Cooperating gene mutations in childhood acute myeloid leukemia with special reference on mutations of \textit{ASXL1, TET2, IDH1, IDH2 and DNMT3A}

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Running title: Gene mutations in childhood AML

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Key Points

- A comprehensive study of 19 gene mutations and their cooperation, including the first report of ASXL1 and TET2 mutations in pediatric AML.
- The development of pediatric AML requires fewer gene mutations than adult AML.
Abstract

Genes involving epigenetic regulators have recently been described in adult AML. Similar studies are limited in children. We analyzed gene mutations and cooperation in pediatric AML with special reference on mutated epigenetic regulators. Nineteen gene mutations including 8 class I genes, 4 class II genes, $WT1$ and $TP53$ (class III), and 5 epigenetic regulator genes (class IV), were analyzed in 206 children with de novo AML. Mutational analysis was performed with PCR-based assay followed by direct sequencing. One hundred seventeen of 206 patients (56.8%) had at least one mutation: 51% class I, 13% class II, 6.8% class III and 5.6% class IV. $FLT3$-ITD was most frequent, 29% of patients had more than one gene mutation. Two patients carried $ASXL1$ mutations, both with t(8;21), 2 had $DNMT3A$ mutations, 2 had $IDH1$ mutations, 1 had $IDH2$ mutation and 3 had $TET2$ mutations. Both patients with $IDH1$ mutations had AML-M0 subtype and $MLL$-PTD. Cooperating mutations with mutated epigenetic regulators were observed in 8 of 10 patients. We conclude that mutated epigenetic regulators were much less than those in adult AML but with frequent cooperating mutations. $ASXL1$, $TET2$ and $IDH1$ mutations were associated with specific genetic subtypes.
Introduction

Comprehensive analyses of gene mutations involving epigenetic regulation in de novo childhood acute myeloid leukemia (AML) have been limited. The ASXL1 (Additional sex comb-like1) gene mapping to chromosome 20q acts as a cofactor of retinoic acid receptor via binding to steroid receptor coactivation-1 and belongs to enhancer of trithorax and polycomb genes that can both activate and repress HOX gene.\textsuperscript{1,2} Very recently, it has been demonstrated that ASXL1 loss-of-function mutations result in loss of polycomb repressive complex 2 (PRC2)-mediated histone H3 lysine 27 (H3K27) tri-methylation which promotes myeloid leukemia transformation.\textsuperscript{3} ASXL1 mutations conferred a poor outcome in adult AML\textsuperscript{4,5} but there have been no reports of ASXL1 mutations in childhood AML. TET proteins encode $\alpha$-ketoglutarate-dependent oxygenases which are involved in the conversion of 5-methylcytosine to 5-hydroxymethylcytosine.\textsuperscript{6} TET2 protein is important for normal myelopoiesis and disruption of TET2 enzymatic activity results in altered DNA methylation and favors myeloid neoplasm transformation.\textsuperscript{7} IDH1 and IDH2 mutations convert $\alpha$-ketoglutarate to 2-hydroxyglutarate which disrupts TET2 function.\textsuperscript{8,9} Somatic mutations of TET2 were identified with microdeletion at 4q24 in myeloid neoplasms by using high resolution single nucleotide polymorphism (SNP) microarrays.\textsuperscript{10,11} TET2 mutations were detected in adult AML with a frequency ranging from 7-23% but with controversial results of their prognostic relevance.\textsuperscript{12} There has been no published reports on TET2 mutations in pediatric AML except an abstract form.\textsuperscript{13} Mutation of the codon 132 of IDH1 gene was firstly identified in an adult AML patient with normal karyotype by whole genome sequencing.\textsuperscript{14} IDH2 mutations in codons R140 and R172 was later reported in adult AML.\textsuperscript{15} IDH1 or IDH2 mutations also occurred rarely in pediatric patients with AML.\textsuperscript{16-18}
DNMT3A which encodes a DNA methyltransferase was first identified by whole genome sequencing in an AML patient with normal karyotype and detected in 22% of adult de novo AML, especially those in the intermediate-risk cytogenetic group.\textsuperscript{19} DNMT3A mutations were rare in pediatric patients with AML: none of the 180 cases in one study,\textsuperscript{20} and only 1.0% and 2.1% of the patients in the other two studies.\textsuperscript{21,22} In this study on 206 pediatric patients with de novo AML, we systematically analyzed the known mutated genes, with numbers of patients and genes examined much more than those we have previously reported,\textsuperscript{23} and including the 5 recently identified genes that encode epigenetic modifiers. We also sought to determine the cooperation among all the mutated genes analyzed.
Materials and methods

