Mutational spectrum and associations with clinical features in patients with acute myeloid leukaemia based on next-generation sequencing

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Abstract. The aim of the present study was to examine the associations between 112 acute myeloid leukaemia (AML)-associated genes and the prognosis and clinical features of AML using bioinformatics analysis in 62 patients with AML. A total of 61 gene mutations were identified, and ≥1 mutations were detected in 96.77% of the patients. A total of 11 frequent mutations were identified, including nucleophosmin 1 (NPM1), Fms related tyrosine kinase 3 (FLT3), DNA methyltransferase 3α (DNMT3A) and Notch 2 (NOTCH2), with a mutation rate of ≥10%. The FLT3 mutation was significantly associated with the white blood cell count at the time of diagnosis, and DNMT3A was significantly associated with the French-American-British subtype and cytogenetics of patients with AML. The FLT3, NPM1 and DNMT3A mutations were significantly associated with a poor overall survival (OS) in patients with AML. In addition, the co-mutation of DNMT3A-CCAAT enhancer binding protein α (CEBPA) was observed to be significantly associated with a poor OS in patients with AML. Furthermore, the functional enrichment analysis revealed that the co-mutations of FLT3-NOTCH2, SETBP1-CREBBP and DNMT3A-CEBPA were significantly enriched in processes of ‘negative regulation of cell differentiation’ and ‘immune system development’, indicating that these mutations may serve crucial roles in the diagnosis and treatment of AML.

Introduction

Acute myeloid leukaemia (AML) is a heterogeneous group of disorders characterized by the clonal proliferation of progenitor cells or primitive hematopoietic stem cells (1). Due to the development of allogeneic hematopoietic stem cell transplantation (allo-HSCT) for patients with AML, in particular the extensive development of haploidentical allo-HSCT in China, the therapeutic efficacy of treatments for AML have significantly improved throughout previous decades (2). However, the treatment of refractory and relapsed patients remains a significant clinical challenge that has yet to be overcome (3).

AML is typically diagnosed using morphologic, immunologic, cytogenetic and molecular biologic (MICM) classification techniques. However, the accumulation of somatically-acquired genetic changes in hematopoietic progenitor cells serves a vital role in the pathogenesis of AML, including gene mutations, copy number alterations and chromosomal translocation, which provides clinicians with a novel method to diagnose AML (4). Due to the successful application of next generation sequencing (NGS), NGS has become widely used in the analysis of clinical and biological heterogeneity of AML in a clinical setting (5). The study conducted by Corces-Zimmerman et al (6) demonstrated that preleukemic mutation in AML affected the regulation of epigenetic systems, and promoted the survival of hematopoietic stem cells via resistance to chemotherapy. In addition, cyclin D1 and cyclin D2 mutations have been identified to be frequently-occurring events in adult patients with AML at t(8;21)(q22;q22), and may serve as additional therapeutic targets for AML. Furthermore, the inhibition of mutant isocitrate dehydrogenase [NADP(+)] 2, mitochondrial via AG-221 or DNA methyltransferase activity by 5-azacytidine has been demonstrated to improve the sensitivity of patients with AML to epigenetic therapy (7,8). These data indicate that mutations in AML exert important functions in the development, treatment and prognosis of AML.

Recently, a spectrum of somatic mutations that were detected by targeted NGS have been identified by Feng et al (9).
This mutation spectrum contained 112 genes and was based on 121 adult patients with acute leukaemia, and has subsequently been used for the analysis of gene mutations and mutation frequency in malignant hematologic disorders (10). In the present study, amongst the 112-gene mutation panel, a total of 61 gene mutations were determined in the 62 patients with AML. Based on these data, single gene mutations and co-mutations in AML were analysed, followed by the associations with clinical features and the prognosis of AML. The aim of the present study was to provide novel information pertaining to the mechanism of action of AML, with particular emphasis on the roles of co-occurrence gene mutations, in order to provide more efficient therapeutics and to guide the individual course of treatment for patients with AML.

Materials and methods

Patients and specimen collection. Bone marrow samples were collected from 62 patients with AML (29 males and 33 females, aged between 15-75 years old) who were diagnosed for the first time at Provincial Hospital affiliated to Shandong University (Jinan, China) from January 2016 to December 2016. The diagnosis and categories of AML were performed according to the criteria recommended by the World Health Organization in 2008 (11), and was combined with the MICM characteristics (12). Bone marrow mononuclear cells were isolated by density gradient centrifugation with 2,000 x g for 15 min at 4°C. The present study was approved by the Ethics Committees of Shandong Provincial Hospital and all participants provided written informed consent. The clinical and pathological information of the 62 patients with AML are summarized in Table I.

DNA isolation. For the bone marrow samples, red blood cells were lysed using Red Blood Cell Lysis buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The remaining cells were subsequently counted, and ~1.0x10^7 karyocytes were used to isolate genomic DNA using the Column Blood DNAOUT kit (Tiandz Inc., Beijing, China) according to the manufacturer’s protocols.

