Mutations of *AML1* in non-M0 acute myeloid leukemia: six novel mutations and a high incidence of cooperative events in a South-east Asian population

Chirayu U. Auewarakul
Amporn Leecharendkeat
Wanna Thongnoppakhun
Chanin Limwongse
Chintana Tocharoentanaphol

Point mutations of *AML1* are uncommon and predominantly reported in a rare minimally differentiated acute myeloid leukemia (M0 AML). Few data exist regarding the frequency of *AML1* mutations in non-M0 cases. We screened 284 consecutive adult Thai patients with *de novo* AML and found that 3.9% had *AML1* mutations. The highest incidence occurred in M6. Six novel mutations were uniquely identified in non-M0 cases. Sixty-four percent of the non-M0 patients with *AML1* mutations had coexisting genetic abnormalities including *FLT3* mutation in 36%. Our study provides evidence to support the model of multiple co-operating events, which could also be critical in the development of leukemia in non-M0 AML patients with mutated *AML1*. The prognostic significance of these novel mutations remains to be determined.

Key words: *AML1* gene, novel *AML1* mutations, co-operative mutations, *de novo* acute leukemia, Thailand.

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From the Department of Medicine (CUA, CL), Department of Immunology (CUA, AL), Faculty of Medicine Siriraj Hospital, Mahidol University, Chulabhorn Cancer Centre, Chulabhorn Research Institute (CUA, CT), Office of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (WT, CL).

Correspondence
Chirayu U Auewarakul, MD, PhD, Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Rd, Bangkoknoi, Bangkok 10700, Thailand.
E-mail: sicaw@mahidol.ac.th/ chirayuaue@yahoo.com

The human *AML1* (*CBFA2/RUNX1*) gene encodes the major subunit of the heterodimeric transcription factor that plays an important role in hematopoiesis.[1] The most common mechanism of *AML1* gene deregulation in acute leukemia is through chromosome translocation.[2] Recently, non-translocation mechanisms of *AML1* deregulation were also described, including point mutations in patients with acute myeloid leukemia (AML) and gene amplifications in patients with acute lymphoid leukemia (ALL).[3] Patients with minimally differentiated AML (M0 AML), a rare subtype of AML associated with a poor prognosis, have been reported to have a high frequency of *AML1* mutations, ranging from 12% to 50%.[4-12] *AML1* mutations have been infrequently detected in other subtypes of AML (M1-M5, M7) and had never been reported in M6 cases. Most mutations involved the amino (N)-terminal region of *AML1*, particularly the runt domain, which is encoded by exons 3-5, and mutations have rarely been found in the carboxy (C)-terminal region.[1-3]

Current data suggest that a single gene mutation alone may not be sufficient to cause acute leukemia.[13] *AML1* mutations belong to class II mutations according to the recently proposed multi-step leukemogenesis model.[14] Class I mutations are exemplified by constitutively activated tyrosine kinases and their downstream effectors, such as *BCR/ABL*, *RAS* and *FLT3*. Aberrations of genes within this class confer a proliferative and/or survival advantage to hematopoietic progenitors. Class II mutations, which cause loss of function of transcription factors through chromosomal translocations such as *AML1/ETO*, *CBFβ/SMMHC*, or *PML/RARα*, as well as point mutations in *AML1* and *C/EBPα*, predominantly interfere with processes of normal hematopoietic differentiation.

As *AML1* mutation is not a common phenomenon in AML, few data are available with respect to the cooperation between mutations in *AML1* (class II mutation) and *FLT3* (class I mutation). In this study, we set out to determine the frequency and type of *AML1* mutations in a large series of Southeast Asian adult AML patients in Thailand, and to search for the existence of cooperative mutations between *AML1* and *FLT3*. Other associated karyotypic abnormalities were also determined.

Design and Methods

Leukemia samples

Consecutive leukemia samples from 284 newly diagnosed adult AML patients were available for complete analysis. This study was a part of a large leukemia project approved by the Ethical Committee for Human Research, Faculty of Medicine Siriraj Hospital. Conventional chromosome banding studies were performed and karyotypes are described according to the International System for Cytogenetic Nomenclature (ISCN).[15] Morphologic, cytochemical and immunophenotypic studies were performed

Table 1. Main features of adult Thai patients with *de novo* AML with *AML1* mutations.

