

# 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  $\geq 1$  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 3 $\alpha$  (*DNMT3A*) and Notch 2 (*NOTCH2*), with a mutation rate of  $\geq 10\%$ . 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  $\alpha$  (*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).

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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  $\sim 1.0 \times 10^7$  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  $\chi^2$  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 v.6.8 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 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  $\geq 2$  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 (*ASXL1*) and DNA methyltransferase 3 $\alpha$  (*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%), *ASXL1* (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 monoblastic or 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

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 Lewis<sup>x</sup>; 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.

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
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	STXBP2	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	DNMT3A	66	PRMT5	83	GATA3	100	PHF6	-	-
16	FANCC	33	SH2B3	50	FGFR3	67	FAM46C	84	KRAS	101	U2AF1	-	-
17	MUM1	34	SAMHD1	51	JAK2	68	TNFAIP3	85	NPM1	102	PRDM1	-	-

binding protein 1 (*SETBP1*; 14.52%), NRAS proto-oncogene, GTPase (*NRAS*; 14.52%), CCAAT enhancer binding protein  $\alpha$  (*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  $\chi^2$  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 *DMT3A* (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

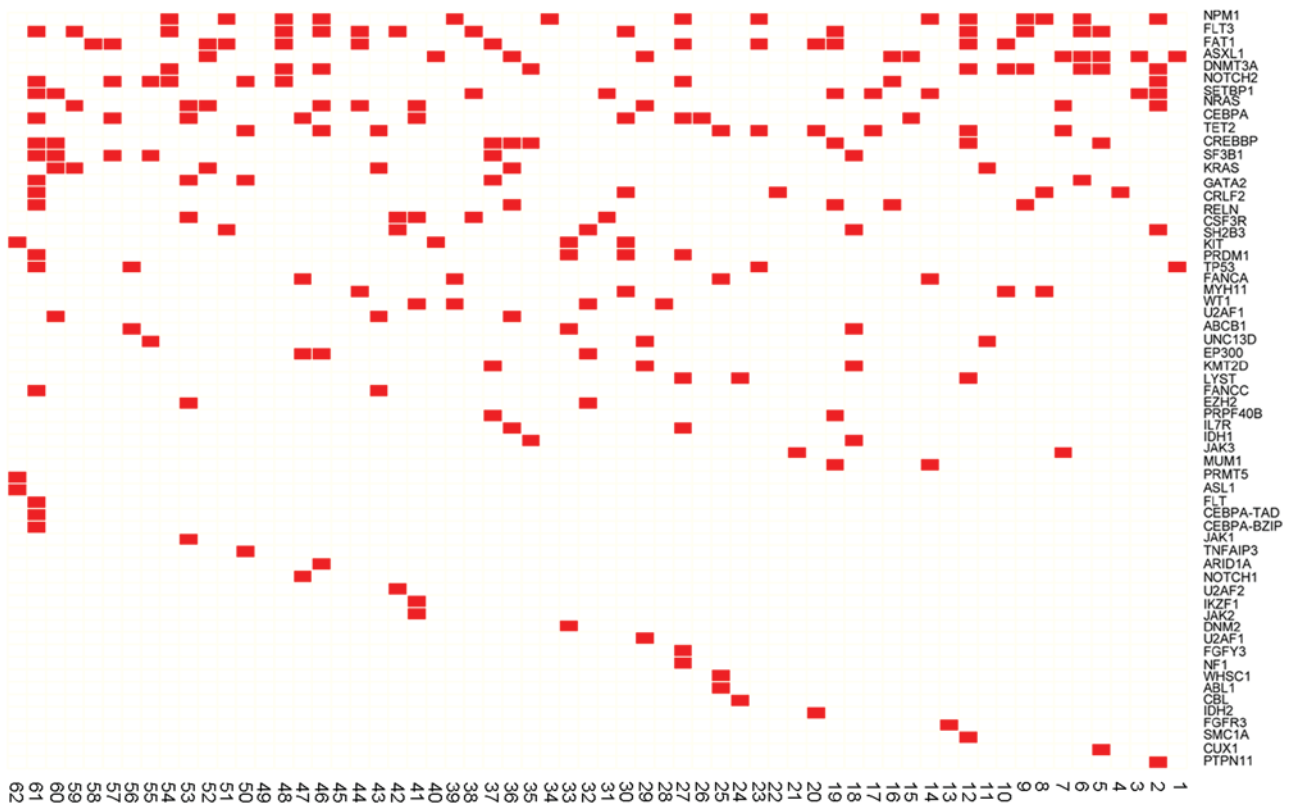


Figure 1. Mutations of 61 genes detected in 62 patients with acute myeloid leukaemia. The x-axis represents the sample number, and the y-axis represents the mutated genes. The red colour indicates the presence of a mutation.