Detection of gene mutations. A specific target panel for malignant hematologic disorders, which covered hotspots or complete coding regions of 112 genes (Table II) known to be recurrently mutated and/or associated with malignant hematologic disorders was used in the present study (9). A DNA library was constructed using Ion Proton™ Ion kits (Ion AmpliSeq™ Library Kit 2.0-96 rxns), according to the manufacturer’s protocol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequent to preparation of the template, the Ion Proton sequencing platform was applied to sequence the exons of these genes using the Ion PI Hi-Q OT2 200 Kit (A26434) and Ion PI Hi-Q Sequencing 200 Kit (A26433). Then, the results were mapped to the National Center for Biotechnology Information hg19 RefSeq with a mean of >97% coverage of the targeted regions at an average depth of 800X. The genetic mutation analysis was completed by Ion Reporter system and Variant Reporter software v2.0 (Thermo Fisher Scientific, Inc.). All putative mutations were compared against multiple databases, including dbsNP (13), 1,000 genomes (14), Polyphen-2 (15), and Catalogue of Somatic Mutations In Cancer (16). The detection rate of 5% mutation frequency was 97-98%.

Statistical analysis of gene mutations. The distribution of detected mutations in the 62 patients was presented using the ggplot2 (version 2.2.1, https://cran.r-project.org/web/packages/ggplot2/) (17) in R software. The mutation frequency of each gene was calculated and the high frequency mutated genes (mutation frequency >10%) were selected for subsequent analysis.

Single gene mutation analysis. Associations between high frequency mutated genes (mutation frequency >10%) and clinical characteristics were analysed using the Pearson’s χ² test (18) in R 3.4.1 software. In addition, high frequency gene mutation profiles were extracted from The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov/). Then, prognosis-associated gene mutations were analysed using Cox univariate regression analysis in a survival package (version 2.40.1; https://cran.r-project.org/package=survival) (19), and the survival results of the high frequency gene mutations were also analysed using Kaplan-Meier survival curves and log-rank tests (20).

Combined gene mutation analysis. Associations between co-mutations with a high frequency and clinical characteristics were analysed using the lm function (https://www.rdocumentation.org/packages/stats/versions/3.4.1/topics/lm) (21) in R 3.4.1 software. The multiple regression model was performed by forced entry linear regression in limma of package R and bilateral P<0.05 was considered statistically significant. The clinical features that were significantly associated with combined gene mutations were subjected to analysis using the Gene Ontology (GO) (22,23) and the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway (24) analyses using Database for Annotation, Visualisation and Integrated Discovery v6.8 software (25,26) with the threshold of P<0.05, which was considered to indicate a statistically significant difference. In addition, the prognosis-associated co-mutations were analysed using the aforementioned method for single gene mutations.

Results

Mutations in patients with AML. A total of 61 gene mutations were detected based on the 112 genetic mutations associated with AML. Among of the 62 enrolled patients, a total of 60 cases (96.77%) presented with at least one mutation, and 52 out of 62 (83.87%) patients exhibited ≥2 mutations. Specifically, 9 cases (14.52%) had 2 mutations, 11 patients (17.74%) had 3 mutations, 15 patients (24.19%) had 4 mutations and 17 patients (27.42%) had >5 mutations (Fig. 1). Nucleophosmin 1 (NPM1), Fms related tyrosine kinase 3 (FLT3), FAT atypical cadherin 1 (FAT1), ASXL transcriptional regulator 1 (ASXLI) and DNA methyltransferase 3α (DNMT3A) were the 5 most frequently identified mutations in patients with AML. Using a cut-off frequency of >10%, a total of 11 high frequency mutations were screened, including NPM1 (22.58%), FLT3 (22.58%), FAT1 (20.97%), ASXLI (17.74%), DNMT3A (16.13%), Notch 2 (NOTCH2; 14.52%), SET
Table I. Clinical and pathological information of 62 patients with AML.

