blood

2013 121: 2988-2995
Prepublished online January 30, 2013;
doi:10.1182/blood-2012-06-436782

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

Der-Cherng Liang, Hsi-Che Liu, Chao-Ping Yang, Tang-Her Jaing, Iou-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

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Regular Article

MYELOID NEOPLASIA

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

Der-Cherng Liang,[1] Hsi-Che Liu,[1] Chao-Ping Yang,[2] Tang-Her Jaing,[2] Iou-Jih Hung,[2] Ting-Chi Yeh,[1] Shih-Hsiang Chen,[2] Jen-Yin Hou,[1] Ying-Jung Huang,[3] Yu-Shu Shih,[4] Yu-Hui Huang,[4] Tung-Huei Lin,[4] and Lee-Yung Shih[3,4]

[1]Division of Pediatric Hematology-Oncology, Mackay Memorial Hospital, Taipei, Taiwan; [2]Department of Hematology-Oncology, Chang Gung Children's Hospital, Taoyuan, Taiwan; [3]Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital, Taipei, Taiwan; and [4]Chang Gung University, Taoyuan, Taiwan

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.

Gene mutations involving epigenetic regulators recently have been described in adult acute myeloid leukemia (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 polymerase chain reaction–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*-internal tandem duplication was most frequent, and 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*-partial tandem duplication. 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. (*Blood*. 2013;121(15):2988-2995)

Introduction

Comprehensive analyses in de novo childhood acute myeloid leukemia (AML) of gene mutations involving epigenetic regulators have been limited. The *ASXL1* (additional sex comb-like 1) 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 the *HOX* gene.[1,2] Very recently, it has been demonstrated that ASXL1 loss-of-function mutations result in the loss of polycomb repressive complex 2–mediated histone H3 lysine 27 trimethylation, which promotes myeloid leukemia transformation.[3] *ASXL1* mutations conferred a poor outcome in adult AML,[4,5] but there have been no reports of *ASXL1* mutations in childhood AML. TET proteins encode α-ketoglutarate-dependent oxygenases, which are involved in the conversion of 5-methylcytosine to 5-hydroxymethylcytosine.[6] TET2 protein is important for normal myelopoiesis, and disruption of TET2 enzymatic activity results in altered DNA methylation and favors myeloid neoplasm transformation.[7] *IDH1* and *IDH2* mutations convert α-ketoglutarate to 2-hydroxyglutarate, which disrupts TET2 function.[8,9]

Somatic mutations of *TET2* were identified with microdeletion at 4q24 in myeloid neoplasms by the use of high-resolution single-nucleotide polymorphism microarrays.[10,11] *TET2* mutations were detected in adult AML with a frequency ranging from 7% to 23%, but the prognostic relevance of these results was controversial.[12] There have been no published reports on *TET2* mutations in pediatric AML except in abstract form.[13] Mutation of the codon 132 of *IDH1* gene was identified first in an adult AML patient with normal karyotype by the use of whole-genome sequencing.[14] *IDH2* mutations in codons R140 and R172 were later reported in adult AML.[15] *IDH1* or *IDH2* mutations also have occurred rarely in pediatric patients with AML.[16-18] Mutation of *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 patients with de novo AML, especially those in the intermediate-risk cytogenetic group.[19] *DNMT3A* mutations have been rare in pediatric patients with AML: in one study that included 180 cases, no patients were found,[20] and only 1.0% and 2.1% of the patients in the other two studies were reported.[21,22] In this study of 206 pediatric patients with de novo AML, we systematically analyzed known mutated genes, examined more patients and genes than we have reported previously,[23] and included 5 recently identified genes that encode epigenetic modifiers. We also

Submitted June 29, 2012; accepted January 22, 2013. Prepublished online as *Blood* First Edition paper, January 30, 2013; DOI 10.1182/blood-2012-06-436782.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

sought to determine the presence of multiple mutated gene combinations within a single individual.

