Risk Factors Affect Accurate Prognosis in ASXL1-mutated Acute Myeloid Leukemia

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Abstract

Background: The epigenetic regulator additional sex combs-like 1 (ASXL1) is an adverse prognostic factor in acute myeloid leukemia (AML). However, the mutational spectrum and prognostic factors of ASXL1-mutated (ASXL1+) AML are largely unknown. We aim to evaluate the risk factors influencing the prognosis of ASXL1+ AML.

Methods: We performed next-generation sequencing (NGS) in 1047 cases of de novo AML and discovered 91 ASXL1+ AML (8.7%). The Log-Rank test and Kaplan-Meier were used to evaluate survival rate, and the Cox regression model was used to analyze multivariate analysis.

Results: In a total of 91 ASXL1+ AML, 86% had one or more co-mutations. The factors that had adverse impact on overall survival (OS) and event-free survival (EFS) are defined as high risk factors, including age ≥60 years, WBC count ≥50×10⁹/L, FLT3-ITD mutations, RUNX1 mutations, and absence of AML1-ETO fusion gene. ASXL1 mutations without any risk factor were classified as single-hit ASXL1+ AML; ASXL1 mutations accompanied with one of the risk factors was referred to be double-hit ASXL1+ AML; ASXL1 mutations with two or more of the risk factors were designated triple-hit ASXL1+ AML. The combination of these risk factors had a negative influence on the prognosis of ASXL1+ AML. The median OS was not attained in single-hit ASXL1+ AML, 29.53 months in double-hit ASXL1+ AML, and 6.67 months in triple-hit ASXL1+ AML (P=0.003). The median EFS was not attained in single-hit ASXL1+ AML, 29.53 months in double-hit ASXL1+ AML, and 5.47 months in triple-hit ASXL1+ AML (P=0.002). Allogenic hematopoietic stem cell transplantation (allo-HSCT) improved the prognosis of double/triple-hit ASXL1+ AML patients.

Conclusion: Our study provided new insights into the mutational spectrum and prognostic factors of ASXL1+ AML patients. Our primary data suggest that the risk factors in ASXL1+ AML contribute to the poor outcome of these patients. The management of ASXL1+ AML patients should be based on the risk factors and allo-HSCT is highly recommended for consolidation.

Background

Acute myeloid leukemia (AML) is a group of hematological malignancies with high heterogeneity [1, 2]. Advances in individualized induction regimens with targeted agents and hematopoietic stem cell transplantation (HSCT) for consolidation have significantly improved the results of AML patients. However, the prognosis in some AML cases remains unsatisfactory. Recurrent chromosomal abnormalities and gene mutations have been implicated in leukemogenesis and are employed in the clinic for risk-adopted AML therapy[3]. The favorable risk factors are t(8;21), inv(16)/t(16;16), t(15;17), and CEBPA double mutations and NPM1 mutations, and the adverse risk factors are t(9;22) and mutations in FLT3-ITD, RUNX1, and AXSL1[4, 5]. It has been established that not all molecular alterations have prognostic and therapeutic implications in AML. The mutations of CEBPA showed a favorable prognostic impact on AML only when the mutation occurred at both alleles[6]. The FLT3-ITD mutations
had a negative prognostic impact when the ratio of the mutant allele to wild alleles was more than 0.5[7, 8]. The beneficial prognostic effects of t(8;21) and inv(16)/t(16;16) can be reversed by co-occurring with c-KIT mutations[9], and the same goes for NPM1 co-occurring with FLT3-ITD mutations[10]. Agents that target mutations, such as midostaurin on FLT3-ITD, can rescue patients from unfavorable outcomes[11, 12]. Based on the understanding of gene mutations in the prognosis of AML, hematologists have used innovative and targeted agents in chemotherapy to improve the outcome of these patients[13]. However, some patients may have multiple gene mutations or risk factors simultaneously. The interaction between mutated genes and other risk factors may affect the prognosis of AML patients. For instance, coexistence of ASXL1 and SRSF2 mutations may increase the risk of death in AML patients[14]. Therefore, it is crucial to make precise risk stratification to guide the managements of AML patients. Further research is needed to determine the interaction of co-mutated genes and clinical risk factors in patients carrying certain mutations, such as ASXL1, on the prognosis and treatment options.

