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RESEARCH ARTICLE

Mutation profiling of 16 candidate genes in *de novo* acute myeloid leukemia patients

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Abstract **This retrospective analysis aimed to investigate the mutation profile of 16 common mutated genes in *de novo* acute myeloid leukemia (AML) patients. A total of 259 patients who were diagnosed of *de novo* AML were enrolled in this study. Mutation profiling of 16 candidate genes were performed in bone marrow samples by using Sanger sequencing. We identified at least 1 mutation in 199 of the 259 samples (76.8%), and 2 or more mutations in 31.7% of samples. *FLT3-ITD* was the most common mutated gene (16.2%, 42/259), followed by *CEBPA* (15.1%, 39/259), *NRAS* (14.7%, 38/259), and *NPM1* (13.5%, 35/259). Concurrence was observed in 97.1% of the *NPM1* mutated cases and in 29.6% of the double mutated *CEBPA* cases. Distinct patterns of co-occurrence were observed for different hotspot mutations within the *IDH2* gene: *R140* mutations were associated with *NPM1* and/or *FLT3-ITD* mutations, whereas *R172* mutations co-occurred with *DNMT3A* mutations only. Concurrence was also observed in 86.6% of epigenetic regulation genes, most of which co-occurred with *NPM1* mutations. The results showed certain rules in the mutation profiling and concurrence of AML patients, which was related to the function classification of genes. Defining the mutation spectrum and mutation pattern of AML will contribute to the comprehensive assessment of patients and identification of new therapeutic targets.**

Keywords leukemia; myeloid; acute; gene; mutation

Introduction

Acute myeloid leukemia (AML) is a group of heterogeneous diseases [1]. Along with the progress in molecular biology regarding hematological malignancies and the use of next generation sequencing technology, more molecular genetic markers have been identified. Different mutations can respectively be of unilateral or multivariate clinical significance in clonality, diagnosis, prognosis assessment, and individual therapy [2,3]. Moreover, the newly published 2016 revision to the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia and lymphoid neoplasms include several molecular genetic markers with well-defined diagnostic and prognostic values, which provide molecular basis for highly precise classification and treatment of diseases, including mutations of *CALR*, *CSF3R*, *SF3B1*, *RUNX1*, and *PCM1-JAK2*. In *de novo* AML, AML with mutated *RUNX1* has been added as a provisional category that has a possibly worse prognosis. AML with biallelic mutated *CEBPA* is specifically defined and associated with improved prognosis, but not single *CEBPA* mutation [4,5]. Furthermore, the hematological malignancy gene mutation profiling is no longer confined to the detection of only a few molecular targets, and the current study and application of leukemia has entered the "mutaome" [6] profile era.

The initiation and progression of tumors result from the mutational synergism of many different functional genes. Current studies have discovered certain rules on the occurrence and mutation pattern of gene mutation. In this study, we analyzed 16 common mutated genes in *de novo* AML patients and investigated the rules of mutation spectrum, thus offering an approach to further explore the molecular biological mechanism of tumorigenesis and find new therapeutic targets.

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Materials and methods

Subjects

From June 2014 to June 2016, a total of 259 patients were diagnosed with AML at Hebei Yanda Lu Daopei Hospital and included 153 males and 106 females aged from 2 to 68 years, with a median age of 23 years. We defined the children group as aged $\leqslant$ 14 years, adult group as aged between 15 and 55 years, and the elderly group as aged $\geqslant$ 55 years. The diagnosis of AML was performed according to the WHO 2008 classification criteria.

DNA extraction

Bone marrow specimens (n = 78) or bone marrow smear specimens (n = 181) of the *de novo* AML patients were collected. The number of white blood cells was measured by using a blood cell counter for each sample before extraction. Additional handling before counting the bone marrow smear samples involved pipetting with purified water repeatedly to dissolve the cells. Moreover, 1.0×10^7/mL karyocytes were acquired to ensure enough genomic DNA output. Genomic DNA were extracted by using a column DNA extraction kit (Tiangen Biotech (Beijing) Co., Ltd., Item No. DP318-03) according to the manufacturer's protocol.

Gene sequencing and mutation analysis

A total of 16 common mutated genes, namely, *ASXL1*, *CEBPA*, *DNMT3A*, *ETV6*, *FLT3*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *NRAS*, *NPM1*, *PTPN11*, *PHF6*, *RUNX1*, *TET2*, and *TP53* were detected of mutation hotspot regions that have already been reported in the literature (Supplementary Table S1). Sanger sequencing was performed by using an AB 3500XL sequencer, whose detection sensitivity is approximately 15%–20%; additionally, *FLT3-ITD* and *NPM1* mutations were detected by fragment length analysis by using an AB 3500XL sequencer with a detection sensitivity of approximately 1%–3%. Gene mutation analysis was performed by using Variant Reporter V1.1 software (Life, America). The evaluation of leukemia-associated somatic mutations was followed by previously reported principles [7].