| Characteristics                              | Mean                        | N   |
|----------------------------------------------|-----------------------------|-----|
| Age at study entry, years (range)            | 43.32 (15-75)               | -   |
| Sex                                          |                             |     |
| Male                                         | -                           | 29/62|
| Female                                       | -                           | 33/62|
| WBC count at diagnosis (range)               |                             | -   |
| WBC ($10^9/l$)                               | 31.35 (0.80-280.70)         | -   |
| Bone marrow blast count (range)              | 63.22 (5.83-99.00)          | -   |
| AML FAB subtype                              |                             |     |
| AML with minimal maturation (M0)             | -                           | 0/62 |
| AML without maturation (M1)                  | -                           | 3/62 |
| AML with maturation (M2)                     | -                           | 14/62|
| Acute myelomonocytic leukemia (M4)           | -                           | 12/62|
| Acute monocytic leukemia (M5)                | -                           | 11/62|
| Acute erythroid leukemia (M6)                | -                           | 3/62 |
| Acute megakaryoblastic leukemia (M7)         | -                           | 1/62 |
| Unclassified                                 | -                           | 18/62|
| Immunophenotype                              |                             |     |
| CD13                                         | -                           | 58/62|
| CD15                                         | -                           | 39/62|
| CD33                                         | -                           | 60/62|
| CD34                                         | -                           | 48/62|
| CD117                                        | -                           | 58/62|
| MPO                                          | -                           | 39/62|
| CD64                                         | -                           | 40/62|
| HLA-DR                                       | -                           | 58/62|
| CD56                                         | -                           | 22/62|
| CD38                                         | -                           | 61/62|
| Cytogenetics                                 |                             |     |
| Abnormal karyotype                           | -                           | 28/62|
| Normal karyotype                             | -                           | 22/62|
| Information missing                          | -                           | 12/62|
| Risk                                         |                             |     |
| High                                         | -                           | 12/62|
| Medium                                       | -                           | 31/62|
| Low                                          | -                           | 10/62|
| Information missing                          | -                           | 9/62 |
| Induction therapy                            |                             |     |
| IA                                           | -                           | 32/62|
| DA                                           | -                           | 10/62|
| Others                                       | -                           | 8/62 |
| Information missing                          | -                           | 12/62|
| Response evaluation                          |                             |     |
| Achieving CR                                 | -                           | 27/62|
| NR                                           | -                           | 19/62|
| Unevaluated                                  | -                           | 8/62 |
| Information missing                          | -                           | 8/62 |
binding protein 1 (*SETBP1*; 14.52%), NRAS proto-oncogene, GTPase (*NRAS*; 14.52%), CCAAT enhancer binding protein α (*CEBPA*; 14.52%), Tet methylcytosine dioxygenase 2 (14.52%) and cyclic adenosine 5'-phosphate response element-binding protein binding protein (*CREBBP*; 14.52%) (Fig. 2A). The distribution of high frequency mutations in clinical characteristics are presented in Fig. 2B. The frequencies and types of variants of the 11 high frequency mutations are presented in Table III.

### Single mutation analysis

In order to examine the significance of acquired genetic mutations in the development of AML, the present study initially analysed the association between single mutations and clinical features, including white blood cell (WBC) count at diagnosis, French-American-British (FAB) subtype (27), and karyotype using Pearson's χ² test. As a result, *FLT3*, *NRAS* and *CEBPA* mutations were significantly associated with WBC count, while *ASXL1* and *DNMT3A* mutations were significantly associated with the FAB subtypes. The *DNMT3A* mutation was also significantly associated with the variation of the karyotype (Table IV). The survival information of 11 high frequency mutations in AML was extracted from TCGA database, and survival prognosis analysis was performed. The results revealed that 3 single mutations were identified to be negatively associated with a poor overall survival (OS) in patients with AML, including *FLT3*, *NPM1* and *DNMT3A* (Fig. 3).

### Combined mutation analysis

The mutation analysis revealed that 56.45% of patients (35/62) exhibited >2 high frequency mutations (Fig. 4), indicating that co-occurrence gene mutations were a common phenomenon in AML. The present study

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**Table I. Continued.**

| Characteristics | Mean | N   |
|-----------------|------|-----|
| Consolidation therapy after CR | | |
| Chemotherapy    | -    | 30/62|
| HSCT            | -    | 14/62|
| Information missing | - | 18/62|

AML, acute myeloid leukaemia; WBC, white blood cell count; FAB, French American British; CR, complete remission; IA, idarubicin + cytarabine. DA, daunorubicin + cytarabine. HSCT, hematopoietic stem cell transplantation; CD13, aminopeptidase N; CD15, sialyl Lewisx; CD33, myeloid cell surface antigen; CD34, hematopoietic progenitor cell antigen CD34; CD117, mast/stem cell growth factor receptor Kit; MPO, myeloperoxidase; CD64, high affinity immunoglobulin gamma Fc receptor I; HLA-DR, human leukocyte antigen-DR isotype; CD56, neural cell adhesion molecule 1; CD38, ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1; CR, complete response; NR, non-remission; HSCT, hematopoietic stem cell transplantation.

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**Table II. Genes closely associated with diseases of the blood system.**

