, little is known regarding the interaction of these risk factors and their cumulative effect on NK-AML outcome. The aims of this study were to: (1) evaluate the role of gene mutations and clinical characterization in predicting the prognosis (especially relapse) of patients with NK-AML (14-60 years of age); and (2) build and validate a GMSN prognostic system for these patients. In this study, based on the analysis of 347 intensively treated patients with NK-AML, we established the GMS model for AML relapse prediction. A high GMS score was independently associated with relapse in patients with NK-AML. Furthermore, we combined clinical factors, MRD, and GMS to generate the prognostic GMSN, which exhibited an improved accuracy of prognostic classification. We found that the incidence of relapse was higher in the GMSN-high than in GMSN-low and -intermediate groups. The GMSN stratified patients based on relapse risk, highlighting the importance of molecular and clinical factors and their interaction with other risk factors.

Various models for AML prognosis have been reported, but most were focused on specific AML cohorts. For example, patients with specific DNA methylation signatures, CEBPA double mutations, or FLT3-ITD mutations(26-28). For NK-AML, a previous study indicated that prognostic indices discriminated among low-, intermediate-, and high-risk patients, without considering the effect of HSCT and MRD as only NPM1, CEBPA, and FLT3 gene mutations were analyzed(8). During AML development, single gene mutations might be affected by individual differences, leukemia heterogeneity, or different consolidation strategies. Moreover, the interaction between multiple genes, such as co-mutations and mutually exclusive mutations, cannot be ignored and could be related to treatment response and relapse. Thus, we assessed the correlations among mutations and found that DNMT3A and FLT3-ITD mutations appeared more commonly in patients harboring mutated NPM1, which is consistent with a recent report(29). RUNX1 mutations were associated with those of EZH2 and SF3B1, which agrees with recent findings(29, 30). CEBPA double mutations were mutually exclusive with NPM1, which is in line with a previous finding(31). These observations support the paradigm of how co-occurring variations can influence
prognosis beyond the effects of a single mutation(29, 32, 33) and indicate the importance of refining NK-AML molecular characterization.

To further evaluate sequencing data for the prediction of relapse, we performed bioinformatics analysis for identifying new biomarkers for risk group classification, and applied the LASSO algorithm to select the best panels of mutational genes for prognostic prediction. Finally, nine gene mutations were included in the GMS; several genetic alterations have been confirmed to affect clinical outcomes in AML(9, 29, 34). Based on transcriptional profiling, a previous study used LASSO to predict initial induction resistance and to develop prognostic biomarkers for AML(35). Moreover, a recent study suggested that a prognostic model based on the immune marker score predicts OS in NK-AML(36). Notably, the cut-off values of transcriptional data frequently limit clinical practice, especially across centers. Unlike the analysis of RNA-sequencing data sets (Gene Expression Omnibus) conducted in the studies described above, we employed NGS to establish the GMS, which was significantly associated with the incidence of relapse. Our model mainly focused on the mutational status (yes or no) of each gene, allowing an easy calculation of GMS based on only nine genes. Importantly, our GMS was an independent prognostic factor for relapse as well as DFS and OS, which was convenient and efficient in discriminating the different outcomes of NK-AML. In addition, we found that the FLT3-ITD-high status was associated with a higher incidence of relapse than the FLT3-ITD-low and wild-type status, and FLT3-ITD-low patients might benefit from allo-HSCT, which is consistent with findings from recent studies(37, 38). MRD is a crucial biomarker in AML and is applied in prognostic monitoring(39, 40). Getta et al. reported that both multicolor flow cytometry and NGS can be used to monitor MRD for AML relapse prediction, but clinical factors were not considered(41).

Another study proposed a prognostic model for the prediction of 3-year OS, with an AUC of 0.74, without considering the MRD status or treatment choice (including HSCT) and analyzing only a few gene mutations(17). Damm et al. proposed a model for NK-AML prognosis that includes several gene mutations and clinical characteristics but lacks treatment response analysis(42). To further improve the performance of genomic analysis-based models, we combined GMS, MRD, and clinical features to build GMSN for relapse prognosis. We recognize that it is crucial to consider both the risk category of the European Leukemia Net guideline and therapy response when applying GMSN scores, so the selected clinical features included risk category, Cycle3rd status, MRD3rd, and consolidation treatment choice. This study demonstrated that the combination of gene mutations, MRD, and treatment choice successfully differentiated high-, intermediate-, and low-risk of relapse patients. Therefore, GMSN has potential as a predictive tool for NK-AML outcomes.