Materials and methods

Patients and samples

Two hundred six consecutive children with de novo AML who were 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 performed at initial diagnosis as has been described previously.[23,24] *MLL* gene rearrangement was screened by cytogenetics, Southern blot analysis, or fluorescent in situ hybridization followed by reverse transcriptase-polymerase chain reaction (PCR) assays or panhandle PCR to detect the *MLL* fusion transcripts as previously described.[25]

The earlier cohort of patients with acute promyelocytic leukemia (APL) was treated with the Taiwan Pediatric Oncology Group (TPOG)-APL-97 protocol, which consisted of all-*trans* retinoic acid followed by idarubicin and cytarabine. The postremission therapy consisted of 6 courses of idarubicin and cytarabine.[26] Since 2001, patients with APL have been treated with the TPOG-APL-2001 protocol, which was modified from the Programa para el Estudio de la Terape´utica en Hemopati´a Maligna (PETHEMA) protocol.[27] The non-APL patients were randomized to TPOG-AML-97A protocol[26] or AML-97B protocol, which was modified from Medical Research Council (MRC) AML 10.

Cell fractionation, DNA and RNA extraction, and cDNA preparation

Mononuclear cells were obtained from bone marrow aspirates at diagnosis and cryopreserved until testing. Genomic DNA (gDNA) and RNA were extracted from freshly frozen cells. RNA was reversely transcribed to complementary DNA (cDNA) with the Superscript II RNase H2 reverse transcriptase kit (Invitrogen Corporation, Carlsbad, CA), as described previously.[28]

Mutational analysis

Detections of gene mutations, including *FLT3*-internal tandem duplication (ITD), *FLT3*-tyrosine kinase domain (TKD), *C-FMS* (exons 6-22), *C-KIT* (exons 7-21), exons 2 and 3 of *NRAS* and *KRAS*, *MLL*-partial tandem duplication (PTD), and the entire coding sequences of C*EBPα* and *RUNX1* were performed as previously described,[23,25,29-31] and the results were updated in the present study.

Detection of *ASXL1* mutation

We performed mutational analysis of *ASXL1* exon 13 (original exon 12) by using the method described by Gelsi-Boyer et al.[32]

Detection of *TET2* mutations

Mutational analysis of *TET2* was performed with a 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 with primers described by Delhommeau et al[10] or Langemeijer et al[11] with some modifications (supplemental Table 1; see the *Blood* Web site). *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 polymorphisms if they were present in complete remission (CR) samples.

Detection of *DNMT3A* mutation

Mutational analysis of *DNMT3A* was performed by PCR assay to amplify the entire coding sequence (exons 2-23) of *DNMT3A*. The PCR products were first screened by denaturing high-performance liquid chromatography (DHPLC; WAVE Transgenomic, Omaha, NE), to which guanine and cytosine clamps were added to the primers to facilitate the detection of mutations, as described previously.[33] Samples with an abnormal DHPLC profile were sequenced directly on both directions. The sequences of primers used for PCR-based analysis are shown in supplemental Tables 2 and 3. DHPLC sensitivity was determined by mixing various quantities of sequence-confirmed mutants with the wild type (5%-50%). The detection limit was 5% in our assay system (supplemental Figure 1), which was more sensitive than that of direct sequencing.

Detection of *IDH1* and *IDH2* mutations

Mutational analyses of exon 4 of *IDH1* and *IDH2* were conducted with gDNA-PCR followed by direct sequencing. The primers used are shown in supplemental Table 4, which cover the coding sequences of exon 4 of *IDH1* and *IDH2* containing the mutational hot spot codons R132, R140, and R172.

Detection of additional gene mutations

The mutational analyses of exons 1-3 (supplemental Table 4) and 7-9 of *WT1*,[34] *TP53* (exons 5-9),[35] *JAK2*V617F,[36] and *NPM1* (exon 11, original exon 12)[37] were performed according to the previously described methods of other investigators with some modifications. Mutational analysis of the entire *PTPN11* coding regions (exons 1-15) was performed using cDNA-PCR assay with primers shown in supplemental Table 4. For patients without available RNA samples, a gDNA PCR assay was performed according to the method of Tartaglia et al.[38] For all the mutational analyses, the detected mutations were confirmed in a second independent analysis, and/or by the use of cDNA samples, and/or by the use of different primers to confirm mutations.

Statistical analysis

The Fisher exact test, the χ^2 analysis, and Wilcoxon rank-sum test were used whenever appropriate to make comparisons between groups. A Kaplan-Meier estimation was used to plot the overall survival (OS) and event-free survival (EFS) of 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 included gene mutations involving signaling transduction and RAS pathways, class II included hematopoietic transcription factor genes, *TP53* and *WT1* comprised class III, and class IV comprised epigenetic regulator genes. The frequencies and distribution of mutated genes and their cooperation are shown in Figure 1. Approximately one-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 was as follows: 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*).