No.	*Age/Sex*	*AML subtype*	*Karyotype*	*WBC/PLT ($\times 10^9$/L)*	*AML1 mutation*	*AML1 Exon/ Intron*	*FLT3 mutation*
003	18/M	M4	46,XY, Normal karyotype	272/112	c.231_2326del GC[a]	Exon 3	ITD
089	21/F	M4	45,XY, -7, 21q+	20/10	c.238C→T	Exon 3	None
094	59/F	M5	46,XX, Normal karyotype	31/93	c.238C→T	Exon 3	ITD & TKD
322	71/M	M1	46,XY [4]/47,XY,+8[11]	72 /124	c.238C→T	Exon 3	ITD
455	58/M	M2	46,XY, Normal karyotype	36.7/3	c.238C→T	Exon 3	None
342	78/M	M1	46,XY, del(5)(q13q22)	9/27	dupTTTAG in IVS3-5a	Intron 3	None
105	53/F	M4	46,XX, del(9)(q13→qter).	168/22	c.416_417487insGTCCAATAG[a]	Exon 4	None
219	24/F	M6	46,XX, Normal karyotype	5/57	c.422_423insTCGAAGTGG[a]	Exon 4	None
006	40/M	M4	46,XX, Normal karyotype	39.7/110	c.341C→Ta	Exon 4	ITD
016	17/F	M5	46,XX, Normal karyotype	154/87	del 17 in IVS5+41[a]	Intron 5	None
159	36/F	M6	46,XX, Normal karyotype	8/112	del 17 in IVS5+41[a]	Intron 5	None

[a]: *novel AML1 gene mutation.*

according to standard methods.[16-18] The European Group for the Immunological Classification of Leukaemias (EGIL) and the French-American-British (FAB) Classification criteria were used for leukemia subclassification.[16-17] Fifty-two blood samples from healthy volunteers were also screened for *AML1* polymorphisms.

AML1 gene mutation analysis

Polymerase chain reaction-(PCR)-single strand conformational polymorphism (SSCP) analyses were used to screen for *AML1* mutations in exons 3, 4 and 5, which correspond to the runt domain.[2,5] PCR was performed with 100 ng of genomic DNA, 1X PCR buffer, 1.5 mmol/L $MgCl_2$, 10 mmol/L dNTP, 10 pmol of each primer, and 0.5 U of Taq DNA polymerase in a total reaction volume of 25 μL. The forward and reverse primer sequences used to amplify the genomic DNA were as follows: 5'-AGCTGTTTGCAGGGTCCTAA-3'/5'-GTCCTCCCACCACCCTCT-3' for exon 3, 5'-CATTGCTATTCCTCTGCAACC-3'/5-CCAT-GAAACGTGTTTCAAGC-3' for exon 4, and 5'-CCAC-CAACCTCATTCTGTTT-3'/5'-AGACATGGTCCCT-GAGTATA-3' for exon 5.[16] PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 seconds, 62°C for 1 min (exon 3) or 57 °C for 1 min (exons 4 and 5) and 72 °C for 1 min, followed by a final elongation at 72 °C for 5 min. The PCR products were denatured by formamide and heat treatment at 95 °C for 10 min, and electrophoresed on a 10% polyacrylamide gel containing 5 % glycerol at 150 V for 3 hours (exons 3 and 5) or 200 V for 2 hours (exon 4). All PCR products with abnormal SSCP bands were confirmed by an independent amplification and SSCP analysis. The PCR products with abnormal bands were sequenced in both directions to identify the type of mutations. Amplified fragments were cut from agarose gels and isolated with QIAquick® (Qiagen, USA) following the manufacturer's instruction. The purified PCR products were directly sequenced with primers as described above with the Big Dye Terminator Cycled Sequencing Kit (Perkin Elmer, USA). *FLT3* mutation analysis was performed according to previously described protocols.[19]

Results and Discussion

The frequency of AML1 mutations

The overall incidence of *AML1* mutations was 3.9%. The main features of 11 patients with known (4 cases) or novel (7 cases) mutations are delineated in Table 1. Most patients were young to middle-aged adults (median age, 43 years), with only two patients aged over 60 years old. The majority of patients with mutations were categorized as having the M4, M5 or M6 subtype of AML. The frequency of *AML1* mutations in various AML subtypes was as follows, 2.7% (2/75) in M1, 1.2% (1/81) in M2, 9.5% (4/42) in M4, 7.7% (2/26) in M5, and 28.6% (2/7) in M6 cases (Table 2). No mutations were found in patients with M0, M3 or M7 AML. None of the 52 healthy volunteers had mutations.