Statistical analysis

The χ^2 test and Fisher exact test were performed with SPSS (version 19.0; SPSS Inc., Chicago, IL) to analyze the gene-gene co-occurrence correlation and the correlation of mutations and age. $P < 0.05$ was considered statistically significant.

Results

Overall gene mutation frequency and hotspots

We identified at least 1 mutation in 199 of the 259 samples (76.8%), and 2 or more mutations in 31.7% of samples, among which, 53 (20.5%), 24 (9.3%), and 5 (2.0%) samples carried 2, 3, and 4 kinds of gene mutations, respectively. A total of 64 combinations of gene mutations were detected. The most frequently mutated two-gene combinations included *NPM1* + *FLT3-ITD*, *DNMT3A* + *NPM1*, *DNMT3A* + *IDH2*, *IDH2* + *NPM1*, and *TET2* + *NPM1*, and these were respectively detected in 9, 9, 6, 6, and 6 samples; moreover, the most frequently mutated three-gene combinations included *DNMT3A* + *NPM1* + *FLT3 (ITD/TKD)* and *DNMT3A* + *TET2* + *NPM1*, and these were respectively detected in 4 and 3 samples. *FLT3-ITD* (16.2%, 42/259) was the most frequently mutated gene, followed by CEBPA (15.1%, 39/259), *NRAS* (14.7%, 38/259), and *NPM1* (13.5%, 35/249), which accounted for 59.5% of the total mutation frequency. The mutation frequencies of other genes are shown in Fig. 1.

A total of 26 kinds of insert-fragment lengths were found in the *FLT3-ITD* mutation, of which the minimum length was 12 base pair (bp), the maximum length was 204 bp, and the common insert lengths were 21 bp, 33 bp, and 57 bp, of which 3 cases carried 2 insert fragments and 2 cases carried 3 fragments. A total of 6 of 8 patients carried *FLT3-TKD* D835 mutations, and the other 2 cases carried D839G and I836_M837insG mutations. A total of 28 patients carried 10 kinds of *KIT* mutations, among which 14 patients carried D816 mutations and 11 patients carried N822K mutations. Moreover, 2 patients carried more than 2 kinds of mutations (2 and 3 mutations, respectively). Furthermore, 28 cases carried *NRAS* mutations, all of which were G12 (19/38), G13 (9/38), Q61 (10/38), and G60 (2/38) mutations, of which 2 cases carried two mutations. A total of 7 cases carried *KRAS* mutations, all of which were G12 (6/7) and G13 (2/7) mutations, of which 1 case carried 2 mutations. A total of 18 cases carried *PTPN11* mutations, of which 15 cases were in exon 3, and 3 cases were in exon 13. A total of 39 cases carried *CEBPA* mutations, of which 12 cases were single-mutated *CEBPA*, 9 cases were at the N-terminal, and 3 cases were at the C-terminal; moreover, 27 cases were double-mutated CEBPA, and all were at the N-terminal and C-terminal. A total of 18 cases carried *RUNX1* mutations scattered in the whole coding area, of which 8 cases were missense mutations and were mainly concentrated on exons 5 and 6, and 7 cases were frameshift mutations and mainly concentrated on exon 9, of which 2 cases carried 2 and 3 mutations at the same time. A total of 35 cases carried *NPM1* mutations, of which 22 cases were A-type mutations, B- and D-type mutations were found in 1