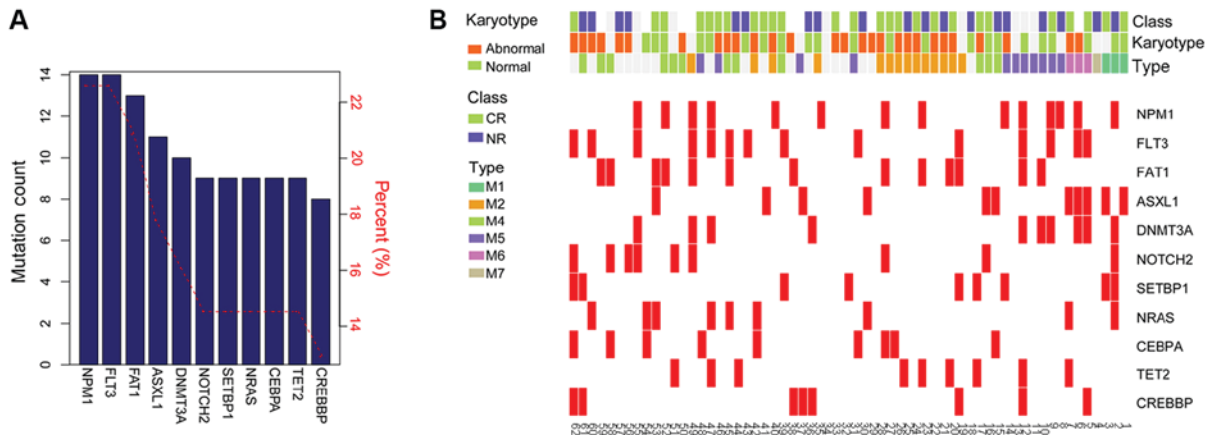


Figure 2. High frequency mutations among 62 patients with acute myeloid leukaemia. (A) Genes whose mutation rates were >10%. The x-axis represents the gene names, and the y-axis represents the number of patients with mutations in these genes. (B) Distributions of gene mutations. The red colour indicates the presence of a mutation. CR, complete remission; NR, non-remission.

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 *FLT3-NOTCH2* and *DNMT3A-CEBPA* were significantly associated with WBC and cytogenetics, respectively, while the *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 *DNMT3A-CEBPA* was significantly associated with a poor OS in patients with AML, and no significant difference was identified in the co-mutation of *FLT3-NOTCH2* due to the small sample size (Fig. 5). However, no information about the co-mutation of *SETBP1-CREBBP* was available in TCGA database; therefore, the present study did not analyse the association between prognosis and the co-mutation *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.W288fs	>10.00	
	p.W288fsX12	>10.00	
	p.W288fs	>10.00	
	p.K193R	5.00	
	p.E245Q	45.02	
	p.W288fs	>10.00	
	p.W288fs	>10.00	
	p.K193R	4.80	
	p.W288Cfs	>10.00	
	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.V5911	50.91	
	p.A4551G	48.07	
	p.R1257q	46.51	
	p.Q587K	9.41	
	p.A4551G	48.14	
	p.Q587K	7.75	
	p.Y4232C	52.24	
ASXL1 (18)	p.V3694I	58.18	
	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	
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	

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.1689F	50.89
	p.I1789F	51.70
	p.1689F	48.41
	p.1689F	48.94
	p.I1689F	50.12
	p.I1689F	51.42
	p.I1689F	49.20
	SETBP1(15)	p.P1563L
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
	p.G12D	4.74
	p.G12C	4.03
	p.G12D	6.61
	p.Q61R	1.80
	p.G13D	4.85
	p.G12D	30.81
	p.G12D	46.83
	p.G12D	1.75
	p.G13V	6.36
CEBPA (15)	p.Q16H	22.49
	p.G32fs	25.35
	p.K313delinsQK	59.26
	p.A66fs	>10.00
	p.A303P	48.16
	p.P23fs	46.12
	p.A72LfsX35	+
	p.L317delinsRL	48.27
	p.P23fs	2.70
	TET2 (15)	p.F868L
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
	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, %
	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 3 $\alpha$ ; *NOTCH2*, Notch 2; *SETBP1*, SET binding protein 1; *NRAS*, NRAS proto-oncogene, GTPase; *CEBPA*, CCAAT enhancer binding protein  $\alpha$ ; *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			
Mutations	WBC (H/L)		P-value
	Mutation	Non-mutation	
<i>FLT3</i>	7/7	10/38	0.04402
<i>NRAS</i>	6/4	11/32	0.009661
<i>CEBPA</i>	5/4	12/41	0.049879

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

C, Cytogenetics			
Mutations	Karyotype, abnormal/normal		P-value
	Mutation	Non-mutation	
<i>DNMT3A</i>	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  $\alpha$ ; *ASXL1*, ASXL transcriptional regulator 1; *DNMT3A*, DNA methyltransferase 3 $\alpha$ .