ASXL1 is the human homologue of the Drosophila Additional sex combs (Asx)[2]. The ASXL family consists of three members (ASXL1, ASXL2, and ASXL3) with conserved domain structures consisting of ASXN, ASXH, ASXM1, ASXM2, and a PHD finger[15]. ASXL1 encodes a chromatin binding protein of the polycomb group and trithorax complex family[16, 17], which may be involved in epigenetic regulation. ASXL1 is located on chromosome 20q11. ASXL1 acts as a coactivator for the retinoid receptors retinoic acid receptor (RAR) and retinoid X receptor through binding with steroid receptor coactivator-1[18]. Moreover, ASXL1 also cooperates with heterochromatin protein-1 and histone H3 demethylase LSD1 to regulate histone methylation and repress retinoic acid-receptor activity[19]. Germ-line mutations of ASXL1 and ASXL3 can be seen in individuals with congenital abnormalities, such as Bainbridge–Ropers syndrome, while somatic truncation mutants of all three ASXL family members are found in human cancer[15]. ASXL1 is frequently mutated in patients with different types of myeloid malignancies, including myelodysplastic syndromes (MDS), myeloproliferative neoplasms, chronic myelomonocytic leukemia, and AML with MDS-related alterations[20]. ASXL1 mutations are commonly associated with aggressive behaviors and a poor clinical prognosis across the spectrum of malignant myeloid diseases[21]. In mouse model experiments, ASXL1 silencing together with oncogenic NRasG12D generates hepatosplenomegaly and progressive anemia, emphasizing ASXL1’s function in myeloid malignancies[2]. Although the adverse prognostic value of ASXL1 mutations in AML is obvious, previous studies often focused on comparing the difference between mutated and wild-type ASXL1 patients. However, the impact of other factors such as variant allele frequency (VAF) and companion gene mutations (co-mutations) on the prognosis of ASXL1+ AML needs to be evaluated.

In this study, we comprehensively investigated the mutational spectrum and prognostic factors of ASXL1+ AML. We also analyzed the interaction of molecular profiles of gene mutation and clinical risk factors on the survival of ASXL1+ AML patients. Our data demonstrated that the addition of risk factors to ASXL1 mutations were associated with the adverse outcome of AML patients. Meanwhile, Allo-HSCT and AML/ETO fusion gene improved the survival of ASXL+ AML patients. In conclusion, our data provide new evidence for precise risk stratification and optimal treatments of ASXL+ AML.
Subjects And Methods

Patients

Between May 2016 and January 2020, 1047 cases of de novo AML were examined with next-generation sequencing (NGS) at the First Affiliated Hospital of Zhengzhou University. A total of 91 cases with ASXL1+ AML were identified and included in the research. The WHO 2016 edition of classification of myeloid neoplasms and acute leukemia [22] was used to make the diagnosis and classification of AML. According to the 2017 ELN guideline for adult acute myeloid leukemia[4], patients were categorized into three risk groups: favorable-risk, intermediate-risk, and adverse-risk. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Following the Declaration of Helsinki, all patients or their legal guardians gave their informed permission.

Treatment protocols

All-trans retinoic acid and arsenic trioxide-based chemotherapy was used for induction and consolidation therapy in individuals with acute promyelocytic leukemia (APL). Induction chemotherapy regimens for non-APL patients included the DA, IA, and MA regimens, which consisted of a standard dose of cytarabine (Ara-C; 100mg/m^2/day for 7 days) combined with daunorubicin (60 mg/m^2/day for 3 days) or idarubicin (12 mg/m^2/day for 3 days) or mitoxantrone (10 mg/m^2/day for 3 days). Patients were given cytarabine (2–3g/m^2, once every 12 h for 3 days)-based chemotherapy after remission. The chemotherapy consolidation for older patients was chosen on an individual basis by the specialists. As part of the consolidation process, 12 patients received allo-HSCT. The actual therapy was chosen based on both the doctor's suggestion and the patient's desire. The final follow-up for surviving patients occurred in January 2021.

