Molecular Genetics of Myeloid Leukemias : From Bench to Bedside

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Abstract

Acute myeloid leukemia (AML) is a genetically diverse hematologic malignancy. Myelodysplastic syndrome (MDS) refers to a group of clonal hematopoietic stem cell disorders which may progress to AML and involve a multistep molecular pathogenesis. In the past several years, we have used a full range of molecular technology, (1) to detect and monitor the most prognostically relevant fusion genes involving core binding factor, retinoic acid receptor α, and *MLL* rearrangements in AML, (2) to characterize the fusion partners of *MLL* gene, and (3) to analyze the mutations of receptor tyrosine kinase/Ras pathway and myeloid transcripts factors in myeloid leukemias.

In AML, the frequencies of t(8;21)/*AML1-ETO*, inv(16)/*CBFβ-MYH11*, and t(15;17)/*PML-RARα* were defined by RT-PCR assays. We used Southern blot analysis to screen *MLL* rearrangement [*MLL*(+)] in de novo AML, RT-PCR to detect common *MLL* fusion transcripts and cDNA panhandle PCR to identify infrequent or unknown *MLL* partner genes. We identified two novel *MLL* fusion transcripts (*MLL-SEPT6* and *MLL-CBL*) and 2 novel fusions of *MLL-ENL*. *MLL*-PTD was the most common genetic lesion in adult patients with *MLL*(+) AML, whereas *MLL*-PTD was rare in childhood AML. We also demonstrated that mutations in *FLT3* or *Ras* genes were highly associated with AML harboring *PML-RARα* or *MLL* rearrangement, supporting the two-hit model of leukemogenesis.

AML1, CEBPα and PU.1 are the three important myeloid transcription factors of granulopoiesis. Our results showed that *CEBPα* mutations were infrequent and *PU.1* mutation was absent in patients with MDS/AML, whereas *AML1* mutations were common in MDS or CMML. We have published the first pediatric AML series with *CEBPα* and *FLT3*/TKD mutations. The incidence of *FLT3*/ITD in childhood AML was lower than adult AML and a high ratio of mutant to wild-type *FLT3* predicted poor prognosis.

The roles of mutations of *FLT3*/ITD, *FLT3*/TKD, N-*ras*/K-*ras* and *CEBP*α genes in de novo AML at diagnosis and relapse, and in the transformation of AML from MDS were also examined. Nine percent of AML patients acquired *FLT3*/ITD mutations at relapse. We observed heterogeneous patterns, loss or gain, of *FLT3*/TKD mutations at AML relapse, suggesting the mutation being a secondary event in a subset of AML patients. One-third of MDS patients acquired *FLT3* or N-*ras* mutations during AML evolution. *FLT3*/ITD was associated with a poor outcome in patients with MDS.

Detection of the genetic abnormalities in AML have been translated into diagnostic approach and patient management. Characterization of novel genetic alterations will continue to provide a bench to bedside translation of molecularly targeted therapy in the future.

Introduction

Acute myeloid leukemia (AML) is now recognized as a very heterogeneous group of genetically diverse hematologic malignancies. The genetic abnormalities of AML influence disease aggressiveness and responses to therapy. The importance of genetics in classification of AML has been recognized in the new World Health Organization (WHO) classification system.[1] Our current understanding of the molecular pathogenesis of AML has been gained through characterization of chromosomal translocation breakpoints and cloning of genes involved in these translocations.[2] Translocations create chimeric genes with novel oncogenic properties.[3] Three classes of chromosomal translocations have been particularly well-characterized at the molecular level in AML: those involving (1) core binding factor (CBF), i.e. *AML1-ETO*/t(8;21) and *CBFβ-MYH11*/inv(16),[4,5] (2) *MLL* (mixed-lineage leukemia) gene rearrangement,[6,7] and (3) the retinoic acid receptor α (*RARα*) in acute promyelocytic leukemia (APL).[8,9]

Approximately 45% of de novo adult AML lack cytogenetic abnormalities.[10] In AML patients who lack cytogenetic abnormalities, efforts to classify these patients molecularly are desperately needed. AML patients with normal karyotype often harbor submicroscopic genetic abnormalities which are discernible only by molecular genetic techniques. Partial tandem duplication of the *MLL* gene (*MLL*-PTD), internal tandem duplication of the *FLT3* gene (*FL3*/ITD), and mutations of *CEBPα* or *NPM1* gene, mainly occur in patients with normal karyotype and have recently been found to be of prognostic significance.[11-14]

Studies from transgenic mouse models and cell lines have shown that fusion genes of *AML1-ETO*, *CBFβ-MYH11* and *PML/RARα* are not sufficient to induce full-blown leukemia.[15-17] Recently, the two-hit model of leukemogenesis in AML has been proposed.[18] Class I mutations that drive proliferation including receptor tyrosine kinases (RTK) and Ras signaling pathway, and Class II mutations that block differentiation comprising mutations or

translocations in hematopoietic transcription factors (HTFs).