Nevertheless, this study had some limitations. First, we mainly used NGS and clinical data to establish the predictive model for NK-AML relapse. Multi-omics, including DNA methylation analysis and proteomics, should be considered in future studies. Second, our study was a pilot study and focused only on the application of NGS. A larger prospective multicenter study should be performed to extensively validate the prognostic value of GMS/GMSN.
Conclusions

This study established the potential of NGS-based GMS scoring as a novel and robust biomarker for the prediction of relapse in NK-AML. The GMSN that integrates GMS, MRD, and clinical characteristics exhibited even greater predictive potential.

Abbreviations

AML, Acute myeloid leukemia; allo-HSCT, allogeneic hematopoietic stem cell transplantation; BM, bone marrow; CR, complete remission; DFS, disease-free survival; GMS, genomic mutation signature; GMSN, genomic mutation signature nomogram; LASSO, least absolute shrinkage and selection operator; MRD, measurable residual disease; NGS, next-generation sequencing; NK-AML, normal karyotype acute myeloid leukemia; NR, no remission; OS, overall survival; PR, partial remission; sAML, secondary acute myeloid leukemia

Declarations

Ethics approval and consent to participate

The study was performed in accordance with the Helsinki Declaration and was approved by the ethical committees of the First Affiliated Hospital of Zhengzhou University, Henan Cancer Hospital, the First Affiliated Hospital of Xinxiang Medical University, and the Huaihe Hospital of Henan University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Jointly Sponsored Project of Henan Medical Science and Technology Research Plan (grant number LHGJ20190040); the National Nature Science Foundation of China (grant numbers 82270225, and 82060327); the Key Scientific Research Projects of Colleges and Universities in Henan Province (grant number 20B320048); the Medical Science and Technology Project of Henan Province (grant number SB201901106); the National Natural Science Foundation of China (grant number 82000163); the Open Funds of State Key Laboratory of Oncology in South China (grant number HN2020-
the Qian Dong Nan Science and Technology Program (grant number qdnkhJz2020-013); and the Science and Technology Foundation of Guizhou Province (grant numbers Qian ke he ji chu-ZK 2021, yi ban 454).

Authors’ contributions

LH, JP, and ZJ designed the study. LH, JP, XL, and JW wrote the manuscript. HZ, XH, WW, MZ, CL, WF, JZ, and LS collected samples and recorded the clinical data. JP, JF, YL, ZB, WL, CH, HS, and HC performed the data analysis. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Editage (www.editage.cn) for English language editing.

References


Figures
Gene mutation landscape and clinical characteristics of *de novo* normal karyotype AML. Each column denotes a patient. Clinical characteristics or different subgroups are color coded and displayed in the upper part: from the first row to the last row, each plot represents a patient's age, gender, risk category, WBC, MRD1st, MRD2nd, MRD3rd, treatment choice, CR status of cycle1st, cycle2nd, cycle3rd, training and validation cohorts, and relapse status, respectively. Gene mutations are indicated in red, whereas the wild type is shown in green in the lower half of the image. The frequency of gene mutations is presented in the middle column on the right. Chemo, chemotherapy consolidation; CR, complete remission; Cycle1st-3rd, response after the 1st~3rd chemotherapy cycle, respectively; GMS, genomic mutation signature; MRD, measurable residual disease; MRD1st-3rd, MRD after the 1st-3rd chemotherapy cycle, respectively; sAML, secondary acute myeloid leukemia; WBC, white blood cell.
Figure 2

Correlations between gene mutations and the origin procedure of the gene model. (a) Correlations between different gene mutations and correlation coefficient in each code. (b) Tuning parameters for gene mutation selection in the LASSO regression model. LASSO, least absolute shrinkage and selection operator.
Figure 3

Outcomes of patients with NK-AML based on GMS. Relapse (a, d), DFS (b, e), and OS (c, f) of patients with NK-AML in the training and validation cohorts according to the GMS score (2-sided log-rank test). DFS, disease-free survival; GMS, genomic mutation signature; NK-AML, normal karyotype AML; OS, overall survival.
Figure 4

Nomogram model combining GMS, MRD, and clinical characteristics to predict the probability of relapse. Cycle3rd, Response after the 3rd chemotherapy cycle; GMS, genomic mutation signature; MRD3rd, measurable residual disease after the 3rd chemotherapy cycle; sAML, secondary acute myeloid leukemia.
Figure 5

Performance of the GMSN model. Efficiency of the GMSN model in predicting relapse (a, d), DFS (b, e), and OS (c, f) in the low-, intermediate-, and high-score groups from the training and validation cohorts (2-sided log-rank test). GMSN: genomic mutation signature nomogram.

Supplementary Files
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- AdditionalFile.docx