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

Clinic characteristics	<i>NPM1</i>	<i>FLT3</i>	<i>FAT1</i>	<i>ASXL1</i>	<i>DNMT3A</i>	<i>NOTCH2</i>	<i>SETBP1</i>	<i>NRAS</i>	<i>CEBPA</i>	<i>TET2</i>	<i>CREBBP</i>
Age at study entry, years	0.490	0.491	0.209	0.153	0.116	0.519	0.985	0.212	0.263	0.637	0.820
Sex, male/female	0.875	0.033	0.590	0.695	0.080	0.760	0.750	0.569	0.750	0.680	0.793
WBC, H/L	0.695	0.034	0.269	0.705	0.902	0.003	0.092	0.108	0.645	0.061	0.577
Bone marrow blast count	0.647	0.114	0.717	0.801	0.777	0.907	0.151	0.698	0.464	0.955	0.412
AML FAB subtype, M0/M1/M2/M4/M5/M6/M7	0.707	0.536	0.442	0.242	0.045	0.811	0.978	0.145	0.739	0.279	0.638
Cytogenetics, abnormal/normal	0.204	0.137	0.735	0.438	0.021	0.277	0.186	0.863	0.001	0.872	0.904
High risk, high/medium/low	0.111	0.971	0.292	0.933	0.826	0.976	0.528	0.929	0.530	0.664	0.163
Response evaluation, CR/NR	0.952	0.529	0.668	0.176	0.148	0.409	0.036	0.793	0.365	0.277	0.003
Consolidation therapy following CR, chemotherapy/hematopoietic stem cell transplantation	0.804	0.532	0.184	0.427	0.535	0.801	0.024	0.916	0.905	0.664	0.046

WBC, white blood cell; AML, acute myeloid leukemia; FAB, French-American-British; CR, complete remission; NR, non-remission; *NPM1*, nucleophosmin 1; *FLT3*, Fms related tyrosine kinase 3; *FAT1*, FAT atypical cadherin 1; *ASXL1*, ASXL transcriptional regulator 1; *DNMT3A*, DNA methyltransferase 3 $\alpha$ ; *NOTCH2*, Notch 2; *SETBP1*, SET binding protein 1; *NRAS*, NRAS proto-oncogene, GTPase; *CEBPA*, CCAAT enhancer binding protein  $\alpha$ ; *TET2*, Tet methylcytosine dioxygenase 2; *CREBBP*, cyclic adenosine 5'-phosphate response element-binding protein binding protein.

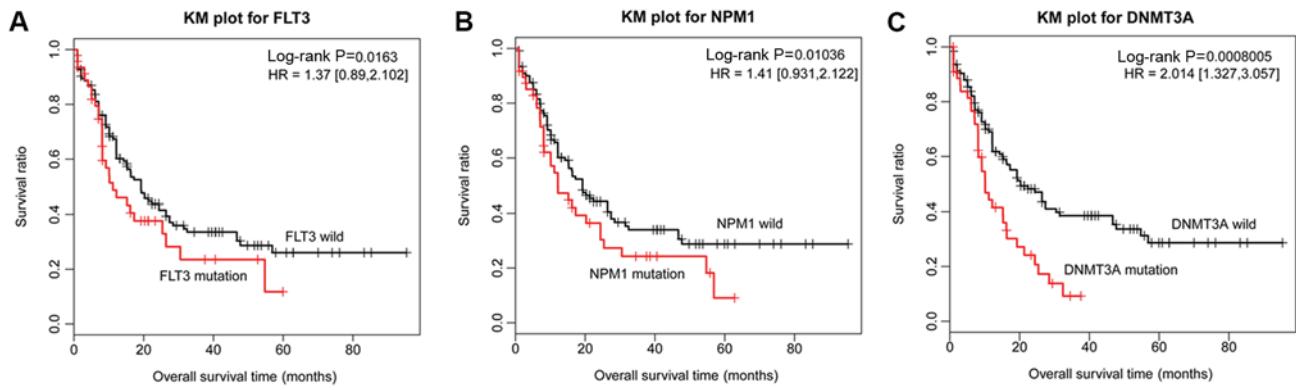


Figure 3. Survival curves of mutated genes based on The Cancer Genome Atlas database. (A) KM survival curve of *FLT3*. (B) KM survival curve of *NPM1*. (C) KM survival curve of *DNMT3A*. KM, Kaplan-Meier; HR, hazard ratio; *FLT3*, Fms related tyrosine kinase 3; *NPM1*, nucleophosmin 1; *DNMT3A*, DNA methyltransferase 3 $\alpha$ .