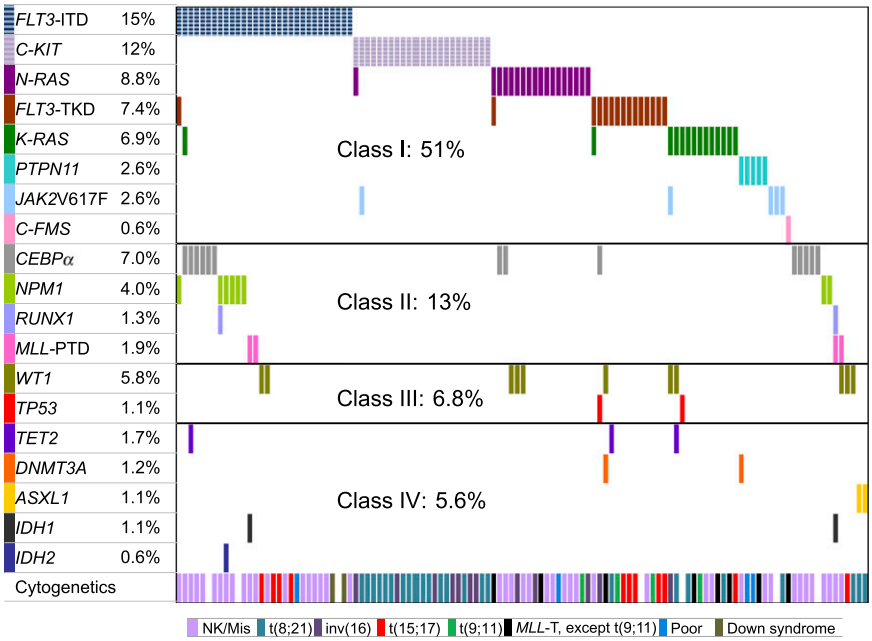

Figure 1. The frequencies and distribution of 19 gene mutations and their cooperativity. Each column represents one individual patient with at least one mutated gene(s) shown by different colored bars. The top 8 genes belong to class I, the next 4 genes class II, *WT*1 and *TP53* class III, and last 5 genes class IV. The last raw represents the cytogenetics for each patient.

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% of patients; 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 1 of the 5 gene mutations involving the epigenetic modifiers. Of the 8 patients who achieved CR, 6 had CR samples available for analysis, and no mutation was detected in any of the 6 CR samples examined, including the 1 patient with missense mutation of *TET2* (F760Y) located outside the conserved regions; this finding indicated that they were somatic mutations.

One patient with a frameshift mutation of *ASXL1* (E635Rfs*15) and another one patient with nonsense mutation of *TET2* (E1364*) that resulted in truncated protein who were expected to have loss of function mutations did not achieve CR. In addition, one patient each with *IDH1* (R132C) and *TET2* (R1359C located in highly conserved region and reported previously[39]) did not have a CR sample available, and 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 clinicohematological features and characteristics of mutated genes of epigenetic modifiers as well as their coexisted 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 subtypes. The frequency of *ASXL1* mutations in pediatric t(8;21) AML was 5.6% (2/36) compared with none of the 141 patients with non-t(8;21) AML ($P = .04$).

In the present series, 4 patients had *MLL*-PTD, and 2 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 also was statistically significant ($P = .003$). Another one patient with FAB M2 subtype and a normal karyotype harbored the *IDH2* mutation (R140Q). Both patients with *DNMT3A* mutations (R882H and W795S) were older than 10 years of age and had FAB M4 or M5, and 1 had an *MLL* translocation (with unknown partner gene) that was detected by fluorescence in situ hybridization analysis but not by conventional cytogenetics. All 3 patients with *TET2* mutations were older than 10 years of age and had FAB M1 or M2; 2 had t(8;21).