Patients and samples
Two hundred and six consecutive children with de novo AML diagnosed at Chang Gung Memorial Hospital, Taoyuan, and Mackay Memorial Hospital, Taipei between December 1995 and June 2011 were enrolled. The study was approved by the Institutional Review Board of Mackay Memorial Hospital and was performed in compliance with the Declaration of Helsinki. The morphologic subtypes were classified according to the French-American-British (FAB) classification system. Immunophenotyping and cytogenetic/genetic analyses were carried out at initial diagnosis as described before.\textsuperscript{23,24} MLL gene rearrangement was screened by cytogenetics, Southern blot analysis, or fluorescent in situ hybridization followed by reverse transcriptase-polymerase chain reaction (RT-PCR) assays or panhandle PCR to detect the MLL fusion transcripts as previously described.\textsuperscript{25} The earlier cohort of patients with acute promyelocytic leukemia (APL) were treated with Taiwan Pediatric Oncology Group (TPOG)-APL-97-protocol consisting of all-trans retinoic acid followed by idarubicin and cytarabine. The post-remission therapy consisted of six courses of idarubicin and cytarabine. Since 2001, patients with APL have been treated with TPOG-APL-2001 protocol which was modified from the PETHEMA protocol.\textsuperscript{26} The non-APL patients were randomized to TPOG-AML-97A protocol\textsuperscript{27} or AML-97B protocol, which was modified from MRC AML10.

Cell fractionation, DNA and RNA extraction, and cDNA preparation
Mononuclear cells were obtained from bone marrow (BM) aspirates at diagnosis and cryopreserved until test. Genomic DNA (gDNA) and RNA were extracted from
freshly frozen cells. RNA was reversely transcribed to cDNA with Superscript II RNase H2 reverse transcriptase kit (Invitrogen Corporation, Carlsbad, CA, USA), as described previously.28

**Mutational analysis**

Detections of gene mutations including FLT3-ITD, FLT3-TKD, C-FMS (exons 6-22), C-KIT (exons 7-21), exons 2 and 3 of NRAS and KRAS, MLL-PTD, and the entire coding sequences of CEBPα and RUNX1 were performed as previously described, and the results have been updated in the present study.23,25,29-31

**Detection of ASXL1 mutation**

Mutational analysis of ASXL1 exon 13 (original exon 12) was carried out using the method described by Gelsi-Boye et al.32

**Detection of TET2 mutations**

Mutational analysis of TET2 was performed by gDNA PCR assay to amplify the whole coding sequences (exons 3–11) of TET2. The PCR products were subjected to direct sequencing. gDNA-PCR was performed using primers described by Delhommeau et al.10 or Langemeijer et al.11 with some modifications (Supplementary Table S1). TET2 missense mutations reported before or occurring in the two conserved regions (amino acids 1134 – 1444 and 1842 – 1921) were deemed somatic TET2 mutations. Otherwise, missense mutations outside the conserved regions were considered polymorphism except confirmed by its absence in complete remission (CR) samples.
Detection of \textit{DNMT3A} mutation