Myelodysplastic syndrome (MDS) refers to a group of clonal hematopoietic stem cell disorders with a tendency to progress to AML. It has been demonstrated that MDS involves a multistep molecular pathogenesis of the disease. Evidence has been accumulated that genetic alterations are closely associated with the progression of MDS to AML,[19-22] MDS serves as a useful model for studying the abnormal genetic events that occur in leukemogenesis. Efforts are being made to determine the prognostic significance of various genetic aberrations in adult patients with MDS.

Over the past several years, we have used a full range of molecular technology, (1) to detect and monitor the most prognostically relevant fusion genes involving *CBF*, *RARα*, and *MLL* rearrangements, (2) to systematically characterize the fusion partners of *MLL* gene, (3) to analyze the mutations of HTFs in patients with myeloid leukemias, (4) to determine the role of genetic aberrations in the progression of AML, and (5) to define the type of cooperating mutations in the hematopoietic RTK/Ras that collaborate with *MLL* rearrangements or AML with mutations of HTFs and to test the hypothesis of two-hit model of leukemogenesis with clinical samples.

Materials and Methods

Samples

Bone marrow (BM) samples from patients with MDS and AML at diagnosis, during follow-up, at AML transformation, or at relapse of AML have been obtained with informed consent and freshly frozen in our cell bank since 1991. It allowed us to examine and characterize the various molecular mutations in a systematic way. Subtypes of AML and MDS were classified according to the French-American-British (FAB) and WHO systems.[1,23,24] Cytochemical study and immunophenotypic analysis were performed at diagnosis of de novo AML and at AML transformation of MDS. Cytogenetic analysis was performed at initial diagnosis of MDS and AML.

The mononuclear cells from BM samples were enriched by Ficoll-Hypaque density gradient centrifugation and cryopreserved until use. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays for detection of fusion transcripts of *AML1-ETO*, *CBFβ-MYH11*, *PML-RARα* and *BCR-ABL* were performed on the diagnostic samples as previously described.[25] Samples positive for t(8;21)/*AML1-ETO*, inv(16)/*CBFβ-MYH11*, t(15;17)/*PML-RARα* , or t(9;22)/*BCR-ABL* were excluded from the examination for *MLL* rearrangement.

Detection of *MLL* rearrangement and common partner genes[26]

Southern blot analysis was performed to detect *MLL* rearrangement. Two sets of multiplex PCR assays were performed with nested protocol and then analyzed with GeneScan analysis for detection of common *MLL* fusion transcripts including *MLL-AF6*/*MLL-AF9*/*MLL-ENLa1* and *MLL-ENLa2*/*MLL-ELL*/*MLL*-PTD. Standard RT-PCR was used for the detection of fusion transcripts of *MLL-AF1*, *MLL-AF4* and *MLL-AF10*.

Cloning of *MLL* fusion transcripts by cDNA panhandle PCR

cDNA panhandle PCR technology was used to identify the infrequent or unknown *MLL* partner genes according to the method of Megonigal et al: with minor modification.[27]

Mutational analysis of hematopoietic transcription factors: *AML1*, *CEBPα* and *PU.1*

DNA PCR and direct sequencing for detection of CEBPα mutations

DNA PCR assay was carried out with two overlapping primer pairs PP1F and PP1R, and PP2F and PP2R which cover the entire coding region of human *CEBPα*.[28] PCR products were purified (Qiagen) and sequenced in both directions. Samples with abnormal or ambiguous sequencing results were subjected to repeated PCR assays with an alternative primer pairs.[29,30]

RT-PCR and direct sequencing for detection of AML1 mutations

We used RT-PCR with 3 primer pairs which cover the coding sequences from exons 3 to 8 of *AML1* gene (GenBank accession number D43969) for the mutational analysis.