Fusion genes detection

Fresh bone marrow samples were collected using an Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulant tube. Mononuclear cells were extracted by density gradient centrifugation. RNA was extracted using the standard TRIzol technique (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Fusion genes were detected by real-time quantitative PCR using Multiplex RT-PCR Fusion Gene Kits (Rightongene, Shanghai, China). A panel of forty-three fusion genes was screened, including MLL-(AF4, AF6, AF9, AF10, AF17, AF1q, AF1p, AFX, ELL, SEPT6, ENL), NUP98-(HoxA11, HoxA13, PMX1), (NPM, F1P1L1, PML, PRKAR1A, STAT5b, NUMA1, PLZF)-RARα, (ETV6, FIPIL1)-PDGFRA, AML1-(ETO, MTG16, MDS1/EV11), TEL-(JAK2, AML1, ABL), NPM-(ALK, MLF), (DEK, SET)-CAN, SIL-TAL1, E2A-HLF, TEL-PDGFRB, TLS-ERG, CBFβ-MYH11, BCR-ABL, E2A-PBX1.
Next-generation sequencing

The mutational hotspots or whole coding regions of 22 genes were assessed by next-generation sequencing, including FLT3, NPM1, KIT, CEBPA, DNMT3A, IDH1, IDH2, TET2, EZH2, RUNX1, ASXL1, PHF6, TP53, SF3B1, SRSF2, U2AF1, ZRSR2, NRAS, CBL, SETBP1, ETV6, and JAK2. The detection was performed utilizing a Rightongene AML/MDS/MPN Sequencing Panel (Rightongene, Shanghai, China) on an Illumina MiSeq System (Illumina, San Diego, CA) high-throughput sequencing platform. The original data after sequencing was analyzed by bioinformatics using NCBI, CCDS, dbSNP (v138), COSMIC, human genome database (HG19) and other databases to determine the pathogenic mutation site. The average depth of the sequencing was 4837.978Kb, detection sensitivity was ~ 5%. Details on variant calling, filtering, and annotation are detailed in our recently published reports[23].

Statistical analysis

SPSS software version 26.0 (Chicago, IL, USA) and GraphPad Prism™ 8.01(San Diego, California, USA) were used for the analyses. Overall survival (OS) is defined as the time from diagnosis to death or the time of the last follow-up. Event-free survival (EFS) is defined as the time from diagnosis to relapse, death, or the time of the last follow-up. The Kaplan-Meier method was used for survival analysis, and the Log-rank test was utilized to assess differences between groups. Multivariable analysis including variables with $P<0.05$ in univariate analysis were performed for OS and EFS. A two-sided $P<0.05$ was regarded as statistically significant.

Results

Clinical features of ASXL1 + AML patients

ASXL1 mutations were found in 8.7% (91 of 1047) of the patients in the whole cohort. The median age of the patients was 50 (33–58) years, with 20 cases older than 60 years and 49 cases being male, as indicated in Table 1. The median white blood cell (WBC) count was 7.5 (2.4–33.3) $\times 10^9$/L, with 18 cases (19.78%) having a value of $\geq 50 \times 10^9$/L. Bone marrow blast percentage of more than 80% was seen in 15 cases (16%). According to the 2017 ELN risk criteria, 27 cases (20%) were favorable-risk AML (including 4 cases of APL), 1 case (1%) was intermediate-risk AML, and 63 cases (69%) were adverse-risk AML. Allo-HSCT was applied in 12 patients (13%). Three cases died within 30 days after induction therapy, and 50 cases (63%) died at the end of the follow-up.
Table 1
Clinical characteristics of ASXL1 + AML

| Characteristics                          | Median (interquartile range) or N (%) |
|------------------------------------------|--------------------------------------|
| Gender male(n[ ])                       | 49(54%)                              |
| Age (years)                              | 50(33–58)                             |
| Age ≥ 60 years(n[ ])                     | 20 (22%)                              |
| Type (APL vs. non-APL)                   | 4(4%)                                |
| ASXL1 type(n[ ])                         |                                      |
| G652S                                    | 38(41.76%)                           |
| G642fs                                   | 11(12.09%)                           |
| H630fs                                   | 8 (8.79%)                             |
| ASXL1 VAF(%)                             | 49.17 (22.9-57.11)                   |
| ASXL1 VAF (≥ 49.17%)                     | 46(51%)                              |
| WBC counts (× 10⁹/L)                     | 7.5 (2.4–33.3)                        |
| WBC counts (≥ 50 × 10⁹/L)                | 18(19.78%)                           |
| HGB counts (g/L)                        | 79(66–93)                             |
| HGB counts (≥ 110 g/L)                   | 13 (14%)                             |
| PLT counts (× 10⁹/L)                     | 48 (20–93)                            |
| PLT counts (≥ 100 vs × 10⁹/L)            | 19 (21%)                             |
| Bone marrow blasts (%)                   | 51 (26–72)                            |
| Bone marrow blasts (≥ 80%)               | 15(16%)                              |
| AML1-ETO                                 | 17 (19%)                             |
| CBFβ-MYH11                               | 3(3%)                                |
| Risk group                               |                                      |
| Favorable                                | 27 (30%)                             |
| Intermediate                             | 1 (1%)                                |
| Adverse                                  | 63(69%)                              |