| No. | Gene name | No. | Gene name | No. | Gene name | No. | Gene name | No. | Gene name | No. | Gene name | No. | Gene name | No. | Gene name | No. | Gene name |
|-----|-----------|-----|-----------|-----|-----------|-----|-----------|-----|-----------|-----|-----------|-----|-----------|-----|-----------|-----|-----------|
| 1   | ABL1      | 18  | MYC       | 35  | SRSF2     | 52  | NF1       | 69  | CCND1     | 86  | PTPN11    | 103 | CSF3R     |
| 2   | BRAF      | 19  | ABCB1     | 36  | BIRC3     | 53  | MAPK1     | 70  | CEBPA     | 87  | STX11     | 104 | EZH2      |
| 3   | CUX1      | 20  | SF1       | 37  | CBL       | 54  | ZRSR2     | 71  | EP300     | 88  | U2AF2     | 105 | IDH2      |
| 4   | FANCA     | 21  | MAFB      | 38  | DNM2      | 55  | IKZF1     | 72  | GATA2     | 89  | CRLF2     | 106 | MAF       |
| 5   | IL7R      | 22  | PRPF40B   | 39  | FAT1      | 56  | TET2      | 73  | KIT       | 90  | TRAF3     | 107 | PAX5      |
| 6   | MPL       | 23  | ECT2L     | 40  | JAK1      | 57  | TAL1      | 74  | NOTCH2    | 91  | BCL6      | 108 | RB1       |
| 7   | PDGFRB    | 24  | WT1       | 41  | MYH11     | 58  | ATM       | 75  | PTEN      | 92  | CREBBP    | 109 | SMC3      |
| 8   | XPO1      | 25  | ALAS2     | 42  | PRF1      | 59  | CDKN1A    | 76  | ARID1A    | 93  | ETV6      | 110 | SF3B1     |
| 9   | ZMYM3     | 26  | RUNX1     | 43  | KMT2D     | 60  | EGFR      | 77  | ADAMTS13  | 94  | IDH1      | 111 | ASXL1     |
| 10  | SUZ12     | 27  | DDX3X     | 44  | SF3A1     | 61  | FLT3      | 78  | FBXW7     | 95  | SH2D1A    | 112 | WAS       |
| 11  | UNC13D    | 28  | FANCG     | 45  | SETBP1    | 62  | JAK3      | 79  | TP53      | 96  | Nras      | -   | -         |
| 12  | WHSC1     | 29  | ITK       | 46  | STXBPB    | 63  | NOTCH1    | 80  | BCL2      | 97  | RAB27A    | -   | -         |
| 13  | AKT1      | 30  | MYD88     | 47  | XIAP      | 64  | RELN      | 81  | LYST      | 98  | EED       | -   | -         |
| 14  | CALR      | 31  | PIK3CA    | 48  | CCND3     | 65  | SMC1A     | 82  | EPHA7     | 99  | DIS3      | -   | -         |
| 15  | CYLD      | 32  | CXCR4     | 49  | DNM3TA    | 66  | PRMT5     | 83  | GATA3     | 100 | PHF6      | -   | -         |
| 16  | FANCC     | 33  | SH2B3     | 50  | GFGR3     | 67  | FAM46C    | 84  | KRAS      | 101 | U2AF1     | -   | -         |
| 17  | MUM1      | 34  | SAMHD1    | 51  | JAK2      | 68  | TNFAIP3   | 85  | NPM1      | 102 | PRDM1     | -   | -         |
subsequently analysed the association between co-mutations of 11 high frequency mutations and clinical features, including age at the time of diagnosis, sex, bone marrow blast proportion, FAB subtype, karyotype and first course therapeutic response using a multiple regression model. Consequently, a total of 3 combined mutations were identified to be markedly associated with the clinical features of AML. Specifically, the combined mutations \textit{FLT3-NOTCH2} and \textit{DNMT3A-CEBPA} were significantly associated with WBC and cytogenetics, respectively, while the \textit{SETBP1-CREBBP} combined mutation was significantly associated with response evaluation and consolidation therapy following complete remission (CR) in AML (Table V). According to the TCGA, among these 3 significant co-mutations, only \textit{DNMT3A-CEBPA} was significantly associated with a poor OS in patients with AML, and no significant difference was identified in the co-mutation of \textit{FLT3-NOTCH2} due to the small sample size (Fig. 5). However, no information about the co-mutation of \textit{SETBP1-CREBBP} was available in TCGA database; therefore, the present study did not analyse the association between prognosis and the co-mutation \textit{SETBP1-CREBBP} in patients with AML.
### Table III. Frequencies and types of variants of 11 high frequency mutations.