Detection of PU.1 mutations

DNA PCR analysis was performed by using 5 exon-specific primer pairs according to those described by Mueller et al:[31]

Detection of *Ras* and activating *FLT3* mutations

The DNA/cDNA PCR assay with direct sequencing for the detection of point mutations

at codons 12, 13 and 61 of the N-*ras* and K-*ras* genes, of *FLT3*/ITD and point mutations at tyrosine kinase domain of *FLT3* (*FLT3*/TKD), and GeneScan-based analysis of the *FLT3*/ITD mutant level were performed as previously described.[22,32,33]

Expand Long Template PCR assay and cloning analysis

For samples carrying more than one mutant, Expand Long Template PCR assay (Roche, Mannheim, Germany) with primers designed specifically to amplify the region of all mutations was carried out to determine whether the multiple mutations located on the same allele or on different alleles.[30,33] The PCR product was subcloned into the pCRII-TOPO vector (Invitrogen), and at least 10 clones were subsequently sequenced for each sample.

Statistical analysis

The Fisher's exact test, the χ^2 analysis, the unpaired t-test, and the Wilcoxon's rank-sum test were used as appropriate to make comparisons between groups. Overall survival was defined as the length of time from diagnosis to death or last follow-up. Event-free survival was defined as the length of time from diagnosis to the date of failure (induction failure, relapse, or toxicity related death) for patients who experienced failure or to the date of last contact for all others. Kaplan–Meier analysis was used to evaluate survival. Differences in survival were assessed using the log-rank test. Statistic analyses were performed using a SPSS software version 8.0 for Windows (SPSS Inc., Chicago, IL). In all analyses, *P* values were two-tailed and values < 0.05 were considered statistically significant.

Results and Discussion

Detection of recurrent genetic abnormalities in AML proposed by WHO classification

Fusion transcripts of favorable chromosomal translocations

Among 935 adult patients with de novo AML, t(8;21)/*AML1-ETO*, inv(16)/*CBFβ-MYH11*, and t(15;17)/*PML-RARα* were detected in 83, 27 and 112 patients, respectively. Cytogenetic analysis failed to detect 11% of patients with t(8;21)/*AML1-ETO*, 17% of patients with inv(16)/*CBFβ-MYH11*, and 13% of patients with t(15;17)/*PML-RARα* (Table 1) . RT-PCR assays were more sensitive than cytogenetic analysis in the detection of these recurrent favorable genetic abnormalities. Our results showed that 59% of patients with APL had L-type *PML-RARα* isoform, 9% had V-type isoform, and 32% had S-type isoform.[33]

Detection of MLL rearrangements and its fusion partners and correlation with FAB subtypes in de novo AML[26]

MLL rearrangement [*MLL*(+)] was identified in 114 patients with de novo AML by using Southern blot analysis; 98 patients were adults and 16 were children. The incidence of *MLL*(+) was 11.3% and 13.0% of adult and pediatric de novo AML, respectively. The frequencies and fusion partners of *MLL*(+) with respect to FAB subtypes are shown in Table 2. *MLL*(+) AML was most frequently found in AML-M5, both in adults (32.7%) and in children (52.6%).

MLL-PTD was distributed in all FAB subtypes except M7. Forty-five patients with *MLL*-PTD had a duplication of exons 2 to 6 of *MLL* resulting in fusion of exon 6/exon 2 (e6e2) and the remaining 18 had e8e2. *MLL-AF6* was more frequently present in M1 and M4, *MLL-AF9* and *MLL-AF10* most frequently occurred in M5, and *MLL-ELL* in M4 or M5.

None of patients with *MLL*-PTD had 11q23 abnormalities. Cytogenetic analysis failed to detect 11q23 abnormalities in 5 of the 13 patients with *MLL*-AF9, in 4 of 10 patients with *MLL-ENL* or *MLL-ELL*. Moreover, for those with detectable 11q23 abnormalities, their *MLL* chromosomal partners could not be precisely identified in 9 patients.

MLL-PTD was significantly more prevalent in adults than in children ($P<0.001$). Adult *MLL*(+) AML patients had a significantly poorer outcome than children and there was no difference in survival between patients with *MLL*-PTD and those with other *MLL* translocations.

Identification of rare or novel MLL partner genes

cDNA panhandle PCR technology was able to identify 5 rare or novel *MLL* fusion transcripts and 2 novel fusions of *ENL* gene. The breakpoints of *MLL*, the partner genes and their chromosomal locations as well as the clinicohematologic features and cytogenetics are shown in Table 3.