APL, acute promyelocytic leukemia; VAF, variant allele frequency; WBC, white blood cell; HGB, hemoglobin; PLT, platelet.
The molecular mutations of \textit{ASXL1} were detected in 30 different nucleotide sites, all of which were located in exon 12, including G652S (41.76%), G642fs (12.09%)\textsuperscript{,} H630fs (8.79%)\textsuperscript{,} S1231F and R693X (5.49%)\textsuperscript{,} N986S (4.40%)\textsuperscript{,} T1139K (3.33%)\textsuperscript{,} G643fs and Y591X (2.20%). The distribution of all nucleotide sites was shown in Additional file 1. Most of the patients carried a single-point mutation, 7 (7.69%) patients carried two-point mutations, and one patient carried three-point mutations (G642fs, G643fs and G645fs). The median VAF value of \textit{ASXL1} mutation was 49.17% (1.02%-79.28%).

\textbf{Companion gene mutations and fusion genes in ASXL1 + AML patients}

One or more co-mutation of genes was observed in 83 patients (86.46%) of \textit{ASXL1} + AML (Fig. 1). \textit{TET2} had the highest mutation frequency (48.35%), followed by \textit{U2AF1} (16.48%), \textit{CEBPA} (15.38%), \textit{NRAS} (14.29%), \textit{FLT3-ITD} (13.19%), \textit{DNMT3A} (10.99%), \textit{IDH2} (8.79%), \textit{RUNX1} (7.69%), \textit{KIT} (6.59%), \textit{SRSF2} (5.49%). Other mutant genes (including \textit{FLT3-TKD}, \textit{ETV6}, \textit{IDH1}, \textit{CBL}, \textit{SETBP1}, \textit{NPM1}, \textit{TP53}, \textit{EZH2}, \textit{SF3B1}, \textit{JAK}) are found in fewer than 5% of \textit{ASXL1} + AML patients; \textit{PHF6} and \textit{ZRSR2} mutations are not seen in \textit{ASXL1} + AML patients.

The fusion genes were screened in 83 of 91 \textit{ASXL1} + AML cases. There were 31 cases (37.35%) with fusion gene mutations, including \textit{AML1-ETO} in 17 cases (20.48%), \textit{PML-RARα} in 4 cases (4.82%), \textit{BCR-ABL}, \textit{MLL-AF9} and \textit{CBFB-MYH11} in 3 cases (3.61%), \textit{MLL-ELL} in 1 case (1.20%). The remaining 52 cases (62.65%) were with negative fusion genes.