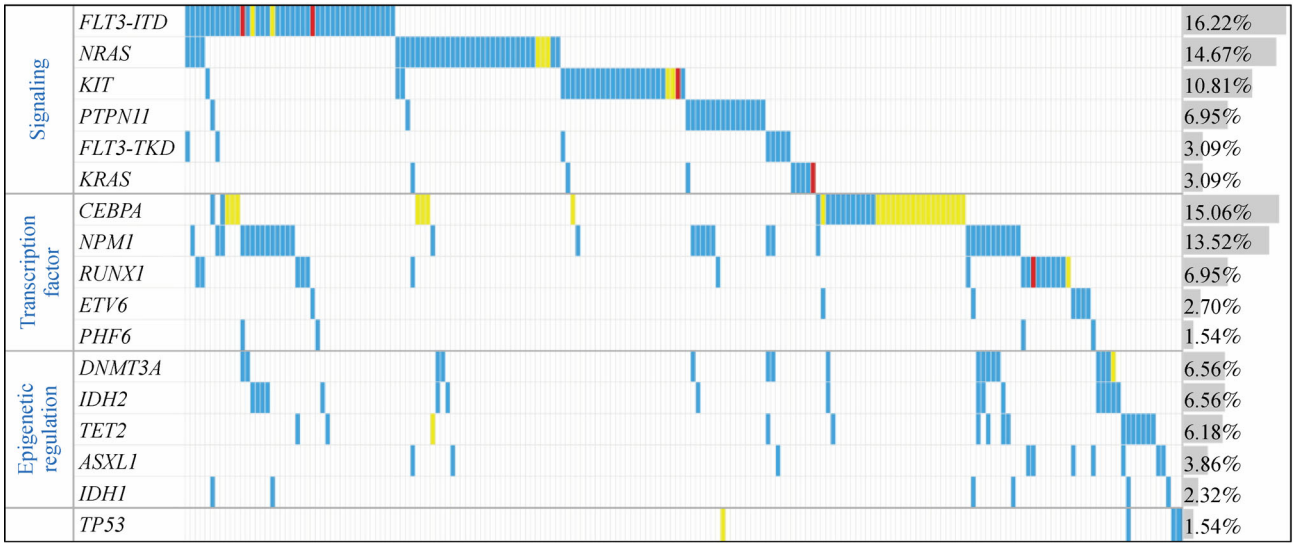

Fig. 1 Mutation spectrum of 16 common mutated genes in AML. Rows in the graph represent individual gene mutations, and the columns represent individual patients in the study. Vertical blue lines indicate the presence of one mutation in a patient. Vertical yellow lines and red lines indicate the presence of double and triple mutations in a patient, respectively.

case, and 4 cases were rare type; furthermore, the remaining 7 cases were not classified owing to being below the detection sensitivity of Sanger sequencing. A total of 10 cases carried *ASXL1* mutations, of which 5 cases were nonsense mutations, 4 cases were frameshift mutations, and 1 case was missense mutation. A total of 17 cases carried *DNMT3A* mutations, of which 16 cases were R882 mutations, 1 case carried two kinds of mutations at the same time. A total of 6 cases carried *IDH1* mutations, and all of which were R132 mutations. A total of 17 cases carried *IDH2* mutations, of which 13 cases were R140 mutations and 4 cases were R172K mutations. Finally, *TET2*, *ETV6*, *PHF6*, and *TP53* mutations were scattered without significant mutation hotspots.

Mutation spectra of genes in different function classifications

The results showed that 142 of the 259 samples (54.8%) carried signal transduction mutations, among which *FLT3-ITD*, *NRAS*, and *KIT* were frequently mutated with 16.2%, 14.7%, and 10.8%, respectively. A total of 103 of the 259 samples (39.8%) carried transcription factor mutations, among which *CEBPA*, *NPM1*, and *RUNX1* were frequently mutated with 15.1%, 13.5%, and 7.0%, respectively. A total of 66 of the 259 samples (25.5%) carried epigenetic regulation mutations, among which *DNMT3A*, *IDH2*, and *TET2* were frequently mutated with 6.6%, 6.2%, and 6.2%, respectively. A total of 4 of the 259 samples (1.5%) carried *TP53* mutations. The gene mutations of different function classifications could co-occur with each other, and we observed 87.9% (58/66), 64.1% (66/103), and 50.7% (72/142) of the epigenetic regulation, transcription factor, and signal transduction mutations to be co-occurring with other gene mutations (Fig. 2A).

Gene-gene co-occurrence

Signal transduction genes (Fig. 2B, Table 1): We identified 29 of 42 cases (69.1%) with *FLT3-ITD* mutation co-occurred with other gene mutations, among which 14 mutated with *NPM1*. *NPM1* was the most frequent concomitant mutation partner of *FLT3-ITD* (33.3% vs. 13.4%, $P < 0.05$). Moreover, *NRAS* and *KRAS* mutations can occur alone or co-occur with other gene mutations, but rarely with *CEBPA* and *NPM1*. Among patients with mutated *KIT*, 21 of 28 cases (75.0%) occurred alone, 7 cases co-occurred with other gene mutations, but rarely with *FLT3-ITD* and *CEBPA*. Furthermore, *PTPN11* mutations co-occurred in 10 of 18 cases (55.6%), among which 5 were *NPM1* mutation (27.8% vs. 12.4%, $P > 0.05$).