Mutational analysis of \textit{DNMT3A} was performed by PCR assay to amplify the entire coding sequences (exons 2 to 23) of \textit{DNMT3A}. The PCR products were first screened by denaturing high-performance liquid chromatography (DHPLC, WAVE Transgenomic, Omaha, NE, USA) with adding GC-clamps to the primers to facilitate mutation detection as described previously.\textsuperscript{33} Samples with abnormal DHPLC profile were directly sequenced on both directions. The sequences of primers used for PCR based analyzed are shown in Supplementary Tables S2 and S3. DHPLC sensitivity was determined by mixing various quantities of sequence-confirmed mutants with wild-type (5\% to 50\%). The detection limit was 5\% in our assay system (Supplementary Figure S1), which was more sensitive than that of direct sequencing.

Detection of \textit{IDH1} and \textit{IDH2} mutations

Mutational analyses of exon 4 of \textit{IDH1} and \textit{IDH2} were carried out by gDNA-PCR followed by direct sequencing. The primers used are shown in Supplementary Table S4, which cover the coding sequences of exon 4 of \textit{IDH1} and \textit{IDH2} containing the mutational hot spot codons R132, R140 and R172.

Detection of additional gene mutations

The mutational analyses of exons 1-3 (Supplementary Table S4) and 7-9 of \textit{WT1},\textsuperscript{34} \textit{TP53} (exons 5-9),\textsuperscript{35} \textit{JAK2}V617F,\textsuperscript{36} and \textit{NPM1}(exon 11, original exon 12)\textsuperscript{37} were performed according to the previously described methods of other investigators with some modifications.
Mutational analysis of the entire \textit{PTPN11} coding regions (exons 1-15) was performed using cDNA-PCR assay with primers shown in Supplementary Table S4. For patients without available RNA samples, gDNA PCR assay was performed according to the method of Tartaglia et al.\textsuperscript{38}

For all the mutational analyses, the detected mutations were confirmed in a second independent analysis, and/or using cDNA samples, and/or using different primers to confirm mutations.

\textbf{Statistical analysis}

Fisher’s exact test, the $\chi^2$ analysis, and Wilcoxon’s rank-sum test were used whenever appropriate to make comparisons between groups. Kaplan-Meier estimation was used to plot overall survival (OS) and event-free survival (EFS) in each subgroup. Comparisons of estimated survival curves were analyzed by the log-rank test. A $P$ value < .05 was considered as statistically significant. SPSS version 17 software (SPSS Inc) was used to perform the statistical analyses.
Results

Frequency and distribution of 19 gene mutations in pediatric de novo AML

We divided the 19 mutated genes into 4 functional classes: class I gene mutations involving signaling transduction and RAS pathways, class II hematopoietic transcription factor genes, *TP53* and *WT1* belonged to class III, and class IV epigenetic regulator genes. The frequencies and distribution of mutated genes and their cooperation are shown in Figure 1. Approximately half of the patients had class I gene mutations, 13% had class II mutations, 6.8% of patients had *TP53* and *WT1* mutations, and 5.6% of patients had class IV gene mutations. The occurrence of classes I, II and III mutations in the whole cohort of children with AML according to the order of frequency were: class I (*FLT3*-ITD, *C-KIT*, *NRAS*, *FLT3-TKD*, *KRAS*, *PTPN11*, *JAK2*V617F, C-FMS); class II (*CEBPα*, *NPM1*, *RUNX1*, *MLL-PTD*); class III (*WT1*, *TP53*). Class IV mutations occurred rarely: *TET2* mutations in 1.7%, *DNMT3A* in 1.2%, *ASXL1* in 1.1%, *IDH1* in 1.1% and *IDH2* in 0.6%; there was no overlap in involvement among the 5 genes. Taken together, 56.8% of patients had at least one mutation among the genes we examined.