\textbf{Risk factors on the prognosis of ASXL1 + AML}

In order to understand the prognostic impacts of clinical features and molecular profiles on the outcomes of \textit{ASXL1} + AML patients, we analyzed the risk factors on OS and EFS including gender (female vs. male), age (≥ 60 vs. < 60 years), \textit{ASXL1} nucleotide sites, \textit{ASXL1} VAF (≥ 49.17% vs. < 49.17%), WBC counts (≥ 50 vs. < 50 × 10\textsuperscript{9}/L), HGB (≥ 110 vs. < 110 g/L), PLT counts (≥ 100 vs. < 100 × 10\textsuperscript{9}/L), bone marrow blasts (≥ 80% vs. < 80%), peripheral blood blasts (≥ 20% vs. < 20%), allo-HSCT (yes vs. no), risk stratification (adverse vs. inter/favorable-risk), \textit{AML1-ETO} fusion gene (positive vs. negative), \textit{CBFB-MYH11} fusion gene (positive vs. negative), and the mutation status of other common AML co-mutation genes. The median follow-up time was 12.93 (0.37–53.53) months. Table 2 and Additional file 2A revealed that older patients (age ≥ 60 years) had a shorter OS ($P$ = 0.034). Higher WBC counts (≥ 50 × 10\textsuperscript{9}/L) were associated with a shorter OS ($P$ = 0.035, Additional file 2C) and EFS ($P$ = 0.006, Additional file 2D). Cases who accepted allo-HSCT had a longer OS ($P$ = 0.024, Additional file 2E) and a better EFS ($P$ = 0.013, Additional file 2F). The adverse risk group had a lower OS ($P$ = 0.005) and EFS ($P$ = 0.004). \textit{AML1-ETO} coexistence was related to a prolonged OS ($P$ = 0.010, Additional file 3A) and EFS ($P$ = 0.013, Additional file 3B). \textit{FLT3-ITD} co-mutation was related to a shorter OS ($P$ < 0.001, Additional file 3C) and EFS ($P$ <
0.001, Additional file 3D). However, neither the \textit{ASXL1} mutation sites nor the \textit{ASXL1} VAF had impacts on EFS or OS.
Table 2
Comparison of EFS and OS between different clinical and molecular characteristic groups in ASXL1 + AML.

| Variables                          | OS  |          |          | EFS |          |
|------------------------------------|-----|----------|----------|-----|----------|
|                                    | χ²  | P-value  | χ²       | P-value |
| Sex (female vs. male)              | 0.69| 0.406    | 0.719    | 0.395|
| Age (≥ 60 vs. <60 years)           | 4.513| 0.034   | 2.96     | 0.085|
| ASXL1 type(n[%])                   |     |          |          |      |
| G652S                              | 0.911| 0.34    | 1.528    | 0.216|
| G642fs                             | 1.243| 0.265   | 1.737    | 0.188|
| H630fs                             | 0.592| 0.442   | 0.214    | 0.643|
| ASXL1 VAF (≥ 49.17% vs.<49.17%)    | 0.005| 0.944   | 0.344    | 0.557|
| WBC counts (≥ 50 vs. <50 × 10⁹/L)  | 4.471| 0.035   | 7.564    | 0.006|
| HGB counts (≥ 110 vs. <110 g/L)    | 0.131| 0.717   | 0.085    | 0.77 |
| PLT counts (≥ 100 vs. <100 × 10⁹/L)| 1.216| 0.27    | 2.674    | 0.102|
| bone marrow blasts (≥ 80% vs.<80%) | 0.611| 0.434   | 0.364    | 0.546|
| peripheral blasts (≥ 20% vs. <20%) | 1.242| 0.537   | 1.939    | 0.379|
| risk group (high-risk vs. low/inter)| 7.719| 0.005   | 8.231    | 0.004|
| allo-HSCT (yes vs.no)              | 5.066| 0.024   | 6.105    | 0.013|
| AML1-ETO (positive vs. negative)   | 6.583| 0.01    | 6.229    | 0.013|
| CBFβ-MYH11 (positive vs. negative) | 0    | 0.993   | 0.018    | 0.894|
| TET2 (mutated vs. wild type)       | 0.738| 0.39    | 1.206    | 0.272|
| FLT3-ITD (positive vs. negative)   | 14.081| 0       | 11.395   | 0.001|
| U2AF1 (mutated vs. wild type)      | 3.056| 0.08    | 2.273    | 0.132|
| CEBPA (mutated vs. wild type)      | 0.954| 0.329   | 0.476    | 0.49 |
| NRAS (mutated vs. wild type)       | 1.338| 0.247   | 0.986    | 0.321|
| DNMT3A (mutated vs. wild type)     | 1.824| 0.177   | 1.205    | 0.272|
| IDH2 (mutated vs. wild type)       | 3.21 | 0.073   | 2.199    | 0.138|

VAF, variant allele frequency; WBC, white blood cell; HGB, hemoglobin; PLT, platelet; allo-HSCT, allogenic hematopoietic stem cell transplantation.
Variables | OS | EFS |
|---|---|---|
| | χ² | P-value | χ² | P-value |
| RUNX1 (mutated vs. wild type) | 0.608 | 0.436 | 0.391 | 0.532 |
| KIT (mutated vs. wild type) | 1.557 | 0.212 | 1.949 | 0.163 |
| SRSF2(mutated vs. wild type) | 0.323 | 0.570 | 0.537 | 0.464 |

VAF, variant allele frequency; WBC, white blood cell; HGB, hemoglobin; PLT, platelet; allo-HSCT, allogenic hematopoietic stem cell transplantation.