Transcription factor genes (Fig. 2C, Table 2): We identified 26 of 39 cases (66.7%) with *CEBPA* mutations occurred alone, and 33.3% (13/39) of *CEBPA* mutations co-occurred; among the 27 patients carrying double-mutated *CEBPA*, 19 cases carried double-mutated *CEBPA* alone, and 8 cases co-occurred with other mutations (3 *FLT3-ITD*, 3 *NRAS*, 1 *KIT*, and 1 *ETV6*). Among patients with mutated *NPM1*, 34 of 35 cases (97.1%) co-occurred with other gene mutations, including 14 with *FLT3-ITD* mutation (40.0% vs. 17.1%, $P < 0.05$), 10 with *DNMT3A* mutation (28.6% vs. 4.3%, $P < 0.05$), 8 with *IDH2* mutations (22.9% vs. 5.5%, $P < 0.05$), 6 with *TET2*

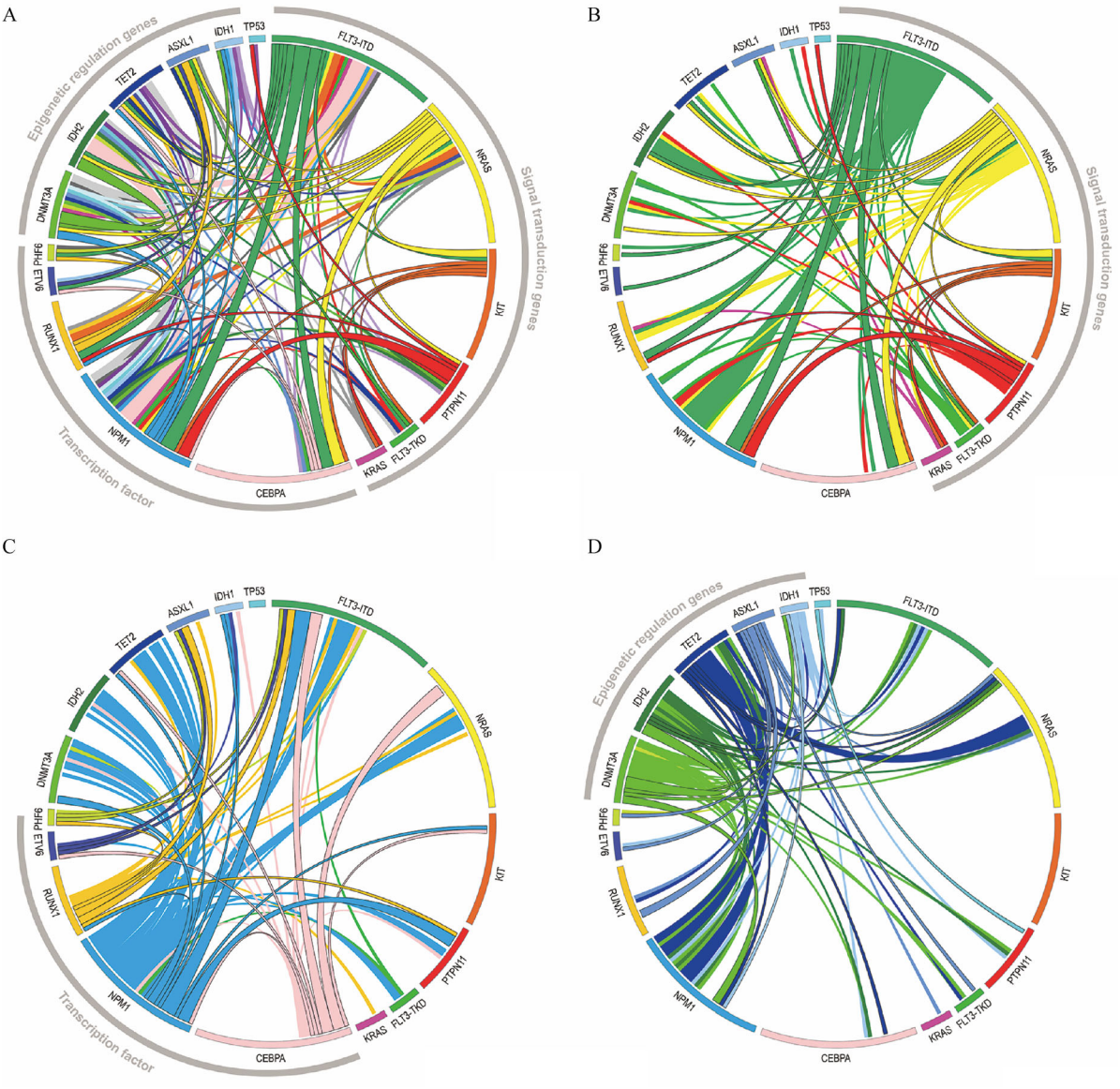

Fig. 2 Gene function classification and co-occurrence of 16 common mutated genes in AML. (A) Co-occurrence of genes within three different function classifications. (B) Co-occurrence of signal transduction genes. (C) Co-occurrence of transcription factor genes. (D) Co-occurrence of epigenetic regulation genes.

mutations, and 3 with *IDH1* mutation. Moreover, *FLT3-ITD*, *DNMT3A*, and *IDH2* are the most common concomitant mutant partners of *NPM1*. *RUNX1* mutation co-occurred in 11 of 18 cases (61.1%), and its frequent mutated partner is *ASXL1* mutation. Furthermore, *ETV6* mutations can occur alone or with other gene mutations. Only 4 cases of *PHF6* mutations were detected, and all of which co-occurred.