Characteristics of patients with gene mutations involving epigenetic regulators and their cooperating mutations

In total, 10 patients harbored one of the 5 gene mutations involving the epigenetic modifiers. Of the 8 patients who achieved CR, 6 had CR samples available for analysis, no mutation was detected in all of the 6 CR samples examined, including the one patient with missense mutation of *TET2* (F760Y) located outside the conserved regions; indicating that they were somatic mutations. One with frameshift mutation of *ASXL1*(E635Rfs*15) and another one with
nonsense mutation of TET2 (E1364*) which resulted in truncated protein and were expected to have loss of function mutations, did not achieve a CR. In addition, one each with IDH1 (R132C) and TET2 (R1359C located in highly conserved region and reported before\(^39\)) did not have CR sample available, they were considered as somatic mutations. The electropherograms of the 5 gene mutations in the 10 cases at diagnosis and the 6 samples in CR are shown in Figure 2. The clinico-hematological features and characteristics of mutated genes of epigenetic modifiers as well as their co-existed mutations in the 10 patients are summarized in Table 1. The 2 patients with ASXL1 mutations had t(8;21)/RUNX1-RUNX1T1 and FAB M2 subtype. The frequency of ASXL1 mutations in pediatric t(8;21) AML was 5.6% (2/36) compared to none of the 141 patients with non-t(8;21) AML (P = .04). In the present series, 4 patients had MLL-PTD, and two of them, both with FAB M0 subtype, had IDH1 mutations (one each of R132C and R132H), there was a strong association of MLL-PTD with IDH1 mutations compared with none of the 174 patients with non-MLL-PTD AML (P < .0001). Likewise, an association of IDH1 mutation with M0 subtype was also statistically significant (P = .003). Another one patient with FAB M2 subtype and normal karyotype harbored IDH2 mutation (R140Q). Both patients with DNMT3A mutations (R882H and W795S) were older than 10 years and had FAB M4 or M5, one of them had MLL translocation (with unknown partner gene) which was detected by FISH analysis but not by conventional cytogenetics. All of the 3 patients with TET2 mutations were older than 10 years and had FAB M1 or M2; two of them had t(8;21).
Correlations between other gene mutations and clinico-hematological features

Of the 19 mutated genes examined, apart from the 5 mutated genes of epigenetic modifiers, 11 of the remaining 14 gene mutations were detected in more than 3 patients; we analyzed the correlation between their clinico-hematological parameters and mutation status. Only 6 mutated genes were found to have clinical correlations as shown in Supplementary Table S5. FLT3-ITD and CEBPα mutations were significantly associated with older age and higher WBC counts. Patients with K-RAS or CEBPα mutations had a significant lower platelet counts. NPM1 mutation was borderline significantly associated with older age. At least one mutated gene was detected throughout all FAB subtypes and cytogenetic risk groups (Supplementary Table S6); however, their frequencies varied among different subgroups, with AML-M6 or M7 and patients with MLL translocations or unfavorable cytogenetic risk groups being less frequent than other subgroups.

Impact of gene mutations on outcomes in pediatric AML

As the number in each subgroup of patients with individual gene mutation was very small, we combined the subgroups based on the functional classes for the outcome analysis. We excluded APL patients in the analysis, there were no significant differences in the EFS or OS according to the mutational status of class I, II, or III in non-APL patients (Supplementary Figures S2A-S4B), though a trend of inferior outcome was observed in patients with class I mutation ($P = .072$ for EFS and $P = .088$ for OS). The 10 patients with class IV gene mutations had a 5-year EFS of 50.0 ±15.8% compared with 44.7 ± 4.4% for other non-APL patients without the mutations ($P = .668$, Supplementary Figure S5A); also no difference in OS was
observed (50.0 ± 15.8% vs. 51.0 ± 4.5%, P = .904, Supplementary Figure S5B).

We further analyzed whether FLT3-ITD had an effect on outcome, no difference was observed (P=.268 for EFS and P=.196 for OS). The presence of C-KIT mutations in core-binding factor (CBF)-AML did not affect the 5-year EFS [P = .726 for t(8;21) and P = .486 for inv(16)]. We found that the patients with MLL-PTD had 5-y EFS of only 33.3±27.2 %. The 2 patients with TP53 mutations had very grave outcomes, both with OS less than 4 months.