| Mutated genes (sample number) | Type of variant (Mutant amino acid) | Frequency, % |
|-------------------------------|-------------------------------------|--------------|
| **NPM1 (11)** | p.W288fs | >10.00 |
| | p.W288fs | >10.00 |
| | p.W288fsX12 | >10.00 |
| | p.K193R | 5.00 |
| | p.E245Q | 45.02 |
| | p.W288fs | >10.00 |
| | p.K193R | 8.00 |
| FLT3 (23) | p.D835γ | 15.47 |
| | p.V491L | 32.25 |
| | ITD | >10.00 |
| | p.A680V | 9.73 |
| | p.D835γ | 41.16 |
| | p.836_837del | 44.29 |
| | ITD | + |
| **FAT1 (21)** | p.V2089I | 54.45 |
| | p.A4551G | 49.27 |
| | p.L2822P | 52.81 |
| | p.V591I | 50.91 |
| | p.A4551G | 48.07 |
| | p.R1257q | 45.61 |
| | p.Q587K | 9.41 |
| | p.A4551G | 48.14 |
| | p.Q587K | 7.75 |
| | p.Y4232C | 52.24 |
| | p.V3694I | 58.18 |
| **ASXL1 (18)** | p.G652S | 50.30 |
| | p.G652S | 51.03 |
| | p.W898X | 22.89 |
| | p.W898X | 42.25 |
| | p.C687X | 41.15 |
| | p.G652S | 51.97 |
| | p.G652S | 57.19 |
| | p.G1954A | 54.18 |
| | p.G652S | 54.72 |
| | p.G652S | 58.47 |
| | p.G652S | 52.21 |
| | p.G652S | 57.60 |
| | p.G652S | 57.19 |
| **DNMT3A (16)** | p.R882H | 42.57 |
| | p.R882H | 43.58 |
| | p.R882C | 47.07 |
| | p.R882H | 44.92 |
| | p.V716D | 45.72 |
| | p.R882C | 52.86 |
| | p.R882P | 31.13 |
| | p.R882C | 42.50 |
| | p.R882H | 49.56 |
| | p.R882C | 47.38 |
| **SETBP1 (15)** | p.P1563L | 20.00 |
| | p.D868N | 1.65 |
| | p.E1466D | 51.09 |
| | p.A1193T | 65.89 |
| | p.E1466D | 54.58 |
| | p.E1466D | 52.11 |
| | p.E1466D | 47.83 |
| | p.R627C | 51.31 |
| | p.R627C | 46.66 |
| **NRAS (15)** | p.G12D | 1.80 |
| | p.G12D | 4.74 |
| | p.G12D | 4.03 |
| | p.G12D | 6.61 |
| | p.G61R | 1.80 |
| | p.G13D | 4.85 |
| | p.G12D | 30.81 |
| | p.G12D | 46.83 |
| | p.G12D | 1.75 |
| | p.G13V | 6.36 |
| | p.Q16H | 22.49 |
| | p.G32fs | 25.35 |
| **CEBPA (15)** | p.K313delinsQK | 59.26 |
| | p.A666fs | >10.00 |
| | p.A303P | 48.16 |
| | p.P23fs | 46.12 |
| | p.A72LfsX35 | + |
| | p.L317delinsRL | 48.27 |
| | p.P23fs | 2.70 |
| **TET2 (15)** | p.F868L | 51.68 |
| | p.S1039L | 48.33 |
| | p.Q1523X | 2.20 |
| | p.I1762V | 47.20 |
| | p.Q324H | 5.88 |
| | p.R550X | 10.64 |
| | p.S1039L | 50.94 |
| | p.R814C | 49.51 |
| | p.S1039L | 50.76 |
| **CREBBP (13)** | p.R1140Q | 9.29 |
| | p.R1140Q | 4.17 |
| | p.V1924M | 41.56 |
| | p.R1140Q | 5.20 |
| | p.R1140Q | 4.35 |

Table III. Continued.

| Mutated genes (sample number) | Type of variant (Mutant amino acid) | Frequency, % |
|-------------------------------|-------------------------------------|--------------|
| **NOTCH2 (15)** | p.I1689F | 47.85 |
| | p.I1689F | 48.99 |
| | p.I1689F | 50.89 |
| | p.I1789F | 51.70 |
| | p.I1689F | 48.41 |
| | p.I1689F | 48.94 |
| | p.I1689F | 50.12 |
| | p.I1689F | 51.42 |
| | p.I1689F | 49.20 |
| **SETBP1 (15)** | p.P1563L | 20.00 |
| | p.D868N | 1.65 |
| | p.E1466D | 51.09 |
| | p.A1193T | 65.89 |
| | p.E1466D | 54.58 |
| | p.E1466D | 52.11 |
| | p.E1466D | 47.83 |
| | p.R627C | 51.31 |
| | p.R627C | 46.66 |
| **NRAS (15)** | p.G12D | 1.80 |
| | p.G12D | 4.74 |
| | p.G12D | 4.03 |
| | p.G12D | 6.61 |
| | p.G61R | 1.80 |
| | p.G13D | 4.85 |
| | p.G12D | 30.81 |
| | p.G12D | 46.83 |
| | p.G12D | 1.75 |
| | p.G13V | 6.36 |
| | p.Q16H | 22.49 |
| | p.G32fs | 25.35 |
| **CEBPA (15)** | p.K313delinsQK | 59.26 |
| | p.A666fs | >10.00 |
| | p.A303P | 48.16 |
| | p.P23fs | 46.12 |
| | p.A72LfsX35 | + |
| | p.L317delinsRL | 48.27 |
| **TET2 (15)** | p.F868L | 51.68 |
| | p.S1039L | 48.33 |
| | p.Q1523X | 2.20 |
| | p.I1762V | 47.20 |
| | p.Q324H | 5.88 |
| | p.R550X | 10.64 |
| | p.S1039L | 50.94 |
| | p.R814C | 49.51 |
| | p.S1039L | 50.76 |
| **CREBBP (13)** | p.R1140Q | 9.29 |
| | p.R1140Q | 4.17 |
| | p.V1924M | 41.56 |
| | p.R1140Q | 5.20 |
| | p.R1140Q | 4.35 |
Functional analysis of combined mutations. To additionally investigate the functions of the combined mutations, the 3 co-mutations were subjected to GO and KEGG pathway analyses. The GO analysis revealed that these 3 co-mutations

Table III. Continued.

| Mutated genes (sample number) | Type of variant (Mutant amino acid) | Frequency, % |
|-------------------------------|------------------------------------|--------------|
| p.R1140Q                      |                                    | 5.21         |
| p.R1140Q                      |                                    | 5.75         |
| p.R1140Q                      |                                    | 6.96         |

NPM1, nucleophosmin 1; FLT3, Fms related tyrosine kinase 3; FAT1, FAT atypical cadherin 1; ASXL1, ASXL transcriptional regulator 1; DNMT3A, DNA methyltransferase 3α; NOTCH2, Notch 2; SETBP1, SET binding protein 1; NRAS, NRAS proto-oncogene, GTPase; CEBPA, CCAAT enhancer binding protein α; TET2, Tet methylcytosine dioxygenase 2; CREBBP, cyclic adenosine 5'-phosphate response element-binding protein binding protein.