FLT3 mutations in myeloid leukemia

Two distinct types of *FLT3*-activating mutations have been described in 30% of patients with AML. The majority of *FLT3* mutations (20~25%) involved *FLT3*/ITD,[34-39] and an additional 5-10% of patients carried a point mutation of Asp835 at exon 20 within the activation loop of TKD of *FLT3* gene (*FLT3*/TKD).[40,41] Both types of mutations induce factor-independent proliferation through constitutive tyrosine phosphorylation and enhance cell proliferation in the experimental system.[40,42-46]

Most of the previous studies on activating mutations of *FLT3* in adult AML were analyzed at initial diagnosis. Paired BM samples at diagnosis and relapse from 108 adult AML patients were analyzed for *FLT3*/ITD mutations and from 120 patients for *FLT3*/TKD

mutations to determine the role of activating mutations of *FLT3* in the relapse of AML.[32,47] Eighty-three patients had wild-type *FLT3* at both diagnosis and relapse, 16 had *FLT3*/ITD at both stages; 8 acquired the mutations and one lost it at relapse. Using GeneScan analysis, we found that *FLT3/*ITD levels at first relapse were significantly higher than those at diagnosis (mean ± SE, 40.5% ± 4.8% versus 17.9% ± 3.6%, P <0.001). The increase in mutation levels at relapse as compared with diagnosis did not correlate with the difference in blast cell percentages at both stages (P=0.777). A hemizygous deletion of wild-type *FLT3* was found in 4 patients at relapse compared to none at diagnosis. Nine of the 11 patients carrying a single mutation at diagnosis relapsed with an identical mutation. All 6 patients with more than one *FLT3/*ITD mutation at diagnosis showed changes in mutation patterns and levels at first relapse; however, each patient retained at least one mutation in the relapse sample. The changes of mutation patterns had implications for the monitoring of minimal residual disease (MRD). Our results suggest that *FLT3*/ITD may contribute as the initial transforming event in AML, and relapse can reflect the selection and outgrowth of a mutant clone or evolution of a new clone harboring this mutation.[32]

The detection of MRD is of growing importance in AML management. As demonstrated here as well as in other studies,[32,39] the vast majority of patients with AML with *FLT3*/ITD lack a previously defined molecular marker of MRD, suggesting that this mutation might serve as a useful target for PCR-based assays. Our data implied that most patients will retain at least one original mutation in their relapse samples. Hence, a patient-specific real-time PCR assay using primer sets that cover the junction regions of the different mutations, permitting the mutant but not wild-type alleles to be amplified, would probably overcome the intrinsic technical difficulties.[48] For patients with more than one *FLT3*/ITD, we suggest that all mutants be monitored during follow-up.

Thirteen patients had Asp835 mutations of *FLT3* (*FLT3*/TKD) at diagnosis, of them 8

lost the mutations at relapse, and 5 patients retained the same mutations at relapse. Another 6 patients acquired Asp835 mutations at relapse. Our results showed that patients with AML had heterogeneous patterns of *FLT3*/TKD mutations, either acquisition or loss of the mutations at relapse. As Asp835 mutations might lose in a subset of patients with AML at relapse; *FLT3*/TKD mutation is not a suitable marker for monitoring MRD or detecting early relapse in patients harboring these mutations at diagnosis. Five samples harbored both *FLT3*/ITD and *FLT3*/TKD mutations that were found on different alleles by cloning analysis in the 3 patients studied. There were no differences in WBC count, FAB subtype, percentage of marrow or circulating blasts between patients with and without Asp835 mutations, whereas the difference in the incidence of Asp835 mutations among cytogenetic/molecular subgroups was statistically significant (P=0.025).[47] The mutation status of *FLT3*/ITD, *FLT3*/TKD and N-*ras* in paired diagnosis and relapse samples of AML is shown in Table 4.[32,47,49]

FLT3/ITD mutation status was evaluated on 198 bone marrow samples obtained from patients with MDS at initial diagnosis.[50] Five patients (2.5%) had *FLT3*/ITD mutations. Four of the 5 patients carrying the *FLT3*/ITD mutations experienced progression of disease to AML, compared with 70 of the 193 patients who did not have *FLT3*/ITD (P= 0.066). In addition, progression to AML was more rapid in patients with *FLT3*/ITD-positive disease than in patients with *FLT3*/ITD-negative disease (P < 0.0001). Patients with *FLT3*/ITD-positive disease also had significantly shorter survival compared with patients who had *FLT3*/ITD-negative disease (P < 0.0001). On multivariate analysis, *FLT3*/ITD was identified as an independent predictor of rapid progression to AML (P = 0.0001) and poor overall survival (P = 0.002).