We observed that 3 patients harboring 3 mutations across classes I and II had 5-year EFS of 6, 8, and 60+ months, respectively. One patient with a combination of 3 classes I, II, and III mutations had no EFS and survived for one month only. Three patients harboring class IV mutations which co-existed with class I and class II mutations had EFS of 87+, 110+ and 167+ months, respectively, suggesting that mutations of epigenetic modifiers did not adversely affect outcome in patients carrying other mutated genes.

Co-existence of gene mutations in pediatric AML

Among the 117 patients with at least one mutated gene, co-existence of more than one gene mutation were detected in 34 patients (29%). The occurrence of gene mutations within the same functional classes was rare. They were mutually exclusive in the 13 patients carrying TP53 and WT1 mutations and in the 10 patients harboring mutated genes of epigenetic regulators. Only 2 of 26 patients carrying class II mutations and 7 of 104 patients carrying class I mutations had gene mutations within the same class. As shown in Figure 2 cooperating mutations across different classes were observed in 30 patients. Combination of classes I and II was detected in 13 patients, I and III in 7 patients, I and IV in 2 patients, II and III in
1, and II and IV in 1. Six patients had multiple mutations, 1 had class I plus II plus III and 2 had class I plus III plus IV, and additional 3 had class I plus II and IV mutations. Cooperating mutations of epigenetic regulator genes with other genes were common and present in 8 patients (Table 1), especially with class I mutations in 7. Of the 3 patients with TET2 mutations; one each co-existed with FLT3-TKD (D835V), FLT3-ITD plus CEBPα mutations, and KRAS (G13R) plus WT1 (R434Hfs*86). In addition to MLL-PTD, the 2 patients carrying IDH1 mutants had co-existed FLT3-ITD and RUNX1 (V333Dfs*242) mutations, respectively. The patient with IDH2 mutation also harbored FLT3-ITD and NPM1 (CTCG duplication, type D) mutations. Of the 2 patients with DNMT3A mutations, one cooperated with PTPN11 (E76K) mutation, and the other co-existed with FLT3-TKD (D835E) and WT1 (A382Gfs*3) mutations. The 2 patients carrying ASXL1 mutations did not have concurrent mutations with other 18 genes analyzed; however, both had RUNX1-RUNX1T1 transcript.
Discussion

Compared with adult AML, there were fewer studies of gene mutations in childhood AML. We had reported a decrease in the frequencies of \( \text{FLT3-ITD} \), \( \text{FLT3-TKD} \) and \( \text{CEBP}\alpha \) in childhood AML, as compared with those in adult AML.\(^{29,31,40} \) The frequencies of \( \text{FLT3} \) and \( \text{CEBP}\alpha \) mutations which differed considerably between children and adults were later confirmed by Children’s Oncology Group.\(^{41,42} \) Others also found the frequency of \( \text{NPM1} \) mutations was 4 times higher in adult AML compared with those of pediatric AML.\(^{43} \) All these findings suggested a different ontogeny between childhood and adult AML.

In the present study, we extended our previous study and systematically analyzed 19 known gene mutations involved in adult myeloid neoplasms in a large cohort of children with de novo AML. If we took the functional groups of gene mutations into consideration, mutations occurring most frequently in childhood AML was class I mutations which involve receptor tyrosine kinases and RAS signaling pathway. Together, they accounted for half of our patients, with \( \text{FLT3-ITD} \) being the most frequent. The frequency of mutations which block hematopoietic differentiation was 13%, mutations involving apoptosis or tumor suppressor genes i.e. \( \text{WT1} \) and \( \text{TP53} \) were 6.9%, with the latter being rare. The mutation frequencies of class I and II were similar to the report by Radtke \textit{et al}, they used SNP array and resequencing of candidate cancer genes in a cohort of 111 children with de novo AML.\(^{44} \) The results of our extended study on pediatric patients again confirmed a great difference in the frequency of gene mutations between childhood and adult AML.

