RESEARCH ARTICLE

FLT3-ITD Mutations in Acute Myeloid Leukemia Patients in Northeast Thailand

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Abstract

The *FLT3*-ITD mutation is one of the most frequent genetic abnormalities in acute myeloid leukemia (AML) where it is associated with a poor prognosis. The *FLT3*-ITD mutation could, therefore, be a potential molecular prognostic marker important for risk-stratified treatment options. We amplified the *FLT3* gene at exon 14 and 15 in 52 AML patients (aged between 2 months and 74 years) from 4 referral centers (a university hospital and 3 regional hospitals in Northeast Thailand), using a simple PCR method. *FLT3*-ITD mutations were found in 10 patients (19.2%), being more common in adults than in children (21.1% *vs.* 14.3%) and more prevalent in patients with acute promyelocytic leukemia (AML-M3) than AML-non M3 (4 of 10 AML-M3 *vs.* 6 of 42 AML-non M3 patients). Duplication sequences varied in size-between 27 and 171 nucleotides (median=63.5) and in their location. *FLT3*-ITD mutations with common duplication sequences accounted for a significant percentage in AML patients in northeastern Thailand. This simple PCR method is feasible for routine laboratory practice and these data could help tailor use of the national protocol for AML.

Keywords: *FLT3*-ITD - acute myeloid leukemia - polymerase chain reaction

Asian Pac J Cancer Prev, **17 (9)**, 4395-4399

Introduction

Genetic abnormalities are critical to the pathogenesis of acute leukemia. The genetic abnormalities could, moreover, be used to (a) confirm a diagnosis, (b) stratify treatment protocols, and (c) target therapy (Auewarakul et al., 2005; Bacher et al., 2010; Yohe, 2015). The mutation of the FMS-like tyrosine kinase 3 (*FLT3*) gene is one of the most frequent genetic abnormalities in acute myeloid leukemia (AML) patients. The *FLT3* gene a member of the class III receptor tyrosine kinase (RTK) is located on chromosome 13 at band q12. It plays an important role in the survival and proliferation of hematopoietic stem cells.

The *FLT3* mutation results in (a) constitutive activation of the receptor, (b) ligand-independent dimerization, and (c) auto-phosphorylation; resulting in uncontrolled proliferation and apoptosis (Gilliland et al., 2002; Stirewalt et al., 2003; Small, 2006; Yohe, 2015). The most frequent type of *FLT3* mutation is internal tandem duplication (ITD) within the juxtamembrane (JM) domain. It is present in approximately 20-30% of adult AML patients and 5-15% of pediatric AML patients (Thiede et al., 2002; Pui et al., 2011; Levis, 2013). The patients with the *FLT3*-ITD mutation have increased risk of relapse, decreased event-free survival and overall survival rates; compared with patients without the *FLT3*-ITD mutations in both adults and children (Thiede et al., 2002; Meshinchi et al., 2006; Small, 2006; Pui et al., 2011; Levis, 2013; Port et al., 2014). The *FLT3*-ITD mutation is currently used as a molecular prognostic marker for risk classification strategies in AML (Estey, 2013). For example, the standard treatment is applied to low-risk patients to reduce toxicity, but the more intensive treatment or bone marrow transplantation is applied to high-risk patients to improve the treatment outcome. The development of risk-based therapy regimens can be applied to improve the survival rate of patients with acute leukemia (Thiede et al., 2002; Weisberg et al., 2009; Kindler et al., 2010; Wiernik, 2010; Levis, 2013; Thai Pediatric Oncology Group, 2014).

The frequency and knowledge of *FLT3* mutations in Thailand remain limited and there has been no study of this mutation among AML patients in Northeast Thailand who may be ethnically different from people in other parts of the country. The molecular analysis methods used to identify genetic abnormalities such as the *FLT3*-ITD mutation are beyond the resources of most hospitals.

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Consequently, the *FLT3*-ITD mutation cannot yet be used for development of any risk-based strategy. In the current study, we determined the frequency of the *FLT3*-ITD mutation using simple PCR and evaluated the pattern of the ITD sequence in AML patients in Northeast Thailand.

Materials and Methods

Specimens

Bone marrow and peripheral blood were used. These samples were taken between June 2014 and June 2016 from 52 AML patients from 4 major referral centers located in Northeast Thailand (i.e., Srinagarind Hospital, Khon Kaen University and 3 regional hospitals; Khon Kaen Hospital, Udonthani Hospital and Maharat Nakhon Ratchasima Hospital). The current study was reviewed and approved by the research ethics committee at each center.