The factors with \( P < 0.05 \) in univariate analyses were included in the multivariate analysis. \( FLT3-ITD \) co-mutation had an independent predictive impact on poor OS (Table 3). Allo-HSCT was an independent protective factor for the OS and EFS of \( ASXL1 \) + AML patients (Table 3).

Table 3
Multivariate analysis for OS and EFS in \( ASXL1 \) + AML.

| Variables | OS | EFS |
|---|---|---|
| | HR (95%CI) | P-value | HR (95%CI) | P-value |
| Age ≥ 60 years | 1.382(0.720–2.653) | 0.331 | |
| Risk group | 1.734(0.655–4.585) | 0.268 | 2.219(0.847–5.813) | 0.105 |
| Allo-HSCT | 0.204(0.061–0.085) | 0.01 | 0.184(0.056–0.605) | 0.005 |
| WBC counts (≥ \( 50 \times 10^9 \)/L) | 1.194(0.566–2.517) | 0.641 | 1.826(0.862–3.867) | 0.116 |
| \( FLT3-ITD \) | 2.894(1.260–6.647) | 0.012 | 1.848(0.810–4.215) | 0.144 |
| \( AML1-ETO \) | 0.611(0.176–2.123) | 0.438 | 0.760(0.231–2.507) | 0.653 |

OS, overall survival; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; allo-HSCT, allogenic hematopoietic stem cell transplantation; WBC, white blood cell.

Then, we assessed the prognosis effect of the aforementioned factors in the adverse-risk group. The survival study revealed that decreased HGB levels (≤ 110g/L), \( FLT3-ITD \) mutations, and \( RUNX1 \) mutations had a negative influence on the OS of \( ASXL1 \) + AML patients (\( P = 0.045, P = 0.047, \) and \( P = 0.027, \) respectively; Additional file 3E-F). These variables had no impact on EFS. Allo-HSCT recipients had a longer OS and EFS (\( P = 0.024 \) and \( P = 0.013, \) respectively). HGB levels < 110g/L and the \( FLT3-ITD \) mutations were found to have an independent predictive influence on poor OS in the multivariate analysis.
Increased number of risk factors may shorten the OS and EFS of ASXL1 + AML patients.

The aforementioned factors that had adverse impact on OS and EFS are defined as high risk factors, including age $\geq 60$ years, WBC count $\geq 50 \times 10^9$/L, FLT3-ITD mutations, RUNX1 mutations, and the absence of AML1-ETO fusion gene. ASXL1 mutations without any risk factor were referred to as single-hit ASXL1 + AML. ASXL1 mutations with one risk factor was referred to as double-hit ASXL1 + AML. ASXL1 mutations with two or more risk factors were referred to as triple-hit ASXL1 + AML. The combination of these risk factors had a negative influence on the prognosis of ASXL1 + AML (Fig. 2). The median OS was not attained in single-hit ASXL1 + AML, 29.53 months in double-hit ASXL1 + AML, and 6.67 months in triple-hit ASXL1 + AML ($P = 0.003$, Fig. 2A). The median EFS in single-hit ASXL1 + AML was not attained in single-hit ASXL1 + AML, 29.53 months in double-hit ASXL1 + AML, and 5.47 months in triple-hit ASXL1 + AML ($P = 0.003$, Fig. 2B).

Allo-HSCT improved the survival of double/triple-hit ASXL1 + AML patients

In our study, 12 patients received allo-HSCT as the consolidation management. Eleven of them carried one or more risk factors in addition to ASXL1 mutations. As shown in Fig. 3, allo-HSCT significantly improved the OS (median 29.53 months vs. 11.33 months, $P = 0.008$, Fig. 3A) and EFS (median 29.53 months vs. 8.53 months, $P = 0.007$, Fig. 3B) in double or triple-hit ASXL1 + AML patients.