The present study showed that the recently identified gene mutations involving the DNA methylation and histone modification were very rare, accounting for approximately one tenth of those reported in adult AML. \( \text{ASXL1} \) mutations have not
been reported in childhood AML, the two mutations we detected were both frame
shift mutations which caused a truncation of C-terminal plant homeodomain of
ASXL1 and leukemia transformation.3 Our result showed a low frequency (1.1%) of
ASXL1 mutations in pediatric AML which was in sharp contrast with those described
in adult AML studies in which the frequencies were 5-10 folds higher.4,5 TET2
mutations in pediatric AML was only reported in an abstract form,13 we detected a
frequency of 1.7% which was much lower than the frequency of 12-22% reported in
adult AML.12 The very low frequencies of IDH1, IDH2 and DNMT3A mutations
detected in the present series were in line with the recent reports of childhood
AML16-18,20 but were in contrast to the findings in adult AML with a reported
frequencies of 15-33% for IDH,12 and 12 to 22 % for DNMT3A.12,19 A recent German
study found 1.0% (2/195) of childhood AML had DNMT3A mutations.21 We also
screened the entire coding sequences, both two mutants were located in the
C-terminal methyltransferase domain where the great majority of the previously
reported mutations in adult series were located.19,45 The mutations of ASXL1, TET2,
IDH1, IDH2 and DNMT3A were mutually exclusive in our patients. Of the 10 patients
with mutated genes of epigenetic regulators, apart from the one with ASXL1
E635Rfs*15 which had been reported in adult AML patients,4,5 and another with the
hot spot of R132C IDH mutation; the remaining 6 patients with IDH1(R132H), IDH2
(R140Q), ASXL1 (P835Dfs*7), DNMT3A missense mutations (W795S and R882H)
or TET2 (F760Y) at diagnosis did not have mutations detected in the CR samples.
Our results indicated that these mutations were somatic acquired and
leukemia-associated.

Although mutations in epigenetic modifiers are particularly rare in pediatric AML,
many known oncogenes and tumor suppressors might contribute, at least in part, to
leukemia transformation through direct or indirect alterations in the epigenetic state. Therefore, additional molecular alterations of disordered DNA methylation, such as promoter hypermethylation with gene silencing, overexpression of DNMT, or \textit{MLL} translocation, may contribute to the leukemogenesis.\textsuperscript{46} Global methylation signature or more extensive genome wide epigenomic research are valuable tools to further uncover the molecular pathway of epigenetic abnormalities, which in turn will provide a rationale molecular basis for therapeutic reversal strategies with dehypermethylating agents or histone deacetylase inhibitors.