Epigenetic regulation genes (Fig. 2D, Table 3): ALL *DNMT3A* mutations co-occurred with other gene mutations, including 10 with *NPM1* (58.8% vs. 13.7%, $P < 0.05$), and 7 with *IDH2* (4 R140Q mutations, 3 R172K mutations) (47.1% vs. 4.9%, $P < 0.05$). *NPM1* and *IDH2* are the most common concomitant mutant partners of *DNMT3A*. *IDH2* mutations co-occurred in 16 of 17 cases (94.1%), including 8 cases with *NPM1* (47.1% vs. 14.8%, $P < 0.05$), and 8 cases with *DNMT3A* (47.1% vs. 4.9%, $P < 0.05$). Moreover, the *NPM1* and *DNMT3A* mutations were the most common concomitant mutant partners of *IDH2*. *IDH1* mutations co-occurred in 6 of 7 cases (85.7%), including 3 cases mutated with *NPM1*. *TET2* mutations co-occurred in 12 of 16 cases (75.0%), and 6 cases were associated with *NPM1* mutations. *ASXL1* mutations co-occurred in 8 of 10 cases (80.0%), and 6 cases were associated with *RUNX1* mutations.

Table 1 Co-occurrence of *FLT3*, *NRAS*, and *KIT* gene mutations

	FLT3-ITD M	*FLT3-ITD* W	*P* value	*NRAS* M	*NRAS* W	*P* value	*KIT* M	*KIT* W	*P* value
	n = 42	*n* = 157		*n* = 38	*n* = 161		*n* = 28	*n* = 171	
FLT3-ITD	–	–	–	4 (10.5%)	38 (23.6%)	*	1 (3.6%)	41 (24.0%)	0.014
CEBPA	5 (11.9%)	34 (21.7%)	*	3 (7.9%)	36 (22.4%)	0.043	1 (3.6%)	38 (22.2%)	0.021
NRAS	4 (9.5%)	34 (21.7%)	*	–	–	–	2 (7.1%)	36 (21.1%)	*
NPM1	14 (33.3%)	21 (13.4%)	0.003	2 (5.3%)	33 (20.5%)	0.027	1 (3.6%)	34 (19.9%)	*
KIT	1 (2.4%)	27 (17.2%)	0.014	2 (5.3%)	26 (16.1%)	*	–	–	–
PTPN11	1 (2.4%)	17 (10.8%)	*	1 (2.6%)	17 (10.6%)	*	0	18 (10.5%)	*
RUNX1	5 (11.9%)	13 (8.3%)	*	3 (7.9%)	15 (9.3%)	*	0	18 (10.5%)	*
DNMT3A	2 (4.8%)	15 (9.6%)	*	2 (5.3%)	15 (9.3%)	*	0	17 (9.9%)	*
IDH2	5 (11.9%)	12 (7.6%)	*	2 (5.3%)	15 (9.3%)	*	0	17 (9.9%)	*
TET2	2 (4.8%)	14 (8.9%)	*	2 (5.3%)	14 (8.7%)	*	0	16 (9.4%)	*
ASXL1	0	10 (6.4%)	*	2 (5.3%)	8 (5.0%)	*	0	10 (5.8%)	*
FLT3-TKD	2 (4.8%)	6 (3.8%)	*	1 (2.6%)	7 (4.3%)	*	1 (3.6%)	7 (4.1%)	*
KRAS	0	8 (5.1%)	*	1 (2.6%)	7 (4.3%)	*	1 (3.6%)	7 (4.1%)	*
ETV6	1 (2.4%)	6 (3.8%)	*	0	7 (4.3%)	*	0	7 (4.1%)	*
IDH1	2 (4.8%)	4 (2.5%)	*	0	6 (3.7%)	*	0	6 (3.5%)	*
PHF6	2 (4.8%)	2 (1.3%)	*	0	4 (2.5%)	*	0	4 (2.3%)	*
TP53	0	4 (2.5%)	*	0	4 (2.5%)	*	0	4 (2.3%)	*

M, mutated; W, wild-type; *n*, number of subjects; *, $P>0.05$.