Screening Method of the FLT3-ITD mutation

We extracted the genomic DNA using the phenol chloroform extraction method. The DNA was amplified for the *FLT3* gene at exons 14 to 15 using the polymerase chain reaction (PCR) method, with the forward primer, 5' GCAATTTAGGTATGAAAGCCAGC 3' and reverse primer, 5' CTTTCAGCATTTTGACGGCAACC 3' (Kiyoi et al., 1997). The PCR reaction was conducted in a 50-μl reactor containing 50-100 ng of genomic DNA, 10 pmol of each primer, 200 μM dNTPs, and 1 Units Taq DNA polymerase (New England Biolab, Inc.,USA) in a buffer comprsing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl2, and 0.01% gelatin. The amplification reaction was carried out in a thermocycler (Biometra; Goettingen, Germany). The PCR condition included the initial denaturation step at 94ºC for 3 minutes followed by 30 cycles at 93ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 1 minute. The amplified product was analyzed on 2.5% agarose gel electrophoresis and visualized under UV-light after ethidium bromide staining. All abnormal PCR products were directly sequenced to confirm and identify the pattern of duplication.

Statistical analysis

The correlation between the *FLT3*-ITD mutation and the *FLT3* wild type including age, initial white blood cell count (WBC), percentage of blast cells in the peripheral blood, hemoglobin level, and platelet count were analysed using the Mann-Whithney U test. Differentiation in the distribution of variables between each subgroup was carried out using the Fisher Exact test. Significance was set at $p<0.05$. SPSS (version 19.0) was used for all analyses.

Results

Patient characteristics

There were 28 male and 24 female patients, ranging in age from 2 months to 74 years (median, 42 years). The *FLT3*-ITD mutations were predominantly found in the female patients (N=6) over against the male patients (N=4); the difference was not significant (p=0.5). The characteristics of all patients are presented in Table 1. The respective correlation between the *FLT3*-ITD and *FLT3* wild type was not significant vis-à-vis age, sex, WBC, hemoglobin level and platelet count. The characteristics of the 10 patients who had the *FLT3*-ITD mutations are shown in Table 2. The WBC of the *FLT3*-ITD patients varied widely (1,990 to 309,100 cells/μl). The WBC count seemed higher in patients with the *FLT3*-ITD mutation, however, the difference was not statistically significant.

FLT3 – ITD mutations by PCR method

The *FLT3* genes at exon 14 to 15 were amplified by PCR. The PCR product of the wild-type *FLT3* gene is at 329 bp; therefore, the product of the *FLT3*-ITD mutation gene would carry more than 329 bp. The sizes of the altered PCR product varied (Figure 1). The *FLT3*-ITD mutations were found in 10 of 52 AML patients (19.2%).

Table 1. Characteristics of All Patients

	Total	*FLT3-ITD* mutation	*FLT3* wild type	P value
No.	52	10	42	
Median Age	42	30.5	45.5	0.4
(range)	(2 mo.-74y)	(9y- 64y)	(2 mo.-74y)	
Sex				
male	28	4	24	0.5
female	24	6	18	
Diagnosis				
AML-non M3 (APL)	42	6	36	
AML-M3 (APL)	10	4	6	
Age (year)				
0-18 (Median 9.5)	14	2	12	
>18 (Median 49.5)	38	8	30	
Median WBC count (x10^9/L)	20.7	30.29	18.2	0.3
(range)	(1.2-309)	(1.99-309)	(1.2-265)	
Median PB blast (%)	50	71.5	43	0.04
(range)	(0-98%)	(8-98%)	(0-97%)	
Median Hemoglobin (g/dL)	8.1	7.3	8	0.4
(range)	(4.8-11.8)	(4.9-10.1)	(4.8-11.8)	
Median platelets count	38.5	42.5	36	0.4
(x10^9) (range)	(8-337)	(20-157)	(8-337)	

Bone Marrow (BM); White Blood Cell (WBC); Peripheral Blood (PB); Acute Promyelocytic Leukemia (APL)

