RESEARCH COMMUNICATION

FLT3 and NPM1 Gene Mutations in Childhood Acute Myeloblastic Leukemia

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Abstract

Mutations of receptor tyrosine kinases are implicated in the constitutive activation and development of human hematologic malignancies. Mutations in fms-like tyrosine kinase 3 (FLT3) gene including internal tandem duplication (ITD) and point mutation in the tyrosine kinase domain (TKD) as well as in nucleoplasmin (NPM1) gene are associated with pathogenesis of acute myeloblastic leukemia (AML). Several reports have demonstrated high incidences of the FLT3 and NPM1 mutations in adult AML patients. Since the pathogenesis of pediatric AML is different from that of adult and the FLT3 and NPM1 mutations have not been well characterized in childhood AML. Therefore, the objective of this study was to determine the frequencies of FLT3 and NPM1 mutations in 64 newly diagnosed childhood AML patients. All blood and bone marrow samples were previously diagnosed with AML by using flow cytometry and/or cytochemistry. FLT3-ITD and FLT3-TKD were detected by PCR and PCR-RFLP methods, respectively. The NPM1 mutation was analyzed by PCR and direct DNA sequencing. The FLT3 mutations were detected in 7 of 64 (11.1%), including FLT3-ITD in 4 of 64 (6.3%) and FLT-TKD in 3 of 62 (4.8%). The NPM1 mutation was not detected in this cohort. By multivariate analysis, white blood cell counts, peripheral blood and bone marrow blast cell counts at diagnosis were significantly higher in children with FLT3-ITD ($P<0.05$). In addition, the median percentage of CD117 was significantly higher in leukemic blast cells with FLT3-ITD than those with wild type ($P=0.01$). We did not find any FLT3 mutations in children aged less than 5 years. The AML M3 cell type was most frequently associated with FLT3 gene mutations (50%). In conclusion, the FLT3 mutations was found in 11.1% but none of NPM1 mutation was detected in Thai children with AML. These data support the hypothesis of different biology and pathogenesis between adult and childhood AML.

Keywords: Acute myeloblastic leukemia - childhood - mutations - FLT3 - NPM1

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Introduction

Acute myeloblastic leukemia (AML) is a genetically heterogeneous clonal disorder caused by the accumulation of acquired somatic genetic alterations in hematopoietic progenitor cells leading to abnormal mechanisms of self-renewal, proliferation and differentiation (Pui et al.,1995; Döhner et al., 2008). Approximately 40% to 50% of adult AML reveals chromosomal abnormalities that incurred significant clinical and prognosis features. Patients with inv(16) and t(15,17) are considered to have good risk cytogenetics while those with 5q-, 7q-, t(9,22), 11q23 rearrangment, and complex karyotypes are at risk of the worst outcomes (Gaidzik et al., 2008). Patients with normal chromosomal finding have been generally classified as an intermediate risk group (Estey et al., 2006; Gaidzik et al., 2008). Recent investigations have demonstrated that several mutations of genes encoding receptor tyrosine kinase, Fms-like tyrosine kinase 3 (FLT3), KIT as well as nucleoplasmin (NPM1) are found in AML with normal karyotype (Falini et al., 2005; Renneville et al., 2008).

FLT3 gene belongs to class III receptor tyrosine kinase and is predominantly expressed on hematopoietic progenitor cells in the bone marrow, thymus, and lymph nodes (Gilliland et al., 2002). Internal tandem duplication of FLT3 gene (FLT3-ITD) is found in approximately 25-45% of adult AML and related with adverse prognosis (Parcells et al., 2006; Rosemary et al., 2008; Ishikawa et al., 2009). In contrast, the frequency of FLT3-ITD is reported to be much lower in children with AML (Zwann et al., 2003; Rachel et al., 2009). The second most common type of FLT3 mutation is the missense point mutation in exon 20, within activation loop of the tyrosine kinase domain (TKD), found in 5-10% of AML

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patients (Yamamoto et al., 2001). Although most affected AML patients have only one type of FLT3 mutation, some patients have both FLT3-ITD and TKD mutations (Kang et al., 2005; Whitman et al., 2008).