Table IV. Associations between mutations and clinical features.

A, WBC count at diagnosis

| WBC (H/L) | Mutations | Mutation | Non-mutation | P-value |
|-----------|-----------|----------|--------------|---------|
| FLT3      | 7/7       | 10/38    | 0.04402      |
| NRAS      | 6/4       | 11/32    | 0.009661     |
| CEBPA     | 5/4       | 12/41    | 0.049879     |

B, AML FAB subtype

| FAB subtype, M1/M2/M4/M5/M6/M7 | Mutations | Mutation | Non-mutation | P-value |
|--------------------------------|-----------|----------|--------------|---------|
| ASXL1                          | 2/1/3/0/3/0 | 1/13/9/11/0/1 | 0.000115 |
| DNMT3A                         | 1/1/0/5/2/0 | 2/13/12/6/1/1 | 0.007636 |

C, Cytogenetics

| Karyotype, abnormal/normal | Mutations | Mutation | Non-mutation | P-value |
|----------------------------|-----------|----------|--------------|---------|
| DNMT3A                     | 2/8       | 26/14    | 0.01446      |

WBC, white blood cell; AML, acute myeloid leukaemia; FAB, French-American-British; FLT3, Fms related tyrosine kinase 3; NRAS, NRAS proto-oncogene, GTPase; CEBPA, CCAAT enhancer binding protein α; ASXL1, ASXL transcriptional regulator 1; DNMT3A, DNA methyltransferase 3α.

Table V. Associations between clinical features and 11 high-frequency mutations by multi-factor analysis.

| Clinic characteristics | NPM1 | FLT3 | ASXL1 | DNMT3A | FAT1 | NOTCH2 | SETBP1 | NRAS | CEBPA | TET2 | CREBBP |
|------------------------|------|------|-------|--------|------|--------|--------|------|-------|------|--------|
| Age at study entry, years | 0.490 | 0.491 | 0.209 | 0.153 | 0.116 | 0.519 | 0.985 | 0.212 | 0.637 | 0.820 | 0.820 |
| Sex, male/female | 0.875 | 0.033 | 0.434 | 0.001 | 0.760 | 0.092 | 0.003 | 0.777 | 0.577 | 0.577 | 0.577 |
| WBC, H/L | 0.695 | 0.034 | 0.269 | 0.705 | 0.902 | 0.108 | 0.645 | 0.061 | 0.645 | 0.061 | 0.645 |
| Bone marrow blast count | 0.647 | 0.114 | 0.717 | 0.801 | 0.907 | 0.151 | 0.698 | 0.464 | 0.955 | 0.464 | 0.955 |
| AML FAB subtype, M0/M1/M2/M4/M5/M6/M7 | 0.400 | 0.491 | 0.209 | 0.153 | 0.116 | 0.519 | 0.985 | 0.212 | 0.637 | 0.820 | 0.820 |
| Cytogenetics, abnormal/normal | 0.400 | 0.491 | 0.209 | 0.153 | 0.116 | 0.519 | 0.985 | 0.212 | 0.637 | 0.820 | 0.820 |
| High risk, high/medium/low | 0.204 | 0.033 | 0.546 | 0.348 | 0.045 | 0.221 | 0.776 | 0.276 | 0.976 | 0.748 | 0.348 |
| Response evaluation, CR/NR | 0.204 | 0.033 | 0.546 | 0.348 | 0.045 | 0.221 | 0.776 | 0.276 | 0.976 | 0.748 | 0.348 |
| Consolidation therapy following CR, chemotherapy/haematopoietic stem cell transplantation | 0.204 | 0.033 | 0.546 | 0.348 | 0.045 | 0.221 | 0.776 | 0.276 | 0.976 | 0.748 | 0.348 |