Correlations between other gene mutations and clinicohematological 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 clinicohematological parameters and mutation status. Only 6 mutated genes were found to have clinical correlations as shown in supplemental Table 5. *FLT3*-ITD and *CEBPα* mutations were significantly associated with older age and greater white blood cell counts. Patients with *K-RAS* or *CEBPα* mutations had a significant lower platelet counts. The *NPM1* mutation was borderline significantly associated with older age. At least one mutated gene was detected throughout all FAB subtypes and cytogenetic risk groups (supplemental Table 6); 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

Because the number in each subgroup of patients with individual gene mutation was very small, we combined the subgroups on the basis of the functional classes for the outcome analysis. We excluded patients with APL from 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 (supplemental Figures 2A-4B), although 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 ($P = .668$, supplemental

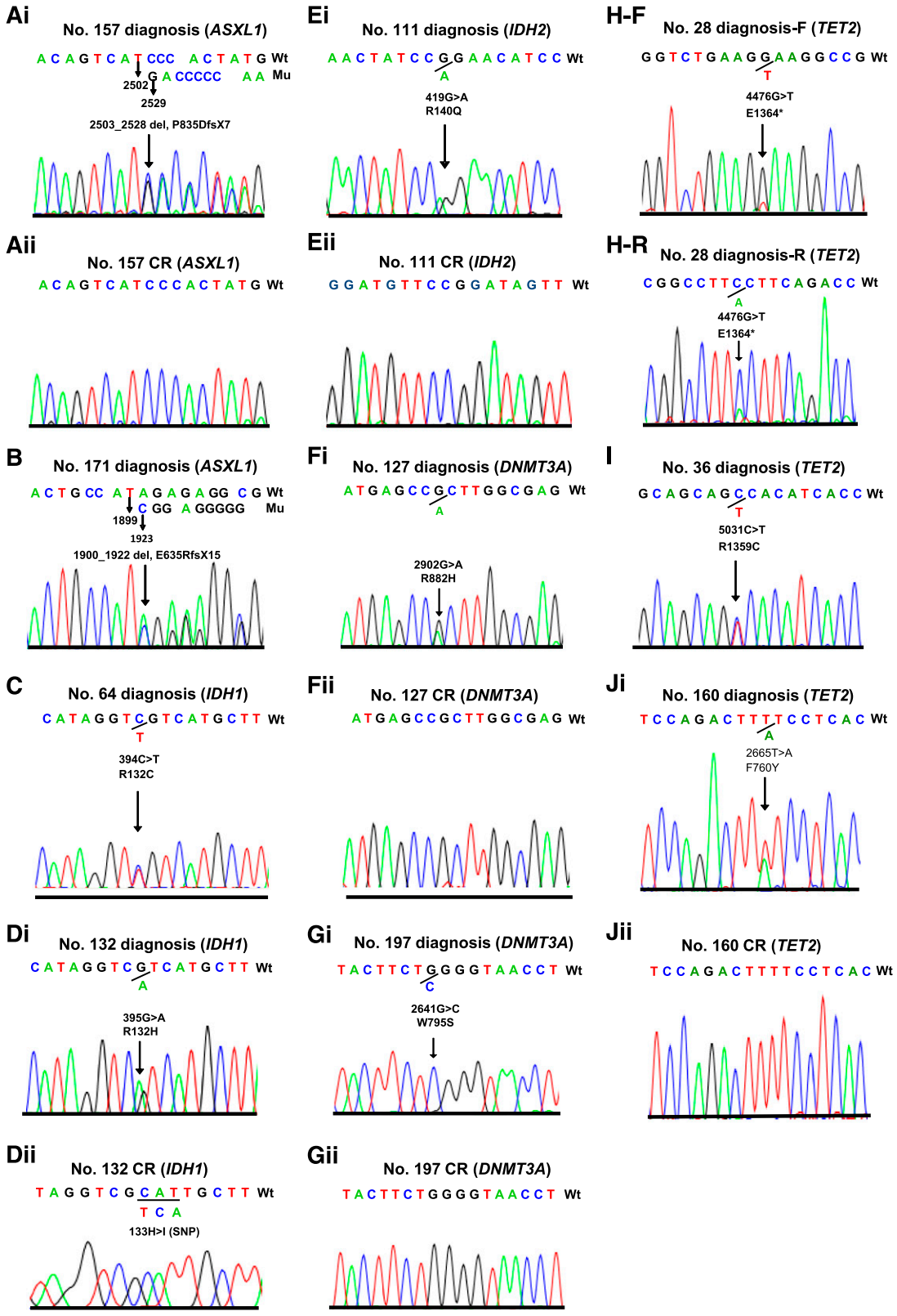

Figure 2. Electropherograms of the 10 pediatric patients with AML carrying 5 mutated genes of epigenetic regulators. Two patients with *ASXL1* mutations (Ai and B), 2 patients with *IDH1* mutations (C and Di), 1 patient with *IDH2* mutation (Ei), 2 patients with *DNMT3A* mutations (Fi and Gi), and 3 patients with *TET2* mutations (H-F reads forward, H-R reads reversely, I, and Ji) at diagnosis, and wild types of the 5 genes in complete remission of the corresponding patients (Aii, Dii, Eii, Fii, Gii, and Jii).