Table 2. Characteristics of Patients with the FLT3-ITD mutation

Case	Age/ Sex	Flow cytometry result	WBC count (cell/μl)	% blasts in PB	Length of FLT3-ITD (base pairs)
1	9/M	AML-non M3	309,100	94	162
2	10/F	AML-M3v	88,380	70	63
3	64/F	AML-M3v	210,000	98	69
4	28 M	AML-M3	1,990	8	69
5	32/F	AML-M3v	11,310	50	45
6	19/ M	AML-non M3 with aberrant CD7	72,800	95	62
7	61/F	AML-non M3	29,900	61	27
8	41/F	AML-non M3	22,400	73	171
9	29/ M	AML-non M3 with aberrant CD7	30,680	75	64
10	46/F	AML-non M3 with aberrant CD7	6,800	79	61

AML-M3variant (AML-M3v); White Blood Cell (WBC); Peripheral Blood (PB): Female (F); Male (M)

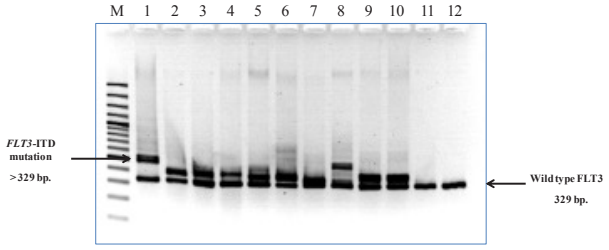

Figure 1. PCR Product on 2.5% Agarose Gel Electrophoresis. M=DNA ladder marker. Lane 1-10; patients with FLT3-ITD mutation. Show wild type FLT3 gene (329 bp.) and mutant ITD (>329 bp), Lane 11-12; normal. Show only wild type FLT3 gene

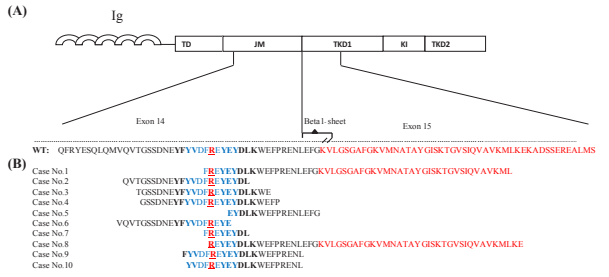

Figure 2. Structure of the *FLT3* Gene. A). Schematic illustration. Immunoglobulin-like domains (Ig), a single teansmebrane domain (TD), juxtamembrane domain (JM), Tyrosine kinase domain-1 (TKD1), kinase insert domain (KI), tyrosine domain-2 (TKD2). B). Site of mutant ITD from 10 AML patients. The duplicated amino acid sequences are shown in alignment

A respective 21.1% (8/38) and 14.3% (2/14) were found to have *FLT3*-ITD mutations in the adult (>18 years) and children with AML.

Sequencing data

Sequencing data from the altered PCR product in the 10 AML patients are summarized in Figure 2. The length of the mutant ITD sequence ranges between 27 and 171 base pairs. The start and end positions of the *FLT3*-ITD sequence were unique in each case. All of the *FLT3*-ITD sequences were mostly restricted to the JM domain in exon 14, and only two cases occurred in exon 15.

Discussion

The mutation of the FMS-like tyrosine kinase 3 (*FLT3*) gene is one of the most frequent genetic abnormalities in acute myeloid leukemia (Levis, 2013; Yohe, 2015). *FLT3*-ITD has been reported associated with poor treatment outcomes (Port et al., 2014; Yohe, 2015). In the current study, we determined the frequency and the pattern of the mutation in AML patients from 4 major hospitals in Northeast Thailand. The people in northeastern Thailand have a different ethnic background as evidenced by their having particular G6PD variants (Nuchprayoon et al., 2002; Louicharoen et al., 2005). We postulated that the prevalence of the *FLT3*-ITD mutation might be different from that reported in other studies.

Recent studies demonstrated that the *FLT3*-ITD mutation was found in 20-30% of AML patients (Thiede et al., 2002; Pui et al., 2011; Levis, 2013). In our study, the *FLT3*-ITD mutation was evident in 21.1% of adult patients and 14.3% of pediatric ones. These results are similar to a study on adult Thai patients (i.e., 24.6% in adult AML patients) (Auewarakul et al., 2005), but higher than the one in pediatric AML patients (6.3%) (Mukda et al., 2011).