We also examined the roles of *FLT3* and N-*ras* mutations in the progression of MDS to AML.[22] Of the 70 paired samples of MDS/AML, 7 patients acquired *FLT3*/ITD during AML evolution. The incidence of *FLT3*/ITD at diagnosis of MDS was significantly lower than that

at AML transformation (3/70 vs. 10/70, *P*<0.001). *FLT3*/ITD(+) patients progressed to AML more rapidly than *FLT3*/ITD(-) patients (2.5±0.5 vs. 11.9±1.5 months, *P*=0.114). *FLT3*/ITD(+) patients had a significantly shorter survival than *FLT3*/ITD(-) patients (5.6±1.3 vs. 18.0±1.7 months, *P*=0.0008). After AML transformation, *FLT3*/ITD was also associated with an adverse prognosis. One patient had *FLT3*/TKD mutation (D835Y) at both MDS and AML stages. Additional three acquired *FLT3*/TKD (one each with D835 H, D835F and I836S) at AML transformation. Five of the 70 matched samples had N-*ras* mutation at diagnosis of MDS compared to 15 at AML transformation (*P*<0.001), one lost and 11 gained N-*ras* mutations at AML progression. N-*ras* mutations had no prognostic impact either at the MDS or AML stage. Together, one-third of MDS patients acquire activating mutations of *FLT3* or N-*ras* gene during AML evolution (Table 4) and *FLT3*/ITD predicts a poor outcome in MDS.[22]

Mutations of myeloid transcription factors *AML1*, *CEBPα* and *PU.1* in patients with de novo MDS, CMML and AML

HTFs represent targets for disruption in MDS or AML, not only by chromosomal translocations but also by mutations in the coding region of these TFs. Point mutations in myeloid TFs may also lead to loss of myeloid differentiation. Transcription factor CCAAT/enhancer binding protein α (CEBPα) is essential for granulocytic differentiation. *CEBPα* mutations were analyzed in 149 patients with de novo AML at both diagnosis and relapse, 15% of patients had mutations at diagnosis and the mutations were only restricted to patients with intermediate cytogenetic risk group.[49] It is of interest to find that the majority of patients with *CEBPα* mutations had biallelic mutations occurring at both N-terminal and bZIP domains. We also demonstrated that 91% of de novo AML harboring *CEBPα* mutations

at diagnosis retained the identical mutant patterns but frequently change in the allelic distribution at relapse, 9% lost *CEBPα* mutations and none acquired the mutations at relapse.[49] *CEBPα* mutations were not involved in the pathogenesis of chronic myeloid leukemia with AML transformation.[51]

Of the 50 paired MDS/AML samples, *CEBPα* mutations were identified in one patient with refractory anemia with excess of blasts (RAEB) and in 3 with chronic myelomonocytic leukemia (CMML) at diagnosis. At AML transformation, 3 patients retained the identical mutant clones as their initial diagnosis, 3 acquired the mutations, and one lost *CEBPα* mutation when she gained *FLT3*/ITD mutation. Together, 7 patients had *CEBPα* mutations throughout the disease course. *CEBPα* mutations had no influence on the time to AML progression or overall survival. Our results showed that *CEBPα* mutations played a role in a subset of patients with MDS, especially in CMML. The mutation status was heterogeneous, exhibiting identical clone, clonal change, or clonal evolution during the progression to AML.[29]

Transcription factor AML1 is essential for normal hematopoiesis. *AML1* mutations have been found in therapy-related MDS but were rarely described in patients with de novo MDS or CMML. Mutation analysis of *AML1* was performed on BM samples from 76 patients with MDS [11 refractory cytopenia with multilineage dysplasia (RCMD), 31 RAEB1 and 34 RAEB2] and 67 patients with CMML. At initial presentation of MDS, 18.4% had *AML1* mutations. *AML1* mutations were detected in 27 of 67 CMML patients (40%). The frequency of *AML1* mutations was significantly higher in patients with CMML than in MDS (P=0.005). Mutations in runt homology domain (RHD) occurred more frequently in CMML than in MDS (P=0.020). CMML patients had a higher frequency of frameshift as compared with MDS patients (P=0.040). Time to AML transformation and overall survival of *AML1*(+) patients did not differ from *AML1*(-) patients in both MDS and CMML groups. Our study

showed that *AML1* mutations were frequently detected in de novo MDS and CMML, especially the latter.[52]

The roles of *AML1*, *CEBPα* and *PU.1* mutations in the progression of MDS into AML remained to be determined, as no matched paired samples of both phases have been analyzed in the previous studies. We analyzed mutations of *AML1* on paired BM samples at initial diagnosis of de novo MDS (8 RCMD, 12 RAEB1, and 17 RAEB2) and at AML transformation. Eight of 37 patients (21.6%) had *AML1* mutations at initial presentation of MDS. Another 2 acquired C-terminal mutations at AML transformation.[53] *PU.1* mutation was not detected in any of the 32 patients examined at both MDS and AML phases.[53] Our study showed that *AML1* mutations were common in patients with MDS/CMML, whereas *CEBPα* mutations were infrequent and *PU.1* mutation was absent. The mutation status of HTFs in paired samples of MDS/AML and AML at diagnosis and relapse is shown in Table 5.