WBC, white blood cell; AML, acute myeloid leukaemia; FAB, French-American-British; CR, complete remission; NR, non-remission; NPM1, nucleophosmin 1; FLT3, Fms related tyrosine kinase 3; NRAS, NRAS proto-oncogene, GTPase; CEBPA, CCAAT enhancer binding protein α; ASXL1, ASXL transcriptional regulator 1; DNMT3A, DNA methyltransferase 3α; FAT1, FAT atypical cadherin 1; ASXL1, ASXL transcriptional regulator 1; DNMT3A, DNA methyltransferase 3α; NOTCH2, Notch 2; SETBP1, SET binding protein 1; NRAS, NRAS proto-oncogene, GTPase; CEBPA, CCAAT enhancer binding protein α; TET2, Tet methylcytosine dioxygenase 2; CREBBP, cyclic adenosine 5'-phosphate response element-binding protein binding protein.
were significantly enriched in 15 biological processes, including ‘hemopoietic or lymphoid organ development’ (P=2.15x10^{-3}), ‘negative regulation of cell differentiation’ (P=1.49x10^{-3}), ‘haemopoiesis’ (P=1.78x10^{-3}) and ‘immune system development’ (P=2.42x10^{-3}). Concomitantly, these 3 co-mutations were also significantly enriched in 3 KEGG pathways, including ‘AML’ (P=0.045), ‘pathways in cancer’ (P=0.023) and the ‘Notch signalling pathway’ (P=0.036; Fig. 6).

**Analysis of the clinical features of patients with combined mutations.** Finally, the present study analysed the common clinical features of patients with these 3 co-mutations. A total of 3 patients with AML were identified to possess the FLT3-NOTCH2 mutation. All of these patients presented with positive aminopeptidase N (CD13), myeloid cell surface antigen CD33 (CD33), myeloid cell surface antigen CD15, sialyl Lewis^a^; CD33, myeloid cell surface antigen; CD34, hematopoietic progenitor cell antigen CD34; CD117, mast/stem cell growth factor receptor Kit; MPO, myeloperoxidase; CD64, high affinity immunoglobulin gamma Fc receptor I; HLA-DR, human leukocyte antigen-DR isotype; CD56, neural cell adhesion molecule 1; CD38, ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1; NR, non-remission; CR, complete response.
Molecular abnormalities in multiples genes are involved in the pathogenesis of AML, and have been demonstrated to affect the overall prognosis of AML (28). In the present study, a total of 11 high frequently mutations were identified. Among them, the mutations of FLT3, NRAS, CEBPA, ASXL1 and DNMT3A were significantly associated with the clinical features of patients with AML. A total of 3 co-mutations, FLT3-NOTCH2, DNMT3A-CEBPA and SETBP1-CREBBP, were identified to be significantly associated with the clinical features and prognosis of patients with AML. Functional enrichment analysis demonstrated that mutations in these genes were significantly enriched in the biological process of immune system development, indicating that these combined mutations may serve a critical role in the development of AML.

Genetic mutations are significantly associated with the prognosis and recurrence of AML (6,29). In previous studies, multiple gene mutations have been identified in AML, including FLT3 (30), GATA2 (31), IDH (32) and CPM1 (33). Among these mutations, FLT3, which is the encoding gene of Fms-like receptor tyrosine kinase 3 receptor, is one of the most frequently-occurring mutations detected in AML (34,35). In the present study, the FLT3 mutation was identified in 22.58% patients; however, this

Figure 5. Survival curve analytical results of co-mutations based on The Cancer Genome Atlas database. (A) Kaplan-Meier survival curve of co-mutation of DNMT3A-CEBPA. (B) Kaplan-Meier survival curve of co-mutation of FLT3-NOTCH2. DNMT3A, DNA methytransferase 3α; CEBPA, CCAAT enhancer binding protein α; FLT3, Fms related tyrosine kinase 3; NOTCH2, Notch 2.

Figure 6. Functional enrichment analysis for co-mutations of Fms related tyrosine kinase 3-Notch 2, DNA methytransferase 3 α-CCAAT enhancer binding protein α and SET binding protein 1-cyclic adenosine 5’-phosphate response element-binding protein binding protein. GO, gene ontology.
SETBP1, which is recurrent in myelodysplastic syndromes (MDS) and often co-exists with cytogenetic markers in the progression of AML (39), was also within the top 10 mutations in the present study, with an occurrence of 14.52%. A previous study demonstrated that the SETBP1 mutation was detected in 17% of patients with secondary AML, which was similar to the results obtained in the present study (40). Cristóbal et al suggested that overexpressed SETBP1 predicted an adverse outcome in patients with AML (41). Taken together, these data demonstrate that gene mutations frequently occur in the development of AML and exert crucial functions in regulating the prognosis of AML. In MDS, the SETBP1 mutation promotes the leukemic transformation of patients with the ASXL1 mutation (42), indicating that the co-mutation of SETBP1 and ASXL1 may serve a promotive role in the development of AML. Notably, ASXL1 was significantly associated with the FAB subtypes in the present study, suggesting that the SETBP1-ASXL1 mutation was associated with the clinical features of patients with AML. In the present study, the co-mutation of SETBP1 and CREBBP was identified in patients with AML, and this was significantly associated with the response evaluation and consolidation therapy following CR, indicating that this co-mutation served an important role in the treatment and prognosis of AML. In addition, the co-mutation of SETBP1 and CREBBP consistently presented with abnormal cytogenetics, and positive CD13, CD33, CD34, CD117, CD56, CD38 and MPO expression, indicating that these features may be utilized as potential biomarkers for the diagnosis of patients with AML who present with the SETBP1 and CREBBP co-mutation. However, the OS was not significantly different in AML patients with or without mutant SETBP1 and CREBBP; therefore, the underlying mechanism of action requires additional investigation.