Figure 4. Combined mutations of 11 high frequency mutations. Different colours represent different mutations, and the length of each coloured line represents the mutation number. The curve in the middle of the circle represents the sample, and the two ends of the curve are the genetic mutations that occurred in a sample.

were significantly enriched in 15 biological processes, including ‘hemopoietic or lymphoid organ development’ ( $P=2.15 \times 10^{-3}$ ), ‘negative regulation of cell differentiation’ ( $P=1.49 \times 10^{-3}$ ), ‘haemopoiesis’ ( $P=1.78 \times 10^{-3}$ ) and ‘immune system development’ ( $P=2.42 \times 10^{-3}$ ). 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

Table VI. Clinical features of 3 patients with concurrent *FLT3* and *NOTCH2* mutations.

Clinical features	Sample ID		
	Sample 2	Sample 9	Sample 15
Age, years	46	58	67
Sex	Female	Male	Male
White blood cell ( $\times 10^9/l$ )	61.51	184.23	21.42
Bone marrow blast count	64.5	89	70
Diagnosis	M4	Unclassified	M5
Immunophenotype			
CD13	+	+	+
CD15	+		+
CD33	+	+	+
CD34	+	+	
CD117	+	+	
MPO	+	+	+
CD64	+	+	+
HLA-DR	+	+	+
CD56	+		
CD38	+	+	+
Cytogenetics	Abnormal	Normal	Normal
Risk	Medium	Medium	Medium
Response evaluation	NR	Unevaluated	CR

*FLT3*, Fms related tyrosine kinase 3; *NOTCH2*, Notch 2; WBC, white blood cell; CD13, aminopeptidase N; CD15, sialyl Lewis<sup>x</sup>; 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.

CD33 (CD33), myeloperoxidase (MPO), high affinity immunoglobulin  $\gamma$  Fc receptor I (CD64), human leukocyte antigen-DR isotype (HLA-DR) and ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1 (CD38) expression (Table VI). In addition, 3 patients

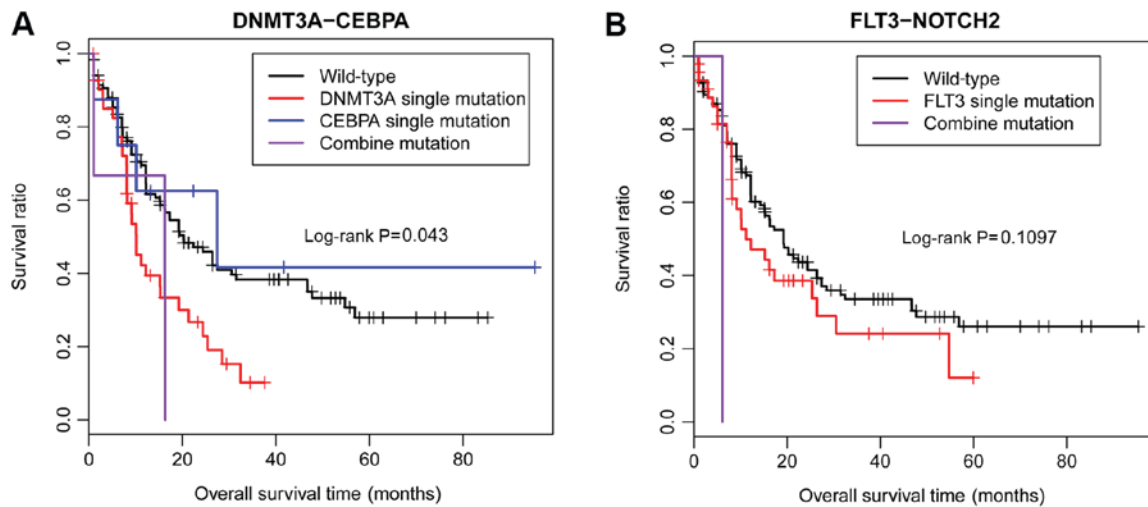


Figure 5. Survival curve analytical results of co-mutations based on The Cancer Genome Atlas database. (A) Kaplan-Meier survival curve of co-mutation of *DNMT3A-CEBPA*. (B) Kaplan-Meier survival curve of co-mutation of *FLT3-NOTCH2*. *DNMT3A*, DNA methyltransferase 3 $\alpha$ ; *CEBPA*, CCAAT enhancer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