Table 1. Clinicohematological features and characteristics of the mutated genes of epigenetic modifiers and their coexisted mutations in 10 pediatric AML patients

Patient no.	Age, y/sex	FAB	Cytogenetics/ genetic subclass	Mutated gene	Amino acid change	Cooperative mutations	Treatment protocol	Event-free survival, mo
157	10/M	M2	45×,-Y,t(8;21)	*ASXL1*	P835DfsX7	Nil	97A	68+
171	2/F	M2	46XX,t(8;12;21)	*ASXL1*	E635RfsX15	Nil	97A	17
64	5/F	M0	47XX,+ 21	*IDH1*	R132C	*MLL*-PTD *RUNX1* (V333Dfs*242)	97B	14
132	11/M	M0	46XY	*IDH1*	R132H	*FLT3*-ITD *MLL*-PTD	97A	87+
111	10/M	M2	46XY	*IDH2*	R140Q	*FLT3*-ITD *NPM1* (type D)	97B	110+
127	11/M	M5	48XY,+11,+20	*DNMT3A*	R882H	*PTPN11* (E76K)	97A	93+
197	14/M	M4	46XY,*MLL* translocation	*DNMT3A*	W795S	*FLT3*-TKD (D835E) *WT1* (A382Gfs*3)	97A	10
28	13/M	M2	45×,-Y,t(8;21)	*TET2*	E1364*	*FLT3*-TKD (D835V)	97B	0
36	14/M	M1	46XY	*TET2*	R1359C	*FLT3*- ITD, *CEBPα* [P46H fs*115 (;) K304_Q305 insL]	97B	167+
160	16/M	M2	45×,-Y,t(8;21)	*TET2*	F760Y	*KRAS* (G13R), *WT1* (R434Hfs*86)	97B	8.9

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

Figure 5A); and also no difference in OS was observed (50.0 ± 15.8% vs 51.0 ± 4.5%, $P = .904$, supplemental Figure 5B).

We further analyzed whether *FLT3*-ITD had an effect on outcome and found no difference ($P = .268$ for EFS and $P = .196$ for OS). The presence of *C-KIT* mutations in core-binding factor-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 a 5-year EFS of only 33.3 ± 27.2%. The 2 patients with *TP53* mutations had very grave outcomes, both with OS of <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 only for 1 month. Three patients harboring class IV mutations that coexisted 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.

Coexistence of gene mutations in pediatric AML

Among the 117 patients with at least one mutated gene, the coexistence of more than one gene mutation was 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, 2 had class I plus III plus IV, and an 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 coexisted 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 coexisting *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 the *PTPN11* (E76K) mutation, and the other coexisted 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 a *RUNX1-RUNX1T1* transcript.

Discussion

Compared with adult AML, there were fewer studies of gene mutations in childhood AML. We previously reported a decrease in the frequencies of *FLT3*-ITD, *FLT3*-TKD, and *CEBPα* in childhood AML compared with those in adult AML.[29,31,40] The frequencies of *FLT3* and *CEBPα* mutations that differed considerably between children and adults were later confirmed by the Children's Oncology Group.[41,42] Other investigators also found that the frequency of *NPM1* mutations was 4 times greater 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 one-half of our patients, with *FLT3*-ITD being the most frequent. The frequency of mutations that block hematopoietic differentiation was 13%, and the frequency of mutations involving apoptosis or tumor suppressor genes, ie, *WT1* and *TP53*, was 6.9%, with the latter mutation being rare. The mutation frequencies of class I and II were similar to the report by Radtke et al,[44] they used single-nucleotide polymorphism array and resequencing of candidate cancer genes in a cohort of 111 children with de novo AML. The results of our extended study on pediatric patients again confirm 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. *ASXL1* mutations have not been reported in childhood AML; the 2 mutations we detected were both frameshift mutations that 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, a finding in sharp contrast with those described in adult AML studies, in which the frequencies are 5- to 10-fold greater.[4,5] *TET2* mutations in pediatric AML only have been reported in an abstract.[13] We detected a frequency of 1.7%, which was much lower than the frequency of 12% to 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 AML[16-18,20] but were in contrast to the findings in adult AML with a reported frequencies of 15% to 33% for *IDH*[12] and 12% to 22% for *DNMT3A*.[12,19] The authors of a recent study from Germany found 1.0% (2/195) of childhood AML had *DNMT3A* mutations.[21] We also screened the entire coding sequences and found that both mutants were located in the C-terminal methyltransferase domain, where the greatest number of 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 patients with AML,[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 *MLL* translocation, may contribute to the leukemogenesis.[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 dehypomethylating 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 *DNMT3A* mutations had AML-M4 or M5, and one of them had an *MLL* translocation that was not present in the 2 reported pediatric patients with *DNMT3A* mutations.[21,22] In adult AML, the *DNMT3A* mutation also was reported to be associated with acute monocytic leukemia.[47] All of our 3 patients with *TET2* mutations and 2 patients with *DNMT3A* mutations were older than 10 years. Both patients harboring *ASXL1* mutations and 2 of the 3 patients carrying *TET2* mutations had t(8;21) AML. The frequency of *ASXL1* mutations in our t(8;21) AML seemed to be greater than those without the mutations. This association was not observed in one adult AML study in which 8% of t(8;21)AML had *ASXL1* mutations compared with 5.3% of entire AML cohort.[5] Whether *ASXL1* mutations are associated with t(8;21) AML remains to be examined in a larger number of t(8;21) patients.