The location and type of AML1 mutations

PCR-SSCP and sequencing analysis of *AML1* mutations are shown in Figure 1.

Seven types of mutations were found. Four patients had a known c.238C→T mutation and seven patients had a novel mutation including c.341C→T, c.231_232dupGC,c.416_417insGTCCAATAG, c.422_423insTCGAAGTGG, dupTTTAG in IVS3-5 and del17 in IVS5+41 (GenBank accession no. D43968). The missense mutations including c.238C→T (case no. 089, 094, 322 and 455) and c.341C→T (case no. 006) resulted in arginine changing to cysteine and serine changing to leucine, respectively.

The insertion of GTCCAATAG nucleotides at c.416_417 (case no. 105) led to the insertion of serine, asparagine and arginine. The insertion of TCGAAGTGG nucleotides at c.422_423 caused the addition of arginine, serine and glycine (case no. 219). We found a novel duplication of TTTAG in intron 3 (dupTTTAG in IVS3-5) of a patient who also had a del (5q) detected by karyotyping. This alteration inserted a new splicing acceptor site. Of the seven cases with novel *AML1* mutations, two cases had the same 17-bp deletion of TACACCCTCCAGGCTGG in intron 5

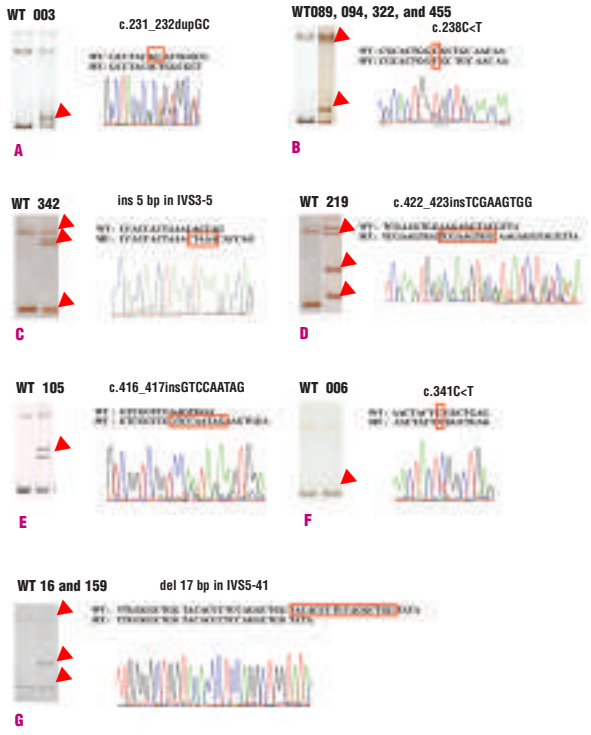

Figure 1. ***AML1*** **mutations detected by PCR-SSCP and sequencing analysis. Shifted PCR-SSCP migration bands are indicated by arrows. Novel mutations were found in case no. 003, 006, 016, 105, 159, 219, and 342 (reverse primer). Known mutations were found in case no. 089, 094, 322 and 455. C, E and G represent sequencing analysis of only mutated** ***AML1*** **alleles as selected from SSCP shifted band.**

(del17 in IVS5+41). All *AML1*-mutated cases were heterozygous because each patient could be shown to have one mutated allele and one normal allele.

Association of AML1 mutation with other genetic abnormalities

Among 11 patients with *AML1* mutations, we found five abnormal karyotypes including trisomy 8, monosomy 7, del(5q), del(9q), and 21q+. Four patients with *AML1* mutations (36%) had coexisting *FLT3* mutations. All four cases had internal tandem duplications (ITD) of *FLT3* mutations, and one case had both ITD and a tyrosine kinase domain (TKD) mutation. One patient with a known AML1 mutation and trisomy 8 was also found to have *FLT3* ITD mutation.