Table 2 Co-occurrence of *CEBPA*, *NPM1*, and *RUNX1* gene mutations

	CEBPA M	*CEBPA* W	*P* value	*NPM1* M	*NPM1* W	*P* value	*RUNX1* M	*RUNX1* W	*P* value
	n = 39	*n* = 160		*n* = 35	*n* = 164		*n* = 18	*n* = 181	
FLT3-ITD	5 (12.8%)	37 (23.1%)	*	14 (40.0%)	28 (17.1%)	0.003	5 (2.8%)	42 (23.2%)	*
CEBPA	–	–	–	2 (5.7%)	37 (22.6%)	0.023	0	39 (21.5%)	*
NRAS	3 (7.7%)	35 (21.9%)	0.043	2 (5.7%)	36 (22.0%)	0.027	3 (16.7%)	38 (21.0%)	*
NPM1	2 (5.1%)	33 (20.6%)	0.023	–	–	–	1 (5.6%)	34 (18.8%)	*
KIT	1 (2.6%)	27 (16.9%)	0.021	1 (2.9%)	27 (16.5%)	*	0	28 (15.5%)	*
PTPN11	1 (2.6%)	17 (10.6%)	*	5 (14.3%)	13 (7.9%)	*	1 (5.6%)	18 (9.9%)	*
RUNX1	0	18 (11.3%)	*	1 (2.9%)	17 (10.4%)	*	–	–	–
DNMT3A	1 (2.6%)	16 (10.0%)	*	10 (28.6%)	7 (4.3%)	0.000	0	17 (9.4%)	*
IDH2	1 (2.6%)	16 (10.0%)	*	8 (22.9%)	9 (5.5%)	0.003	0	17 (9.4%)	*
TET2	1 (2.6%)	15 (9.4%)	*	6 (17.1%)	10 (6.1%)	*	1 (5.6%)	15 (8.3%)	*
ASXL1	0	10 (6.3%)	*	0	10 (6.1%)	*	3 (16.7%)	7 (3.9%)	*
FLT3-TKD	0	8 (5.0%)	*	3 (8.6%)	5 (3.0%)	*	0	8 (4.4%)	*
KRAS	0	8 (5.0%)	*	0	8 (4.9%)	*	1 (5.6%)	8 (4.4%)	*
ETV6	1 (2.6%)	6 (3.8%)	*	1 (2.9%)	6 (3.7%)	*	0	7 (3.9%)	*
IDH1	1 (2.6%)	5 (3.1%)	*	3 (8.6%)	3 (1.8%)	*	0	6 (3.3%)	*
PHF6	0	4 (2.5%)	*	1 (2.9%)	3 (1.8%)	*	1 (5.6%)	3 (1.7%)	*
TP53	0	4 (2.5%)	*	0	4 (2.4%)	*	0	4 (2.2%)	*

M, mutated; W, wild-type; *n*, number of subjects; *, $P>0.05$.

Gene hotspot co-occurrence

Mutational hotspots were present in some genes, which could be single or multiple. Among the 16 genes detected, 2 hotspots were found in the *IDH2* gene, namely R140 and R172. The two most common concomitant mutant partners of *IDH2* were *NPM1* and *DNMT3A*, among which R172 mutation only co-occurred with *DNMT3A* mutation (50.0% vs. 0%, $P < 0.05$), whereas R140 mutation could co-occur with *NPM1* or *DNMT3A* mutation. However, mutations that co-occurred with *NPM1* were all R140 mutations. Through analyses of *NRAS* and *KIT*,

Table 3 Co-occurrence of *DNMT3A*, *IDH2*, and *TET2* gene mutations

	DNMT3A M	*DNMT3A* W	*P* value	*IDH2* M	*IDH2* W	*P* value	*TET2* M	*TET2* W	*P* value
	n = 17	*n* = 182		*n* = 17	*n* = 182		*n* = 16	*n* = 183	
FLT3-ITD	2 (11.8%)	40 (22.0%)	*	2 (11.8%)	40 (22.0%)	*	2 (12.5%)	40 (21.9%)	*
CEBPA	1 (5.9%)	38 (20.9%)	*	1 (5.9%)	38 (20.9%)	*	1 (6.3%)	38 (20.8%)	*
NRAS	2 (11.8%)	36 (19.8%)	*	2 (11.8%)	36 (19.8%)	*	2 (12.5%)	36 (19.7%)	*
NPM1	10 (58.8%)	25 (13.7%)	0.000	8 (47.1%)	27 (14.8%)	0.003	6 (37.5%)	29 (15.8%)	*
KIT	0	28 (15.4%)	*	0	28 (15.4%)	*	0	28 (15.3%)	*
PTPN11	1 (5.9%)	17 (9.3%)	*	1 (5.9%)	17 (9.3%)	*	0	18 (9.8%)	*
RUNX1	0	18 (9.9%)	*	0	18 (9.9%)	*	1 (6.3%)	17 (9.3%)	*
DNMT3A	–	–	–	8 (47.1%)	9 (4.9%)	0.000	3 (18.8%)	14 (7.7%)	*
IDH2	8 (47.1%)	9 (4.9%)	0.000	–	–	–	2 (12.5%)	15 (8.2%)	*
TET2	3 (17.6%)	13 (7.1%)	*	2 (11.8%)	14 (7.7%)	*	–	–	–
ASXL1	0	10 (5.5%)	*	0	10 (5.5%)	*	1 (6.3%)	9 (4.9%)	*
FLT3-TKD	2 (11.8%)	6 (3.3%)	*	0	8 (4.4%)	*	1 (6.3%)	7 (3.8%)	*
KRAS	0	8 (4.4%)	*	0	8 (4.4%)	*	0	8 (4.4%)	*
ETV6	0	7 (3.8%)	*	0	7 (3.8%)	*	0	7 (3.8%)	*
IDH1	0	6 (3.3%)	*	0	6 (3.3%)	*	1 (6.3%)	5 (2.7%)	*
PHF6	1 (5.9%)	3 (1.6%)	*	0	4 (2.2%)	*	0	4 (2.2%)	*
TP53	0	4 (2.2%)	*	0	4 (2.2%)	*	1 (6.3%)	3 (1.6%)	*