Genetic abnormalities in childhood AML

Collaborating with Taiwan Pediatric Oncology Group, we reported the frequencies of common fusion transcripts in Childhood AML in Taiwan (Table 6).[25] We examined the *FLT3*/ITD mutations in childhood AML.[57] The incidence of *FLT3*/ITD in childhood AML was lower than that of adult AML and the ratio of mutant to wild-type *FLT3* was a predictor of poor prognosis. All 3 patients with a mutant *FLT3* to wild-type ratio of greater than 2.0 died within 8 months after diagnosis, two of them failed to achieve complete remission. Our results showed that the mutant *FLT3* to wild-type ratio, but not the presence of *FLT3*/ITD itself, may serve as a potential marker to improve risk-assessement in childhood AML.[56] We also published the first pediatric series, in the world, of *FLT3*/TKD and *CEBPα* mutations in childhood AML.[30,58] *FLT3*/TKD mutations occurred in 3 of 91 patients, an incidence significantly lower than that of *FLT3*/ITD (14 of 91 patients, 15.4%) in the same cohort of

patients. None of them had both *FLT3*/TKD and *FLT3*/ITD mutations. The frequencies of mutations of *FLT3*, *Ras* and *CEBPα* in childhood AML is shown in Table 7.

Cooperating mutations in AML

We investigated the collaboration of *FLT3* mutations with *PML/RARα* in APL and found that *FLT3*/ITD or *FLT3*/TKD mutations were present in 36.4% of patients with *PML/RARα*.[33] The mutations had no prognostic impact. *FLT3*/ITD frequently was associated with S-type *PML/RARα* and with the M3v form of APL.[33] Cooperating mutations of *MLL* rearrangements with other genetic aberrations were further explored. Sixty-one percent of adult *MLL*(+) AML and 37% of childhood *MLL*(+) AML had cooperating mutations of RTK/*Ras* (Table 8). In childhood AML, *Ras* mutations were analyzed according to the presence or absence of *MLL* rearrangements. *Ras* mutations were detected in 17.7% of children with AML. *MLL*(+) AML was not associated with N-*ras* mutations. K-*ras* mutations were detected in 3 of 17 patients with *MLL*(+) AML compared with 5 of 113 *MLL*(-) AML (P=0.069).[59] We observed that *MLL*-PTD was strongly associated with *FLT3*/ITD mutation with a frequency of 45%.[60] The frequencies of *FLT3*/ITD and K-*ras* were significantly higher in patients with *MLL*-PTD than those with *MLL*-T (P<0.001). There was no difference in the mutation status of *FLT3*/TKD or N-*ras* between *MLL*-PTD and *MLL*-T groups. The findings of high incidence of RTKs/*Ras* mutations in AML patients with *MLL* rearrangements support the two-hit hypothesis for the pathogenesis of AML.

Conclusion and Future Directions

During the past 5 years, we used a full range of molecular biologic techniques to identify the most relevant genetic aberrations in patients with de novo AML. Detection of fusion transcripts derived from chromosomal translocations including *AML1-ETO*, *CBFβ-MYH11*, *PML-RARα* and *MLL* rearrangements allowed risk-stratification of AML and guiding the clinician in selecting treatment options. Real-time quantitative RT-PCR assays are used to monitor MRD and define molecular complete remission as well as to detect early relapse. Identification of submicroscopic mutations of genes involving RTK/*Ras* pathway (*FLT3*/ITD, *FLT3*/TKD and *Ras*) and TFs (*AML1*, *CEBPα*, and *PU.1*) showed that cooperating mutations were present in a considerable proportion of AML samples which supported the two-hit model of leukemogenesis. By analyzing paired samples of AML at both diagnosis and relapse, and MDS at initial diagnosis and at AML transformation, we defined the roles of *FLT3*/ITD, *FLT3*/TKD, *Ras*, *CEBPα* and *AML1* in the progression of AML.

The characterization of genetic alterations provides a bench to bedside translation of molecularly targeted therapy for myeloid leukemais as already have been exemplified by the BCR-ABL tyrosine kinase inhibitor which is revolutionizing treatment of chronic myeloid leukemia. Recently, the insights into the molecular pathogenesis of AML have led to the development of more specific targeted agents. Global clinical trials have been currently underway to examine the therapeutic effectiveness of compounds targeting specific molecular lesions in AML. Novel molecular genetic aberrations suitable for therapeutic targeting will continue to be discovered. These advances will hopefully result in further improving outcome of patients with AML.