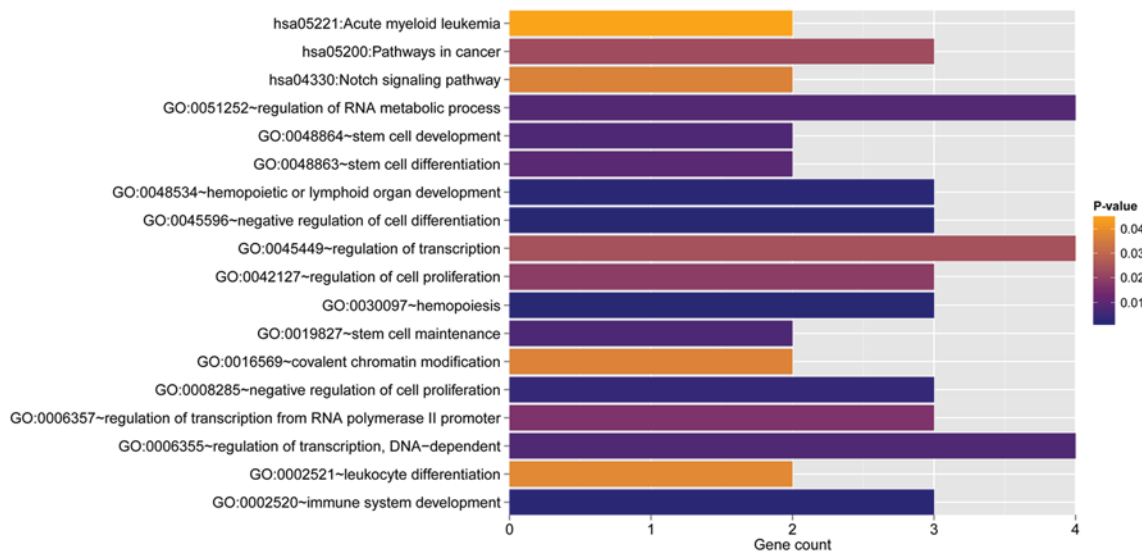


Figure 6. Functional enrichment analysis for co-mutations of Fms related tyrosine kinase 3-Notch 2, DNA methyltransferase 3 $\alpha$ -CCAAT enhancer binding protein  $\alpha$  and SET binding protein 1-cyclic adenosine 5'-phosphate response element-binding protein binding protein. GO, gene ontology.

were identified to possess the *SETBP-CREBBP* mutation, and presented with positive CD13, CD33, hematopoietic progenitor cell antigen CD34 (CD34), mast/stem cell growth factor receptor Kit (CD117), MPO, HLA-DR, neural cell adhesion molecule 1 (CD56) and CD38 expression, and abnormal cytogenetics (Table VII). However, no patients in our study were identified as having the *DNMT3A-CEBPA* co-mutation.

**Discussion**

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

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

Clinical features	Sample ID		
	Sample 2	Sample 3	Sample 44
Age, years	46	23	33
Sex	Female	Male	Female
WBC (10 <sup>9</sup> /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<sup>x</sup>; 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.

frequency was decreased compared with the previously described rate (~30%) (36). This discrepancy may be due to differences in the ethnicity, geographical region and sample size of the investigated subjects between these studies. In the present study, the *FLT3* mutation was also identified to be negatively associated with the survival of patients with AML based on data from TCGA database, which was consistent with the results obtained by a previous study (35). *NOTCH2* receptor signalling was demonstrated to govern the differentiation of dendritic cells in the spleen and intestine (37). Additionally, *NOTCH2* also controls the rate of generation of long- and short-term repopulating stem cells in mice (38). In the present study, a *NOTCH2* mutation was detected in 14.52% of patients with AML, and the co-mutation of *FLT3-NOTCH2* was significantly associated with the variation of WBCs in patients with AML. Additional analysis revealed that patients with the *FLT3-NOTCH2* co-mutation also exhibited positive CD13, CD33, MPO, CD64, HLA-DR and CD38 expression, indicating that *FLT3-NOTCH2* may serve critical roles in the regulation of the immune response.

*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

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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