We also observed a strong association between the *IDH1* mutation and *MLL*-PTD or AML-M0 subtype. In one of the recent reported pediatric series on *IDH* mutations, 2 of the 7 *IDH1*-mutated patients and 1 of 9 patients with *IDH2* mutations harbored *MLL*-PTD.[18] One of 3 children with *IDH1* mutations was associated with AML-M0 in another study.[17] The patient with *IDH2* mutation had a coexisting *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 bone marrow. Patients with *FLT3*-ITD and *CEBPα* mutations were older and had greater white blood cell counts, 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 present more frequently in normal karyotypes, and *C-KIT* mutations were strongly associated with core-binding factor AML in children.

It had been hypothesized that the development of AML is associated with at least a 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).[48] With the subsequent discovery of the increasing number of mutated genes and their cooperating mutations, a mechanism of multistep leukemogenesis was suggested.[49] 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, 7 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 2 patients with *ASXL1* mutations did not have other coexisted mutated genes, but both carried *RUNX1/RUNX1T1* with disruption of RUNX1 at C-terminal region, which, like *RUNX1* mutations, would result in a dysregulated hematopoietic transcription factor and impair differentiation, a finding functionally confirmed in mouse models.[50]

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.[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 and found no significant difference except a trend of adverse outcome in patients carrying *FLT3*-ITD. Class IV mutations in adult AML conferred an inferior outcome.[4,5,12,39,45] One half of our patients with class IV mutations experienced long-term EFS, 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 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

The authors thank Ms Ting-Yu Huang for secretarial assistance.

This study was supported by grants from Mackay Memorial Hospital, Taipei, Taiwan (MMH-E-99009 and MMH-E100009), grants from the National Science Council, Taiwan (NSC-94-2314-B-195-003

and NSC-96-2314-B-195-006-MY3), and grants from National Health Research Institute (NHRI-EX96-9434SI) and Department of Health, Taiwan (DOH100-TD-C-111-006).

Authorship

Contribution: D.-C.L. and L.-Y.S. designed the experiment; D.-C.L., L.-Y.S., and H.-C.L. analyzed the data and wrote the paper; Y.-J.H., Y.-S.S., Y.-H.H., and T.-H.L. performed the experiments; and D.-C.L., H.-C.L., C.-P.Y., T.-H.J., I.-J.H., T.-C.Y., S.-H.C. and J.-Y.H. provided patient samples and clinical data.

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

Correspondence: Lee-Yung Shih, Division of Hematology-Oncology, Department of Internal Medicine, Chang Gung Memorial Hospital,199 Tung-Hwa North Road, Taipei 105, Taiwan; e-mail: sly7012@adm.cgmh.org.tw.