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Table 1. Frequencies of Fusion Genes Derived from Favorable Cytogenetics in de novo Adult AML
(N=935, CGMH 1991-2004)

Subtype	RT-PCR(+)	Cytogenetics			
		ND	(+)	(-)	Sensitivity
t(8;21) / *AML1-ETO*	83 (9%)	19	57	7	89%
inv(16) / *CBFβ-MYH11*	27 (3%)	9	15	3	83%
t(15;17) / *PML-RARα*	112 (12%)	37	65	10	87%
Total	222 (24%)				

ND: not done

Table 2. Frequencies of *MLL* Rearrangements and Distribution of *MLL* Fusion Partners According to FAB Subtypes in AML

FAB	No.	*MLL*(+) (%)	*MLL-PTD*	*MLL-AF6*	*MLL-AF9*	*MLL-AF10*	*MLL-ELL*	*MLL-ENL*	Others*
M0	59	6 (10.2)	4	0	0	0	0	1	1
M1	166	25 (15.1)	17	3	3	1	0	0	1
M2	390	29 (7.4)	25	1	0	0	1	0	2
M4	139	24 (17.3)	12	3	2	0	5	0	2
M5	68	26 (38.2)	3	1	9	7	3	2	1
M6	17	2 (11.8)	2	0	0	0	0	0	0
M7	18	2 (11.1)	0	0	0	1	0	1	0
Unclassified	5	0 (0)							
Total	988	114 (11.5)	63	8	14	9	9	4	7

*Including one case each of *MLL-AF1*, *MLL-AF4*, *MLL-MSF*, *MLL-LARG*, *MLL-LCX*, *MLL-SEPT6* and *MLL-CBL*.

Table 3. Clinical and Genetic Characteristics of 7 Patients with de novo AML and Rare/Novel *MLL* Fusion Transcripts

Patient	Age (yrs)	Sex	FAB subtype	Cytogenetics	*MLL* breakjunction	Partner gene	Partner chromosome
1	28	F	M1	50XX,+22,+3mar	exon 6	*CBL*	11q23
2	0.7	M	M4	46XY	exon 8	*SEPT6*	Xq24
3	77	F	M5a	53XX,+6,+8,+8,+9,+11,+13,+22	exon 6	*LARG*	11q23
4	54	M	M2	ND	exon 5	*LCX*	10q22
5	23	F	M4	47XX,t(11;17)(q22;q12),+19	exon 5	*MSF*	17q25
6	1.2	M	M5a	46XY,t(11;19)(q23;p13)	exon 8	*ENLa4**	11p13.3
7	66	F	M0	46XX,add(21)q22	exon 7	*ENLa4**	11p13.3

ND: not done, *exon 6 of *ENL*

Table 4. Mutation Status of Receptor Tyrosine Kinase/Ras Signaling Pathway in Paired Diagnosis/Relapse Samples of AML And MDS/AML

Genes	No. of patients	WT→WT	Mut→Mut	WT→Mut	Mut→WT	Reference
AML Dx/Rel						
FLT3/ITD	108	83	16	8	1	Shih et al.[32]
FLT3/TKD	120	101	5	6	8	Shih et al.[47]
N-*ras*	149	132	16	0	1	Shih et al.[49]
MDS/AML						Shih et al.[22]
FLT3/ITD	70	60	3	7	0	
FLT3/TKD	65	61	1	3	0	
N-*ras*	70	53	5	11	1	

Dx: at diagnosis, Rel: at relapse, WT: wild-type, Mut: mutation

Table 5. Mutation Status of Myeloid Transcription Factors in Paired Samples of MDS/AML and AML Dx/Rel

Genes	No. of patients	WT→WT	Mut→Mut	WT→Mut	Mut→WT	Reference
MDS/AML						
AML1	37	27	8	2	0	Shih et al.[53]
CEBPα	50	43	3	3	1	Shih et al.[29]
PU.1	32	32	0	0	0	Shih et al.[53]
AML (Dx)/(Rel)						
CEBPα	149	127	20	0	2	Shih et al.[49]

Dx: at diagnosis, Rel: at relapse, WT: wild-type, Mut: mutation

Table 6. Comparison of Frequencies of Common Genetic Abnormalities in Childhood AML Between Taiwan and the Western Countries (%)