NPM1 is a ubiquitously expressed chaperone protein that shuttles rapidly between the nucleus and cytoplasm. It plays a key role in ribosome biogenesis, centrosome duplication, genomic stability, cell cycle progression, and apoptosis (Falini et al., 2009). The mutations in exon 12 of the NPM1 are the most common genetic abnormality in adult AML which represents in approximately 24-45% of all AML cases and up to 60% of AML with normal karyotype (Falini et al., 2005; Döhner et al., 2005; Weina et al., 2006). In children, NPM1 mutations were present in 0-4% with karyotype abnormalities and 0-26% with normal karyotype (Cazzaniga et al., 2005; Brown et al., 2007; Braoudaki et al., 2010). These mutations cause the cytoplasmic localization of NPM and abrogate its function, resulting in accelerated leukemogenesis (Falini et al., 2009; Brown et al 2010).

The data of the prevalence of FLT3 and NPM1 mutations and their clinical significance have been mostly reported in adult AML. The objective of this current study was to evaluate the prevalence of FLT3 and NPM1 mutations in childhood AML in association with patients' clinical characteristics.

Results

Patients' samples

A total of 64 pediatric patients were diagnosed with AML at the departments of Pediatrics, Srinakarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen and Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. The diagnosis of AML was made using FAB classification morphology, cytochemistry, and immunophenotyping. Leukemic blasts were obtained from blood and bone marrow at diagnosis. Sufficient amount of DNA for analysis of FLT3-ITD, FLT3-TKD and NPM1 gene mutations were available of 64, 62 and 62 samples, respectively. The research protocol was approved by the Research Ethics Committee on Human Experimentation of both Medical schools.

Molecular analysis of FLT3-ITD and TKD mutations

To detect FLT3 mutations, genomic DNA was isolated from leukemic blasts using QIAamp DNA blood kits (Germany). Polymerase chain reaction (PCR) for exons 11 and 12 was performed on DNA using the previously published sequences (Kiyoi et al., 1997). In brief, 1 μl DNA (50 ng) was amplified in a volume of 50 μl containing 50 mM $MgCl_2$, 2.5 mM each of dATP, dTTP, dCTP, dGTP deoxynucleotide triphosphate (dNTP), 10 μM each of the oligonucleotide primers, 5 U/ μl of Taq polymerase (Invitrogen, USA) and 10X PCR buffer. Denaturing, annealing, and extension steps were performed at 95°C for 1min, 63.1°C for 1 min, and 72°C for 1 min, respectively, for 30 cycles on a Mastercycler gradient 5331 (Eppendrof, Germany). The PCR product was run through 2% agarose gel. For detection of FLT3-TKD mutations at codons 835 and 836, genomic DNA was amplified using the same PCR condition as above and the previously described primers (Yamamoto et al., 2001). The 25 μl of amplified products were digested with 10U EcoRV (Fermentas, MA, USA) for overnight at 37°C and then run onto 3% agarose gel.

Molecular analysis of NPM1 exon 12 mutations

PCR amplification of NPM1 exon 12 was carried out using primers NPM1-F and NPM1-R, as previously described (Falini et al., 2005). The total reaction and condition were the same as for the detection of FLT3 gene mutations. PCR products were purified by standard methods and directly sequenced with primer NPM1-R2 as previously described (Döhner et al., 2005).

Immunophenotype analysis

In all cases, immunophenotypic analyses of bone marrow leukemic blasts were performed using whole blood lysis method on 4-color flow cytometric analyses with a Coulter Epic XL cytometer (Beckman Coulter, Maimi, FL, USA). Leukemic blasts were gated according to dim CD45 vs low-side scatter and analyzed further using a panel of monoclonal antibodies including CD34, CD33, CD45, CD7, CD56, CD117, CD11b, HLA-DR, CD41, CD14, CD15, CD13, CD19, CD10 and CD20. For all of the markers, expression was considered positive if 20% or more of the population expressed antigen.