Discussion

Previous researches found that ASXL1 mutations were recurrent in 5–20% of AML patients\[24–29\]. These mutations are heterozygous and result in ASXL1 mutants with a C-terminal truncation\[21\]. This ASXL1 mutation pattern is characterized by dominant-negative or gain-of-function mutations \[30\]. ASXL1 gain-of-function mutations have been linked to poor outcomes in AML patients \[27, 29, 31, 32\]. Based on the adverse outcome of ASXL1 + AML patients, ASXL1 mutations were recognized as a stratification criterion for AML in the 2017 ELN guideline\[4\]. However, given the prevalence and adverse outcome of ASXL1 mutations in AML, it is critical to identify the molecular landscape of ASXL1 + AML patients for establishing precise risk stratification in this subgroup of AML. This study investigated several key issues related to ASXL1 + AML, and discovered that the addition of other risk factors to ASXL1 mutations worsens the adverse outcome of ASXL1 + AML patients.

The majority of ASXL1 mutations in our study were found on codon 12, which is consistent with earlier reports\[25, 33\]. The most prevalent mutation was a guanine duplication (c.1934dupG) that results in a frameshift (p.Gly646TrpfsX12)\[29, 34, 35\]. These ASXL1 mutations in AML patients are regarded as gain-of-function with a negative prognosis \[30\]. In our study, the distribution of mutation sites was quite diverse, with G652S, G642fs, and H630fs having the highest occurrence. This diversity of mutations
might be attributed to differences in the selected population and races. There was no statistical difference in OS and EFS across different mutated nucleotides, which was consistent with a recent study[35]. Furthermore, we found that ASXL1 VAF did not correlate with survival.

Age ≥ 60 years and WBC counts ≥ 50 × 10^9/L are typically linked with unfavorable risk and poor outcome in AML patients[4]. In our cohort of ASXL1 + AML, age ≥ 60 years also had a detrimental influence on OS. WBC account ≥ 50 × 10^9/L had a negative impact on OS and EFS. Allo-HSCT, which was formerly thought to be the cure for AML, showed a survival advantage, particularly in individuals with double or triple-hit ASXL1 + AML.

Most primary MDS patients with ASXL1 mutations (85%) have concurrent mutations of genes at the time of diagnosis[36]. The mutational profiles of ASXL1 + AML are complicated and multiple molecular interactions may exist. We observed that 86.46% of ASXL1 + AML patients had additional gene mutations. Detailed investigation in the roles of co-occurred mutations is necessary for ASXL1 + AML patients. A previous study showed that RUNX1 mutation promotes leukemogenesis of myeloid malignancies in ASXL1 + leukemia[37] and is associated with adverse prognoses of patients with de novo AML[38]. In our study, RUNX1 did not have effects on OS and EFS in the overall prognostic analysis, but it was associated with shorter OS in high-risk ASXL1 + AML patients. This suggests that RUNX1 mutation does not have prognostic significance in ASXL1 + AML and may be involved in the leukemogenesis of this subtype of AML. In addition, FLT3-ITD mutation is recognized as a poor prognostic factor that is associated with short OS, EFS and DFS[38–40]. The ASXL1, FLT3-ITD, and RUNX1 mutations have been identified as major risk factors in AML patients by the ELN guidelines[4]. In this study, FLT3-ITD mutations were also associated with a shorter OS and EFS in ASXL1 + AML patients and correlated with a shorter survival time in high-risk ASXL1 + AML patients. This finding suggests that FLT3-ITD mutations exacerbate the poor prognosis of ASXL1 + AML. The AML1-ETO fusion gene results from the chromosomal translocation t(8;21), and is usually related to good response to induction therapy, as well as high complete remission rates in AML patients[41]. Our findings showed that the AML1-ETO fusion gene was similarly associated with a prolonged OS and EFS in ASXL1 + AML patients. These data demonstrate that the complex molecular interactions may affect the prognosis of ASXL1 + AML patients.