We found that the 5 mutated genes of epigenetic modifiers were associated with some interesting clinical characteristics in our 10 patients. Notably, both patients with \textit{DNMT3A} mutations had AML-M4 or M5 and one of them had \textit{MLL} translocation which was not present in the two reported pediatric patients with \textit{DNMT3A} mutations.\textsuperscript{21,22} In adult AML, \textit{DNMT3A} mutation was also reported to be associated with acute monocytic leukemia.\textsuperscript{47} All of our 3 patients with \textit{TET2} mutations and two patients with \textit{DNMT3A} mutations were older than 10 years. Both of the two patients harboring \textit{ASXL1} mutations and 2 of the 3 patients carrying \textit{TET2} mutations had t(8;21) AML. The frequency of \textit{ASXL1} mutations in our t(8;21) AML seemed to be higher than those without the mutations. This association was not observed in one adult AML study in which 8\% of t(8;21)AML had \textit{ASXL1} mutations compared with 5.3\% of entire AML cohort.\textsuperscript{5} Whether \textit{ASXL1} mutations were associated with t(8;21) AML remained to be examined in a larger number of t(8;21) patients. We also observed a high association between \textit{IDH1} mutation and \textit{MLL-PTD} or AML-M0 subtype. In one of the recent reported pediatric series on \textit{IDH} mutations, 2 of the 7 \textit{IDH1} mutated patients and one of 9 patients with \textit{IDH2} mutations harbored \textit{MLL-PTD}.\textsuperscript{18} One of 3 children with \textit{IDH1} mutations was
associated with AML-M0 in another study. One of our two patients with IDH2 mutation had co-existed NPM1 mutation. We correlated the mutation status of individual gene of other functional classes with age, sex, complete blood counts and percentage of blasts in BM. Patients with FLT3-ITD and CEBPα mutations had older age and higher WBC count; whereas KRAS and CEBPα mutations are associated with lower platelet counts. We observed gene mutations occurred less frequently in AML-M6 or M7 as well as in unfavorable cytogenetic risk groups. Similar to adult AML, FLT3-ITD and NPM1 mutations were more frequently present in normal karyotype and C-KIT mutations were strongly associated with core-binding factor AML in children.

It has been hypothesized that the development of AML was associated with at least two-hit process with the cooperation of activation mutations of signaling pathway (class I mutations) and mutations of hematopoietic transcription factors which block differentiation (class II mutations). With subsequent discovery of the increasing number of mutated genes and their cooperating mutations, a mechanism of multistep leukemogenesis was suggested. In the present study, we observed that cooperation of mutated genes of epigenetic regulators with other known gene mutations was very common, occurring in 8 of 10 pediatric AML patients, 6 with class I (5 FLT3, 1 KRAS and 1 PTPN11) and 4 with class II (2 MLL-PTD, 1 CEBPα and one each for RUNX1 and NPM1) mutations, and 2 with WT1 mutations. The two patients with ASXL1 mutations did not have other co-existed mutated genes; but both carried RUNX1/RUNX1T1 with disruption of RUNX1 at C-terminal region which likes RUNX1 mutations would result in dysregulated hematopoietic transcription factor and impair differentiation, a finding functionally confirmed in mouse models.
Regarding the prognostic significance of gene mutations in pediatric AML, we and others found that FLT3-ITD was most common among the mutated genes examined,\textsuperscript{20,44} we failed to find a significant difference in outcome between patients with and without FLT3-ITD. The number of other individual mutated genes in class I, II or III was too small and the distribution among subgroups was very heterogeneous, which precluded a meaningful analysis of the prognostic significance. We thus analyzed any of class I, II, or III genes versus no mutation in each class of genes, no significant difference was observed except a trend of adverse outcome in patients carrying FLT3-ITD. Class IV mutations in adult AML conferred an inferior outcome,\textsuperscript{4,5,12,39,45} half of our patients with class IV mutations had a long-term EFS survival; although there were no significant differences in OS and EFS between those with and without mutations because of the small number of patients carrying these mutations. Together, the frequency of gene mutations was much less in pediatric AML as compared with those in adult patients. The clinical and prognostic relevance of gene mutations on childhood AML remains to be determined by a larger cohort of pediatric patients.
Acknowledgments

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Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


Table 1. Clinico-hematological features and characteristics of the mutated genes of epigenetic modifiers and their co-existed mutations in 10 pediatric AML patients.