Statistical analysis

The relationships of clinical characteristics among FLT3-ITD, FLT3-TKD and NPM1 mutations were analyzed in 64 childhood patients with AML including those with FAB type M3. Differences of median variables of age, peripheral white blood cell (WBC) counts, peripheral blood blast counts, bone marrow blast counts, platelet counts and CD markers expression were analyzed with non-parametric method using the Mann-Whitney U test. Analysis of frequencies was performed using the Fischer's exact test for 2 x 2 tables or the chi-square test for larger tables. For all analyses, the P values were 2 tailed with less than 0.05 considered statistically significant.

Results

Patients' characteristics

The series included 64 pediatric patients who were diagnosed with having AML from two institutions with a median age of 8 years (2 months-15 years). There were 35 males (54.7%) and 29 females (45.3%). FAB subtypes were 4 M0 (6.3%), 9 M1 (14.1%), 27 M2 (42.2%), 4 M3 (6.3%), 4 M4 (6.3%), 8 M5 (12.5%), 2 M6 (3.1%), 4 M7 (6.3%) and 2 biphenotypic leukemia (3.1%). The initial clinical and laboratory characteristics of the patients are shown in Table1.

Molecular analysis of FLT3 gene mutations

FLT3 gene mutations were detected in 7 of 64 patients (11.1%) including 4 FLT3-ITD (6.3%) and 3 FLT3-TKD (4.8%). Our study showed a trend towards higher age of the patients with FLT3 mutations than the ones with wild

Table 1. Clinical and Laboratory Characteristics and FLT3 Mutations in Childhood AML

		FLT3-ITD	FLT3-TKD	FLT3-wt
No.(%)		4 (6.3)	3 (4.8)	57 (89.1)
Median age, y (range)		10 (6-12)	12 (11-15)	8 (<1-15)
Sex, No. (%)				
Male 38 (59.4)		1 (25)	2 (66.7)	35 (61.4)
Female 26 (40.6)		3 (75)	1 (33.3)	22 (38.6)
Median BM blasts, %, (range)		100 (72-100)*	78 (74-78)	78 (74-78)
Median PB blasts, %, (range)		81 (60-95)*	17 (10-47)	37 (0-99)
Median WBC counts, $x10^9$, (range)		79 (20-218)*	10 (6-12)	16 (0.8-230)
Median platelet count $x10^9$, (range)		34 (7-364)	33 (23-36)	39 (3-552)
Median hemoglobin, g/dL, (range)		8 (6-8)	10 (8-13)	8 (4-20)
FAB, No. (%)	M0	0 (0)	0 (0)	4 (100)
	M1	2 (22)	0 (0)	7 (78)
	M2	0 (0)	2 (7.4)	25 (92.6)
	M3	1 (25)	1 (25)	2 (50)
	M4	0 (0)	0 (0)	4 (100)
	M5	1 (12.5)	0 (0)	7 (87.5)
	M6	0 (0)	0 (0)	2 (100)
	M7	0 (0)	0 (0)	2 (100)
Immunophenotype, Median % of markers (range)				
Progenitor markers				
	CD34	81 (50-96)	66 (61-80)	61 (0-98)
	HLA-DR	94 (0-98)	77 (0-81)	76 (0-99)
	CD117	88 (78-95)*	77 (0-87)	67 (0-98)
Myeloid maturation markers				
	CD13	24 (0-64)	51 (35-71)	51 (0-97)
	CD33	89 (79-98)	89 (86-90)	85 (0-99)
	CD11b	27 (0-99)	0 (0-61)	0 (0-99)
	CD15	0 (0-64)	40 (0-51)	0 (0-64)
	CD14	0	0	0 (0-98)

Bone marrow (BM), white blood cell count (WBC), peripheral blood (PB), *statistically significant difference (P<.05)

type (P=0.07). The FLT3 gene mutations were found in 2 of 4 patients (50%) with acute promyelocytic leukemia (APL) and 5 of 58 (8.6%) non APL subtypes. FLT3-ITD positive patients presented with significantly higher WBC counts and percentages of peripheral blood and bone marrow blast compared to FLT3 wild type patients. The leukemic blast cells with FLT3-ITD significantly expressed CD117 higher than wild-type blasts (P=0.01), whereas the expression of other progenitor markers (CD34 and HLA-DR) was not significantly different. Aberranted absence of CD13 was found in 2 FLT3-ITD with 1 of M3 and 1 of M5a FAB subtypes.