The incidences of *AML1* mutations in AML, previously reported from Japan, France and the United Kingdom, are summarized in Table 2.[3,5-6,8-12] The overall incidence of *AML1* mutation of 3.9% in this series was lower than that in the French studies (8-10%)[3,5] but comparable to that in the Japanese series (1.7-6%).[8-9,11-12] The highest incidence of *AML1* mutations in the Thai M6 subgroup of AML is strikingly different from the predilection for M0 subgroup in the French and Japanese series. Since very few Thai patients were identified as having M0 AML and none was found to carry *AML1* mutation, larger numbers of M0 cases will be needed to determine the true incidence of *AML1* mutation in Thai M0 AML. As no other reports have described *AML1* mutations in M6 patients, our study represents the first series to report two cases of M6 AML with *AML1* mutations, both with a novel mutation.

The crystal structure of the AML1 protein was recently determined and provides a structural basis for understanding the impact of *AML1* mutations.[20] In this study, eight mutations were found to cluster within the runt domain, whereas one mutation was clustered in intron 3 and two in intron 5. Two cases with insertions of three additional amino acids at c.416_417 and c.422_423 could be predicted to interfere with the folding structure of the protein product. The novel duplication of *TTTAG* at the end of intron 3 replaces the

Table 2. **Incidence of** ***AML1*** **mutations in AML patients from various countries.**

Study	*Total no. of cases reported*	*Frequency of AML1 mutation in all subtypes (%)*	*Frequency of AML1 mutation in specific subtypes (%)*							
			M0	*M1*	*M2*	*M3*	*M4*	*M5*	*M6*	*M7*
Europe										
France										
Preudhomme C *et al.*[5]	131	8	22	0	7	0	0	0	0	0
Roumier C *et al.*[3]	414	10	22	0	3	7	0	3.1	0	2.8
Roumier C *et al.*[6]	59	N/A	27	N/A	N/A	N/A	N/A	N/A	N/A	N/A
United Kingdom										
Langabeer SE *et al.*[7]	61	N/A	12	N/A	N/A	N/A	N/A	N/A	N/A	5
Asia										
Japan										
Osato M *et al.*[8]	109	5	33	0	0	8	4	8.3	0	N/A
Taketani T *et al.*[9]	100	6	50	14	0	0	6	0	0	0
Matsuno N *et al.*[10]	51	N/A	16	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Harada H *et al.*[11]	131	4.7	22	5	3.5	0	0	0	0	0
Nakao Me *t al.*[12]	58	1.7	0	0	7.7	0	0	0	0	0
Thailand										
This study	***284***	***3.9***	***0***	***2.7***	***1.2***	***0***	***9.5***	***7.7***	***28.6***	***0***

N/A; data not available.

authentic splicing acceptor site generating a premature stop codon. The truncated protein could be predicted to lose its DNA binding ability. Further studies at the expressed protein level should be performed to confirm the predicted functional alterations. The novel deletion in intron 5 (del17 in IVS5+41) could be argued to occur from a rare polymorphism because the deleted sequences were distant from the splicing site of both exons 5 and 6. However, no mutations or polymorphisms were detected in 52 normal donors in this study.

Although the precise mechanism that initiates and propagates the leukemic clone in M0 and non-M0 cases is not known, we speculate that some common cooperative mechanisms may exist. However, a unique mechanism that determines the final phenotype of most non-M0 cases remains to be characterized because half of non-M0 AML patients did not have specific chromosome or molecular markers. It would be of interest to study the gene expression profile of patients with *AML1* mutations, with or without *FLT3* mutations, in M0 and non-M0 phenotypes, in order to determine the set of genes that may be different.

In conclusion, our study represents the first large series of *AML1* mutations ever reported from the region of Southeast Asia and the largest in Asia. Several novel mutations were uniquely identified in Thai patients with *de novo* non-M0 AML with a high incidence of additional genetic abnormalities. The clinical significance of these mutations requires further studies in a larger population.

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