References

1. Cho YS, Kim EJ, Park UH, et al. Additional sex comb-like 1 (ASXL1), in cooperation with SRC-1, acts as a ligand-dependent coactivator for retinoic acid receptor. *J Biol Chem.* 2006;281(26): 17588-17598.
2. Fisher CL, Pineault N, Brookes C, et al. Loss-of-function Additional sex combs like 1 mutations disrupt hematopoiesis but do not cause severe myelodysplasia or leukemia. *Blood.* 2010;115(1): 38-46.
3. Abdel-Wahab O, Adli M, LaFave LM, et al. ASXL1 mutations promote myeloid transformation through loss of PRC2-mediated gene repression. *Cancer Cell.* 2012;22(2):180-193.
4. Chou WC, Huang HH, Hou HA, et al. Distinct clinical and biological features of de novo acute myeloid leukemia with additional sex comb-like 1 (ASXL1) mutations. *Blood.* 2010;116(20):4086-4094.
5. Pratcorona M, Abbas S, Sanders MA, et al. Acquired mutations in ASXL1 in acute myeloid leukemia: prevalence and prognostic value. *Haematologica.* 2012;97(3):388-392.
6. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science.* 2009;324(5929):930-935.
7. Ko M, Huang Y, Jankowska AM, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature.* 2010; 468(7325):839-843.
8. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell.* 2010;18(6):553-567.
9. Xu W, Yang H, Liu Y, et al. Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. *Cancer Cell.* 2011;19(1):17-30.
10. Delhommeau F, Dupont S, Della Valle V, et al. Mutation in TET2 in myeloid cancers. *N Engl J Med.* 2009;360(22):2289-2301.
11. Langemeijer SM, Kuiper RP, Berends M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet.* 2009; 41(7):838-842.
12. Shih AH, Abdel-Wahab O, Patel JP, et al. The role of mutations in epigenetic regulators in myeloid malignancies. *Nat Rev Cancer.* 2012;12(9):599-612.
13. Kutny MA, Alonzo TA, Gerbing RB, et al. TET2 SNP rs2454206 (I1762V) correlates with improved survival in pediatric acute myelogenous leukemia, a report from the Children's Oncology Group. *Blood.* 2010;116(21):418a. Abstract 949.
14. Mardis ER, Ding L, Dooling DJ, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med.* 2009;361(11): 1058-1066.
15. Ward PS, Patel J, Wise DR, et al. The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell.* 2010;17(3):225-234.
16. Ho PA, Alonzo TA, Kopecky KJ, et al. Molecular alterations of the IDH1 gene in AML: a Children's Oncology Group and Southwest Oncology Group study. *Leukemia.* 2010;24(5):909-913.
17. Andersson AK, Miller DW, Lynch JA, et al. IDH1 and IDH2 mutations in pediatric acute leukemia. *Leukemia.* 2011;25(10):1570-1577.
18. Damm F, Thol F, Hollink I, et al. Prevalence and prognostic value of IDH1 and IDH2 mutations in childhood AML: a study of the AML-BFM and DCOG study groups. *Leukemia.* 2011;25(11):1704-1710.
19. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med.* 2010;363(25):2424-2433.
20. Ho PA, Kutny MA, Alonzo TA, et al. Leukemic mutations in the methylation-associated genes DNMT3A and IDH2 are rare events in pediatric AML: a report from the Children's Oncology Group. *Pediatr Blood Cancer.* 2011;57(2):204-209.
21. Thol F, Heuser M, Damm F, et al. DNMT3A mutations are rare in childhood acute myeloid leukemia. *Haematologica.* 2011;96(8):1238-1240.
22. Hollink IH, Feng Q, Danen-van Oorschot AA, et al. Low frequency of DNMT3A mutations in pediatric AML, and the identification of the OCI-AML3 cell line as an in vitro model. *Leukemia.* 2012;26(2):371-373.
23. Shih LY, Liang DC, Huang CF, et al. Cooperating mutations of receptor tyrosine kinases and Ras genes in childhood core-binding factor acute myeloid leukemia and a comparative analysis on paired diagnosis and relapse samples. *Leukemia.* 2008;22(2):303-307.
24. Liang DC, Shih LY, Yang CP, et al. Molecular analysis of fusion transcripts in childhood acute myeloid leukemia in Taiwan. *Med Pediatr Oncol.* 2001;37(6):555-556.
25. Shih LY, Liang DC, Fu JF, et al. Characterization of fusion partner genes in 114 patients with de novo acute myeloid leukemia and MLL rearrangement. *Leukemia.* 2006;20(2):218-223.