FLT3-TKD point mutations were found in 3 of 62 (4.8%) children with AML (2 M2 and 1 M3 FAB subtype). Their initial WBC counts at diagnosis were 5,700, 10,000 and 12,130/ μL, respectively. No patient demonstrated conjunction of both FLT3-ITD and TKD mutations.

Analysis of NPM1 mutations

NPM1 gene mutation of exon 12 was not detected in our present cohort. A single nucleotide polymorphism (22808 G>A) located in the 3' untranslated region of the gene was however identified in 3 of 62 (4.8%) children with AML.

Discussion

Childhood AML is a clinically, biologically, and genetically heterogeneous disease characterized by various genetic defects (Pui., 1995). Our study demonstrated that 6.3% of childhood AML had FLT3-ITD and 4.8% had FLT3-TKD mutations. None of our patients harbored both ITD and TKD mutations. Taken together, a total of 11.1% of childhood AML processed an FLT3-activating mutation. Similar to the previous reports on adult AML which has demonstrated a lower frequency of FLT3-TKD (6.3-7.7%) compared to FLT3-ITD (20.4-25%) (Yamamoto et al., 2001; Kang et al., 2005; Wang et al., 2005; Brown et al., 2009). The frequency of FLT3-ITD among children is much lower than adult, approximately 5-7% in Asia (Iwai et al., 1999; Kang et al., 2005) and 11-27% in Europe (Arrigoni et al., 2003; Zwann et al., 2003; Brown et al., 2007). Most studies of children with AML showed that FLT3-ITD mutations are associated with older age, higher WBC counts, FAB subgroup M1, M2, and M3, and normal cytogenetics (Thiede et al., 2002; Zwann et al., 2003; Brown et al., 2007; Thiede et al., 2009). Clinical characteristics of our patients with FLT3-mutations showed that no one had age less than 5 years and most of them (86%) were diagnosed with AML at over 10 years. Similar to the previous studies, this study suggested that FLT3-ITD were associated with leukocytosis, high percentages of marrow and peripheral blood blasts at initial examination. However, no correlation was observed between FLT3-TKD mutations and the number of leukemic blasts in bone marrow or peripheral blood. All patients with FLT3-TKD had WBC counts within normal range. These data support the conclusion that FLT3-ITD is associated with poor prognostic characteristics but not TKD (Yamada et al., 2005; Mead et al., 2007).

Immunophenotyping is a useful tool for assigning leukemic blast cells to myeloid or lymphoid lineage and identifying the leukemic subtype. Immunophenotyping in adult AML demonstrated that AML cells with FLT3-ITD less commonly expressed progenitor markers such as CD34 and CD117 (Munoz et al., 2003; Mori et al., 2007). In contrast, our series showed that all cases with FLT3 mutations highly expressed CD34 and CD117. This finding may imply that FLT3-ITD mutation in childhood AML might occur during the early phase of myeloid differentiation.

NPM1 gene mutations have been described in both adult and pediatric patients with variety of prevalence and association with prognostic significance (Rachel et al., 2009). The most common NPM1 mutation (>95%) is a 4-bp insertion at position 960 of exon 12 (Falini et al., 2005; Weina t al., 2006). NPM1 is mutated in a large proportion (30-50%) of adult AML cases with normal karyotype (Thiede et al., 2007; Falini et al., 2009). In contrast, the frequencies of NPM1 mutations in children with AML from Europe and the United states ranged from 6.4 to 8.4% (Cazzaniga et al., 2005; Brown et al., 2007). The studies in Asian population revealed lower frequencies of NPM1 mutations which ranged from 0 to 2.1% (Chou et al., 2006; Shimada et al., 2007). In this study, we could not find any NPM1 gene mutation

in Thai pediatric AML. Our data support the hypothesis that NPM1 mutation may occur less frequently in Asians compared to Caucasians. Therefore, NPM1 mutations may not be significantly involved in the pathogenesis of AML in Thai children.

In conclusion, the FLT3 mutations were found in 11.1% of Thai children with AML. Our study supported the hypothesis of the difference of AML between adult and children in the view of molecular pathogenesis.

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