M, mutated; W, wild-type; *n*, number of subjects; * $P>0.05$.

no evidence indicated that different mutation hotspots were present with different concurrence rules.

Gene mutation and age

The total gene mutation frequency and mutation frequency of carrying more than one gene were higher in the adult and elderly groups than in the children group (82.5%, 85.0% vs. 66.7%, $P < 0.05$; 49.2%, 58.8% vs. 25.0%, $P < 0.05$). Moreover, the single gene mutation frequency was higher in the children group than those in other two groups (75.0% vs. 42.0%, 41.2%, $P < 0.05$). Among different gene functional groups, the mutation frequency of kinase genes in the children group was higher than that in the adult group (78.1% vs. 55.9%, $P < 0.05$), the mutation frequency of transcription factor genes in the adult group was higher than that in the children group (57.6% vs. 29.7%, $P < 0.05$), and the mutation frequency of epigenetic regulation genes was higher in the adult and elderly groups than that in the children group (31.4%, 41.1% vs. 10.9%, $P < 0.05$). Among different gene mutations, the frequency of *KIT* mutation was higher in the children group than in the adult group (25.0% vs. 7.6%, $P < 0.05$), the frequency of *CEBPA* and *DNMT3A* mutations were higher in the adult group than in the children group (*CEBPA* 26.3% vs. 10.9%, $P < 0.05$; *DNMT3A* 11.9% vs. 1.6%, $P < 0.05$), and the frequency of *NPM1* and *IDH2* mutations were higher in the adult and elderly group than in the children group (*NPM1* 22.0%, 29.4% vs. 6.3%, $P < 0.05$; *IDH2* 10.2%, 23.5% vs. 0%, $P < 0.05$).

Discussion

We selected 16 genes that are commonly mutated in AML, including genes that clearly guide diagnosis and prognosis stratification, such as *NPM1*, *CEBPA*, *FLT3-ITD*, *TP53*, *ASXL1*, and *RUNX1*; genes with definite or potential guidance for therapy, such as *FLT3*, *KIT*, *DNMT3A*, *TET2*, and *IDH1/2*; and other common genes that can assist in clonal judgments, such as *NRAS*, *KRAS*, *PTPN11*, *ETV6*, and *PHF6*. By detecting 16 common mutated genes in AML, 76.8% of newly diagnosed AML patients were observed to carry at least one gene mutation. Targeted sequencing of 16 genes can assist nearly 80% of patients find molecular etiology and help with adjuvant diagnosis, prognosis stratification, and treatment in a cost-effective manner compared with whole genome sequencing (WGS) or whole exome sequencing (WES).

Despite our understanding of the pathobiology and genomic landscape of AML having improved substantially, the chemotherapy-directed management has remained largely unchanged in the past 40 years [8]. In recent years, various novel agents have demonstrated clinical activity, such as FLT3 inhibitors and epigenetic modifiers (IDH inhibitors, DNMTs inhibitors, HDAC inhibitors) [8,9]. In our research, 54.8% and 25.5% of the samples carried signal transduction genes and epigenetic regulation gene mutations, respectively, among which 52.3% of the patients who carried *FLT3*, *KIT*, *DNMT3A*, *IDH1/2*, and *TET2* mutations could benefit from these novel agents.