Currently, clinical diagnoses and risk assessments for AML are mostly based on cytogenetic and genomic changes[4]. The prognosis for AML patients varies substantially, particularly for those with normal karyotype[31]. With the application of NGS in the clinical practice, we can better understand the complex roles and prognostic impacts of molecular mutations of genes in AML. According to the multiple-hit theory of genetic alterations in lymphoma and multiple myeloma, we further analyzed the additional risk factors for the survival of ASXL1 + AML patients. The results showed that the more risk factors, the shorter the OS and EFS for ASXL1 + AML patients. The application of allo-HSCT significantly improved the prognosis of ASXL1 + AML patients[35]. This was also applicable to the double-hit/triple-hit patients defined in our study, further confirming the importance of allo-HSCT in the treatment of AML patients.
Our research had several limitations. First, our study was retrospective and prone to selection biases. Second, owing to technical limitations, certain gene mutations may go undetected. Prognostic implications of some gene mutations may be overlooked. Third, the small sample sizes of several subgroups resulted in relatively low statistical power. Because of these constraints, our findings require confirmation in a larger and prospective population.

Conclusion

This study provides new insights into the mutational spectrum and prognostic factors of ASXL1+ AML patients. The results demonstrate that increasing risk factors are associated with adversary prognosis of ASXL1+ AML patients. Our research further emphasizes the necessity of having the precise risk stratification for ASXL1+ AML patients.

Abbreviations

ASXL1: additional sex combs-like 1; Asx: Additional sex combs; AML: acute myeloid leukemia; APL: acute promyelocytic leukemia; MDS: myelodysplastic syndromes; allo-HSCT: Allogenic hematopoietic stem cell transplantation; HSCT: hematopoietic stem cell transplantation; RAR: retinoic acid receptor; NGS: next-generation sequencing; EDTA: Ethylene Diamine Tetraacetic Acid; VAF: variant allele frequency; WBC: white blood cell; HGB: hemoglobin; PLT: platelet; OS: overall survival; EFS: event-free survival.

Declarations

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Authors’ contributions

YFL and SJW designed the study and revised the manuscript. LXL collected and analyzed the data. YF analyzed the data and drafted the manuscript. YFL, YJL and SJW revised the manuscript. ZZW partially collected the data. CW and ZXJ critically viewed and supervised the study. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of our research are available from The First Affiliated Hospital of Zhengzhou University, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available.

Ethics approval and consent to participate

The project has been approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University and each participant has signed written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

| Chromatin-modifying | ASXL1 | EZH2 | TET2 | DNMT3A | IDH2 | IDH1 |  |
|---------------------|-------|------|------|--------|------|------|---|
| DNA methylation     |       |      |      |        |      |      | 100% |
| RAS                 |       |      |      |        |      |      | 48.35% |
| Tyrosine Kinase     |       |      |      |        |      |      | 10.99% |
| Spliceosome-complex |       |      |      |        |      |      | 3.30%  |
| Transcription factors |     |      |      |        |      |      | 8.79%  |
| NPM1                |       |      |      |        |      |      | 14.29% |
| Tumor suppressor    |       |      |      |        |      |      | 3.30%  |

**Figure 1**

The mutational landscape of 91 ASXL1+ AML patients. The landscape displayed all genetic anomalies for each subject. A single patient instance was represented by the boxes in one column. Mutations were color coded according to mutation type. The frequency distribution of all aberrations was depicted by the histogram on the right.
Figure 2
Comparison of OS (A) and EFS (B) in single-hit, double-hit and triple-hit ASXL1+ AML. The factors that had adverse impact on OS and EFS are defined as high risk factors, including age $\geq 60$ years, WBC count $\geq 50 \times 10^9/L$, FLT3-ITD mutations, RUNX1 mutations, and absence of AML1-ETO fusion gene. ASXL1 mutations without any risk factor were classified as single-hit ASXL1+ AML. ASXL1 mutations together with any of the risk factors was referred to be double-hit ASXL1+ AML. ASXL1 mutations along with any two or more of the risk factors were designated triple-hit ASXL1+ AML.

Figure 3
Transplantation can improve OS (A) and EFS (B) of ASXL1+ AML patients. The factors that had adverse impact on OS and EFS are defined as high risk factors, including age $\geq 60$ years, WBC count $\geq 50 \times 10^9/L$, FLT3-ITD mutations, RUNX1 mutations, and absence of AML1-ETO fusion gene. ASXL1 mutations without any risk factor were classified as single-hit ASXL1+ AML. ASXL1 mutations together with any of
the risk factors was referred to be double-hit ASXL1+ AML. ASXL1 mutations along with any two or more of the risk factors were designated triple-hit ASXL1+ AML.

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