DNMT3A is essential for the differentiation of hematopoietic stem cells and its mutations have been identified in 4-22% of AML cases (43,44). In the present study, the DNMT3A mutation was identified in 16.12% of patients with AML. The present study also demonstrated that the DNMT3A mutation was significantly associated with the WBC count; however, it was not associated with other mutations in the patient cohort. The DNMT3A mutation was also revealed to be negatively associated with the prognosis of AML, which was consistent with the results obtained by a previous study (45). Although no DNMT3A-CEBPA co-mutation was identified in the present study, the data from TCGA database demonstrated that the co-mutation of DNMT3A-CEBPA was significantly associated with a poor prognosis in patients with AML. Therefore, additional investigations examining the association between the co-mutation of DNMT3A-CEBPA and clinical features should be performed, with a larger patient cohort.

As a result of previous in-depth investigations, several signalling pathways have been demonstrated to be involved in the development and prognosis of AML: The study by Quintás-Cardama et al (46) demonstrated that mutations in the tumor protein p53 pathway are associated with the lowest survival rates in patients with AML. Ufkin et al (47) hypothesized that miR-125a regulated cell proliferation and apoptosis in AML via the ErbB pathway. In the present study, the mutated genes that were significantly associated with

Table VII. Clinical features of 3 patients with concurrent SETBP1 and CREBBP mutations.

| Clinical features | Sample ID |
|------------------|-----------|
|                  | Sample 2  | Sample 3  | Sample 4  |
| Age, years       | 46        | 23        | 33        |
| Sex              | Female    | Male      | Female    |
| WBC (10⁹/l)      | 61.51     | 33.72     | 6.23      |
| Bone marrow blast count | 64.5 | 63     | 45        |
| Diagnosis        | M4        | Unclassified | M2        |
| Immunophenotype  |           |           |           |
| CD13             | +         | +         | +         |
| CD15             | +         | +         | +         |
| CD33             | +         | +         | +         |
| CD34             | +         | +         | +         |
| CD117            | +         | +         | +         |
| MPO              | +         | +         | +         |
| CD64             | +         | -         | -         |
| HLA-DR           | +         | +         | +         |
| CD56             | +         | +         | +         |
| CD38             | +         | +         | +         |
| Cytogenetics     | Abnormal  | Abnormal  | Abnormal  |
| Risk             | Medium    | High      | Low       |
| Response evaluation | NR      | NR        | CR        |

SETBP1, SET binding protein 1; CREBBP, cyclic adenosine 5'-phosphate response element-binding protein binding protein; WBC, white blood cell; CD13, aminopeptidase N; CD15, sialyl Lewis x; CD33, myeloid cell surface antigen; CD34, hematopoietic progenitor cell antigen CD34; CD117, mast/stem cell growth factor receptor Kit; MPO, myeloperoxidase; CD64, high affinity immunoglobulin gamma Fc receptor I; HLA-DR, human leukocyte antigen-DR isoform; CD56, neural cell adhesion molecule 1; CD38, ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1; CR, complete response; NR, non-remission.
clinical features were also subjected to functional enrichment analysis. The data revealed that these genes were significantly enriched in the biological processes of ‘negative regulation of cell differentiation’ and ‘immune system development’. Curran et al suggested that targeting the innate immune system may serve as an underlying therapy for AML (48). Additionally, the co-mutations were significantly enriched in the ‘Notch signalling pathway’. Takam Kamga et al (49) demonstrated that Notch signalling enhanced bone marrow stromal cell-mediated chemoresistance in AML, and the activation of Notch antagonizes DNA-binding protein Ikaros-based tumor suppression in T-cell ALL (50). These data indicated that these clinical features and mutations of the associated genes may promote the development of ALL via dysregulating the differentiation of hematopoietic cells and the immune response.

In conclusion, FLT3, NOTCH2, and DNMT3A were the 3 mutations with the highest frequencies identified in AML. Specifically, the mutations in FLT3 and DNMT3A were significantly associated with a poor prognosis in patients with AML. In addition, co-mutations of FLT3-NOTCH2 and SETBP1-CREBBP were significantly associated with the clinical features of patients with AML, and may serve a critical role in AML, via regulating the differentiation of hematopoietic cells and the immune response. Genome sequencing is an important method for the detection of mutations in patients with AML, which may provide useful information in understanding the mechanism of AML, which would assist in guiding individual treatment strategies.

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Availability of data and materials

The software packages and raw data used to support the results of the present study are available from the corresponding author upon request.

Authors' contributions

YL (first author), XinW and HX made substantial contributions to the conception and design of the present study, and drafted the manuscript. XLiu, CZ and WZ performed the data acquisition. XG, DY and XLv performed the data analysis and interpretation. YL (11th author), MD and XiaW contributed to the design of the study, and performed the bioinformatic analysis. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committees of Shandong Provincial Hospital. All participants provided written informed consent.

Patient consent for publication

All participants provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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