Previous studies have discovered that the combination of gene mutations has certain rules, and these are closely related to gene function classification [10]. This study has been observed in similar patterns, suggesting that in the initiation and progress of AML, the synergistic effect of gene mutations with different kinds of functions is invariable. In addition, the different mutation hotspots within the same gene have different concomitance rules, and *DNMT3A* mutations could co-occur either with R140 or R172 mutations; whereas *IDH2* R172 mutations only co-occurred with *DNMT3A* mutations. Moreover, *NPM1* and/or *FLT3-ITD* mutations only co-occurred with R140 mutations, which suggests that the occurrence of *NPM1* and *FLT3-ITD* mutation clones was later than those of *DNMT3A* and *IDH2* mutations clone, and different mutation hotspots may function differently, and their synergistic mutation types may also be different. Papaemmanuil *et al.* found that different hotspots within the *NRAS* gene have different concurrence rules, and *NPM1* mutations are often accompanied by *NRAS* G12/13 mutations while being mutually exclusive with the *NRAS* Q61 mutations [10]. However, in our study, only 2 of 38 patients with *NRAS* mutations co-occurred with *NPM1* mutations, which were Q61R and G13D mutations. No evidence in this study indicated that different mutation hotspots within the *NRAS* gene have different concurrence rules.

We observed distinct gene mutation profiles in different age groups; moreover, the mutation frequency of kinase genes in children was higher, and the mutation frequency of transcription factor genes and epigenetic regulation genes were higher in the adult and elderly groups. The results were consistent with other large studies. Papaemmanuil *et al*. reported a large AML group that mainly included adults and elderly patients, in which the three largest subgroups were mutations of *NPM1*, *CEBPA*, and chromatin and RNA-splicing genes [10].

The initiation of tumors is the result of abnormal interactions of a number of genes. At present, some clues point to pre-leukemia clones being related to leukemia onset. Shlush *et al.* detected recurring *DNMT3A* and *IDH2* mutations in hematopoietic stem cells (HSCs) of AML patients and persisted in remission samples. In the allograft model, HSCs carrying those mutations showed significant proliferative advantages than those without mutations [11]. In other studies, clonal mutations in epigenetic regulation genes, such as *DNMT3A*, *TET2*, *IDH1/2*, and *ASXL1*, were detected in approximately 10% of healthy elderly people aged more than 65 years, and these patients exhibited a risk to progress to hematologic malignancies [12,13]. In our study, 86.6% of the epigenetic regulation genes co-occurred with other mutations and the most frequently concomitant mutation partner was *NPM1*, which is consistent with previous studies [10,14]. The results suggested that mutations in epigenetic regulation genes often occur at the early stage of the disease and is not sufficient to transform the disease into hematological malignancies independently. Mutations that occur relatively late in *NPM1* or *FLT3-ITD* may eventually lead to tumorigenesis and clinical-observed phenotypes. Therefore, co-occurrence with different mutated genes will determine the phenotype of hematological malignancies, and this will assist in clinical diagnoses; for example, mutations in epigenetic regulation genes concomitant with *JAK2* mutations could lead to MPN, whereas concomitant with *SF3B1* mutations could lead to MDS with increased sideroblasts.

Additionally, different concomitant mutant partners have potential effects on the prognosis. *NPM1* and *CEBPA* mutations are recurrent genetic abnormalities of AML according to the WHO 2008 and 2016 revision editions of the Classification of Hematologic Malignancies, which has distinct diagnostic classification and prognostic value. In normal cytogenetics AML patients, the *NPM1* or *CEBPA* biallelic mutation alone is associated with favorable prognosis [15,16]. However, from clinical observations, some patients still show poor prognosis, thereby suggesting the possibility of other genetic-aberrant mutations that affect prognosis. In this study, 97.1% of the *NPM1* mutations and 29.6% of the double-mutated *CEBPA* co-occurred with other genetic mutations. *NPM1* usually co-mutates with *FLT3-ITD*, *DNMT3A*, and/or *IDH2*. Reports have shown a three-way interaction among *NPM1*, *DNMT3A*, and *FLT3-ITD*, and the deleterious prognostic effects of *FLT3-ITD* is significantly greater when both *DNMT3A* and *NPM1* are mutated [10,17]. Double-mutated *CEBPA* that is concomitant with *TET2* mutation has been associated with impaired overall survival (OS) [18,19]. However, the effect of other mutations on the prognosis of *NPM1* or *CEBPA* biallelic mutations remains unclear. Therefore, further weight-coefficient and multivariate analyses are needed for processing in large-scale samples.

Different genes, different mutations in the same gene, and combinations of gene mutations may have different effects on the initiation, progression, and prognoses of diseases. Defining the mutation spectrum and mutation pattern of AML will contribute to the comprehensive assessment of patients and identification of new therapeutic targets.

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Compliance with ethics guidelines

Yang Zhang, Fang Wang, Xue Chen, Wenjing Liu, Jiancheng Fang,

Mingyu Wang, Wen Teng, Panxiang Cao, and Hongxing Liu declare that there are no conflicts of interest in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the *Helsinki Declaration* of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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