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<th>Patient no.</th>
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<td>DNMT3A</td>
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<td>FLT3-TKD (D835E)</td>
<td>WT1 (A382Gfs*3)</td>
<td>97A</td>
</tr>
<tr>
<td>#28</td>
<td>13/M</td>
<td>M2</td>
<td>45X,-Y,t(8;21)</td>
<td>TET2</td>
<td>E1364*</td>
<td>FLT3-TKD (D835V)</td>
<td>97B</td>
<td>0</td>
</tr>
<tr>
<td>#36</td>
<td>14/M</td>
<td>M1</td>
<td>46XY</td>
<td>TET2</td>
<td>R1359C</td>
<td>FLT3-ITD, CEBPα</td>
<td>[P46H fs*115 (;) K304_Q305 insL]</td>
<td>97B</td>
</tr>
<tr>
<td>#160</td>
<td>16/M</td>
<td>M2</td>
<td>45X,-Y,t(8;21)</td>
<td>TET2</td>
<td>F760Y</td>
<td>KRAS (G13R),</td>
<td>WT1 (R434Hfs*86)</td>
<td>97B</td>
</tr>
</tbody>
</table>

AML indicates acute myeloid leukemia; PTD, partial tandem duplication of MLL; FAB, French-American-British classification; ITD, internal tandem duplication; and TKD, tyrosine kinase domain.
Legends

Figure 1. The frequencies and distribution of 19 gene mutations and their cooperativity. Each column represents one individual patient with at least one mutated gene (s) shown by different colored bars. The top 8 genes belong to class I, the next 5 genes class II, WT1 and TP53 class III, and last 5 genes class IV. The last raw represents the cytogenetics for each patient.

Figure 2. Electropherograms of the 5 gene mutations of ASXL1 (A-1 and B), IDH1 (C and D-1), IDH2 (E-1), DNMT3A (F-1 and G-1), and TET2 (H-F reads forward, H-R reads reversely, I, and J-1 ) in the 10 pediatric AML patients at diagnosis and in complete remission of the corresponding patients (A-2, D-2, E-2, F-2, G-2, and J-2).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3-ITD</td>
<td>15%</td>
</tr>
<tr>
<td>C-KIT</td>
<td>12%</td>
</tr>
<tr>
<td>N-RAS</td>
<td>8.8%</td>
</tr>
<tr>
<td>FLT3-TKD</td>
<td>7.4%</td>
</tr>
<tr>
<td>K-RAS</td>
<td>6.9%</td>
</tr>
<tr>
<td>PTPN11</td>
<td>2.6%</td>
</tr>
<tr>
<td>JAK2V617F</td>
<td>2.6%</td>
</tr>
<tr>
<td>C-FMS</td>
<td>0.6%</td>
</tr>
<tr>
<td>CEBPα</td>
<td>7.0%</td>
</tr>
<tr>
<td>NPM1</td>
<td>4.0%</td>
</tr>
<tr>
<td>RUNX1</td>
<td>1.3%</td>
</tr>
<tr>
<td>MLL-PTD</td>
<td>1.9%</td>
</tr>
<tr>
<td>WT1</td>
<td>5.8%</td>
</tr>
<tr>
<td>TP53</td>
<td>1.1%</td>
</tr>
<tr>
<td>TET2</td>
<td>1.7%</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>1.2%</td>
</tr>
<tr>
<td>ASXL1</td>
<td>1.1%</td>
</tr>
<tr>
<td>IDH1</td>
<td>1.1%</td>
</tr>
<tr>
<td>IDH2</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

**Figure 1**

- **Class I:** 51%
- **Class II:** 13%
- **Class III:** 6.8%
- **Class IV:** 5.6%

Other annotations include:
- NK/Mis
- inv(16)
- t(15;17)
- t(9;11)
- MLL-T, except t(9;11)
- Poor
- Down syndrome
Figure 2
Cooperating gene mutations in childhood acute myeloid leukemia with special reference on mutations of \textit{ASXL1, TET2, IDH1, IDH2} and \textit{DNMT3A}

Der-Cherng Liang, Hsi-Che Liu, Chao-Ping Yang, Tang-Her Jaing, Jou-Jih Hung, Ting-Chi Yeh, Shih-Hsiang Chen, Jen-Yin Hou, Ying-Jung Huang, Yu-Shu Shih, Yu-Hui Huang, Tung-Huei Lin and Lee-Yung Shih