26. Liang DC, Chan TT, Lin KH, et al. Improved treatment results for childhood acute myeloid leukemia in Taiwan. *Leukemia.* 2006;20(1): 136-141.
27. Sanz MA, Martín G, Rayón C, et al. A modified AIDA protocol with anthracycline-based consolidation results in high antileukemic efficacy and reduced toxicity in newly diagnosed PML/RARalpha-positive acute promyelocytic leukemia. PETHEMA group. *Blood.* 1999;94(9):3015-3021.
28. Shih LY, Huang CF, Wu JH, et al. Internal tandem duplication of FLT3 in relapsed acute myeloid leukemia: a comparative analysis of bone marrow samples from 108 adult patients at diagnosis and relapse. *Blood.* 2002;100(7):2387-2392.
29. Liang DC, Shih LY, Hung IJ, et al. FLT3-TKD mutation in childhood acute myeloid leukemia. *Leukemia.* 2003;17(5):883-886.
30. Kuo MC, Liang DC, Huang CF, et al. RUNX1 mutations are frequent in chronic myelomonocytic leukemia and mutations at the C-terminal region might predict acute myeloid leukemia transformation. *Leukemia.* 2009;23(8):1426-1431.
31. Liang DC, Shih LY, Huang CF, et al. CEBPalpha mutations in childhood acute myeloid leukemia. *Leukemia.* 2005;19(3):410-414.
32. Gelsi-Boyer V, Trouplin V, Adélaïde J, et al. Mutations of polycomb-associated gene ASXL1 in myelodysplastic syndromes and chronic myelomonocytic leukaemia. *Br J Haematol.* 2009; 145(6):788-800.
33. Shih LY, Kuo MC, Kuo CY, et al. Emerging kinetics of BCR-ABL1 mutations and their effect on disease outcomes in chronic myeloid leukemia patients with imatinib failure. *Leuk Res.* 2013; 37(1):43-49.
34. King-Underwood L, Renshaw J, Pritchard-Jones K. Mutations in the Wilms' tumor gene WT1 in leukemias. *Blood.* 1996;87(6):2171-2179.
35. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *J Clin Oncol.* 2001;19(5):1405-1413.
36. Baxter EJ, Scott LM, Campbell PJ, et al; Cancer Genome Project. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet.* 2005;365(9464): 1054-1061.
37. Falini B, Mecucci C, Tiacci E, et al. GIMEMA Acute Leukemia Working Party. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med.* 2005; 352(3):254-266.
38. Tartaglia M, Kalidas K, Shaw A, et al. PTPN11 mutations in Noonan syndrome: molecular spectrum, genotype-phenotype correlation, and phenotypic heterogeneity. *Am J Hum Genet.* 2002;70(6):1555-1563.
39. Chou WC, Chou SC, Liu CY, et al. TET2 mutation is an unfavorable prognostic factor in acute myeloid leukemia patients with intermediate-risk cytogenetics. *Blood.* 2011;118(14):3803-3810.
40. Liang DC, Shih LY, Hung IJ, et al. Clinical relevance of internal tandem duplication of the FLT3 gene in childhood acute myeloid leukemia. *Cancer.* 2002;94(12):3292-3298.
41. Meshinchi S, Alonzo TA, Stirewalt DL, et al. Clinical implications of FLT3 mutations in pediatric AML. *Blood.* 2006;108(12): 3654-3661.
42. Ho PA, Alonzo TA, Gerbing RB, et al. Prevalence and prognostic implications of CEBPA mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. *Blood.* 2009;113(26):6558-6566.

43. Brown P, McIntyre E, Rau R, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. *Blood.* 2007;110(3):979-985.

44. Radtke I, Mullighan CG, Ishii M, et al. Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. *Proc Natl Acad Sci USA.* 2009;106(31):12944-12949.

45. Thol F, Damm F, Lüdeking A, et al. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *J Clin Oncol.* 2011; 29(21):2889-2896.

46. Piekarz RL, Bates SE. Epigenetic modifiers: basic understanding and clinical development. *Clin Cancer Res.* 2009;15(12): 3918-3926.

47. Yan XJ, Xu J, Gu ZH, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet.* 2011;43(4):309-315.

48. Kelly LM, Gilliland DG. Genetics of myeloid leukemias. *Annu Rev Genomics Hum Genet.* 2002;3:179-198.

49. Renneville A, Roumier C, Biggio V, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia.* 2008;22(5):915-931.

50. Rosenbauer F, Tenen DG. Transcription factors in myeloid development: balancing differentiation with transformation. *Nat Rev Immunol.* 2007;7(2):105-117.