Method	t(8;21)/*AML1-ETO*	t(15;17)/*PML-RARα*	inv(16)/*CBFβ-MYH11*	t(9;11)/*MLL-AF9*	Reference
Cytogenetics	17	4	9	8	Leblanc et al.[54]
Molecular	16	9	6	11	Behm et al.[55]
Cytogenetics	12	7	12	7	Martinez-Climent et al.[56]
Molecular	18	14	6	6	Liang et al.[25]

Table 7. Mutations of *FLT3*, *Ras* and *CEBPα* Genes in Childhood AML

Mutation	No.(+)/No. examined (%)	Reference
FLT3/ITD	14/91 (15.4%)	Liang et al.[57]
FLT3/TKD	3/91 (3.3%)	Liang et al.[58]
N-*ras*	16/130 (12.3%)	Liang et al.[59]
K-*ras*	8/130 (6.2%)	Liang et al.[59]
CEBPα	7/117 (6.0%)	Liang et al.[30]

Table 8. Cooperating Mutations of Receptor Tyrosine Kinases/Ras Genes in AML with *MLL* Rearrangements

RTK/Ras	Adults (N=112)	Children (N=19)
FLT3/ITD	36	1
FLT3/TKD	16	2
N-*ras*	12	3
K-*ras*	12	3
c-*KIT*	2	0
c-*FMS*	0	0
Total (%)	61	37

骨髓性白血病的分子遺傳學：由實驗研究至臨床應用

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急性骨髓性白血病(AML)在基因學上變化多端，骨髓造血不良症候群(MDS)係一群單源性造血幹細胞疾患，經由多步驟分子生物病變，可惡化為急性骨髓性白血病。過去數年來，我們用全方位分子生物科技來(1)檢驗原發性急性骨髓性白血病 core binding factor，*RARα*和 *MLL* 基因重組，並作微量偵測，(2)鑑認 *MLL* 融合拍檔基因，(3)分析骨髓性白血病之酪胺酸激酶受體、Ras 和造血轉錄因子基因的突變。

在急性骨髓性白血病，以反轉錄聚合酶連鎖反應定出 t(8;21)/*AML1-ETO*, inv(16)/*CBFβ-MYH11*, 和 t(15;17)/*PML-RARα*等的發生率。我們以南方墨點法篩檢 *MLL* 基因重組，以反轉錄聚合酶連鎖反應檢測常見的 *MLL* 融合基因，以 cDNA 鍋柄式聚合酶連鎖反應來鑑認不常見或不明的 *MLL* 拍檔基因。我們鑑認了兩個新的 *MLL* 融合基因(*MLL-SEPT6* 和 *MLL-CBL*)，及兩個新的 *MLL-ENL* 斷裂點。*MLL* 基因部份縱列複製在成人具 *MLL* 基因重組的急性骨髓性白血病是最常見的，而在兒童很少。我們分析具有 *PML-RARα* 或 *MLL* 基因重組的急性骨髓性白血病細胞，發現有高頻率的 *FLT3*/*Ras* 基因突變，可為引起急性骨髓性白血病發病的二次打擊模式的佐証。

AML1、CEBPα、PU.1 是三種最重要的促成顆粒性白血球分化的轉錄因子。在骨髓造血不良症候群或轉化成急性骨髓性白血病病人，無 *PU.1* 基因突變，也少見 *CEBPα*基因突變。而 *AML1* 基因突變在骨髓造血不良症候群或慢性骨髓單核球性白血病則常見。我們發表了全世界第一個兒童急性骨髓性白血病的 *CEBPα*和 *FLT3*-TKD 基因突變論文。

我們也研究 *FLT3*-ITD, *FLT3*-TKD, N-*ras*/K-*ras*, 在急性骨髓性白血病復發時，及骨髓造血不良症候群轉化為急性骨髓性白血病時的角色。我們觀察到 *FLT3*-ITD 突變對一些急性骨髓性白血病病人病程的惡化扮演重要角色。而 *FLT3*-TKD 基因突變，在復發時型態多樣，表示這些突變是屬於次發性的變化。骨髓造血不良症候群病人在轉化成急性骨髓性白血病過程，有三分之一獲得 *FLT3* 或 N-*ras* 基因突變。骨髓造血不良症候群病人有 *FLT3*-ITD 者，預後差。兒童急性骨髓性白血病的 *FLT3*-ITD 比成人少，突變型與原型比值高的，預後差。

檢驗和監測這些基因異常和定量，已應用於診斷和治療。即時定量反轉錄聚合酶連鎖反應用以偵測微量殘餘白血病，以界定是否達到分子緩解，並可偵知早期復發。將來，更多新的基因變異的研究和發現，可提供研發分子標靶治療，應用於臨床。