In Asian populations, the *FLT3*-ITD mutation varies between 10 and 21%. Similar frequencies of the mutation have been reported in Indian, Taiwanese, and Japanese patients (viz., 19.1%, 20.8%, and 20%), respectively (Ahmad et al., 2010; Xu et al., 2012) and (Suzuki et al., 2007). By contrast, a lower frequency of the mutation in AML patients was reported in Saudi Arabia (14.4%) (Elyamany et al., 2014), Malaysia (13%) (Yunus et al., 2015), and Pakistan (11.5%) (Ishfaq et al., 2012). The frequency of the mutation in AML patients was higher among people in western countries (20-24%) (Thiede et al., 2002; Moreno et al., 2003). The differences in the frequency in AML patients may be due to differences in the study sample size, ethnicity, or environmental factors. Indeed, our sample size was relatively small so our results do not likely represent the true frequency.

The *FLT3*-ITD mutation is generally more common in adults than in children with AML (Kottaridis et al., 2001; Thiede et al., 2002; Moreno et al., 2003; Zwaan et al., 2003; Mukda et al., 2011; Levis, 2013). In our study, ITD mutations occurred in 21.1% of adults (>18 years) and 14.3% of children; however, the number of children with AML was relatively small (N=14). The difference in ITD mutations between adults and children suggests that the molecular pathogenesis may differ by age group.

Among the various types of AML, an *FLT3*-ITD mutation is more frequent in acute promyelocytic

leukemia (APL) (Kainz et al., 2002; Noguera et al., 2002; Schnittger et al., 2002; Thiede et al., 2002; Auewarakul et al., 2005; Mukda et al., 2011). By comparison, the *FLT3*-ITD mutation is present in approximately 20-45% of APL patients (Poire et al., 2014). The prognostic significance of the *FLT3*-ITD mutation in APL remains controversial (Molica et al., 2015). In the current study, the *FLT3*-ITD mutation in the APL group was more prevalent (40%) than in the non-APL group (14%).

As for patient characteristics, there was no significant difference among patients with or without the *FLT3*-ITD mutation with respect to age, sex, hemoglobin level, and platelet count (Kainz et al., 2002; Noguera et al., 2002; Schnittger et al., 2002; Thiede et al., 2002; Auewarakul et al., 2005; Mukda et al., 2011). In our study, patients with the *FLT3*-ITD mutation trended to have higher WBC counts than those without the mutation (median WBC count 30.3 x 10^9/l vs 18.2 x 10^9/l), however, the difference is not statistically significant.

The *FLT3*-ITD mutation disrupts the auto-inhibitory function of the juxtamembrane (JM) domain, resulting in ligand-independent activation of the *FLT3* receptor (Stirewalt et al., 2003; Small, 2006; Annesley et al., 2014; Yohe, 2015). The *FLT3*-ITD mutation commonly occurs in the juxtamembrane (JM) domain. The region of duplication occurs frequently at amino acid Y591 to Y599 (YVDFREYEY) which is the most frequent duplicate region found in the *FLT3*-ITD mutation (Vempati et al., 2007). Approximately 30% of the *FLT3*-ITD mutation may occur outside the JM domain-commonly within beta1-sheet of the tyrosine kinase domain-1 (TKD1). Kayser et al. demonstrated that the ITD site in the beta1 sheet is associated with the inferior complete remission rate, decreasing the relapse free survival, and overall survival rate compared to the other insertion sites (Kayser et al., 2009). In our study, the mutation was located mostly within the JM domain in exon 14 (70%), except for another 30% involving the beta1 sheet of the TKD1. In addition, the duplication sequences had at least one amino acid overlapped within stretch the Y591 to Y599 (YVDFREYEY) region. Arginine 595 (R595) in the region of the stretch Y591 to Y599 (YVDFREYEY) region has been reported to play a critical role in activation of STAT5 and in the transforming potential of the *FLT3*-ITD mutants (Vempati et al., 2007). Notably, arginine 595(R595) duplication was found in nine out of ten ITD sequences in our study.

Regarding the size of the ITD, previous studies reported that the size of the mutant ITD sequence could vary from a few base pairs to over 1,000 (Ahmad et al., 2010; Yohe, 2015). Some studies suggested that an increase in duplication size was associated with a decreasing overall survival rate (Stirewalt et al., 2006); however, a correlation between prognosis and duplication size is moot (Yohe, 2015). In our study, the duplication sequences varied in size from 27 to 171 nucleotides (median 63.5); there were only 2 cases with an ITD size above 100 bp and that involved the beta1 sheet of the TKD1. Owing to the short duration of our study, we are not able to demonstrate or extrapolate the outcome.

In the current study, the survival rate could not be evaluated; so, its correlation with a prognosis cannot be determined. A larger population is needed to evaluate the influence of the insertion site and the size of the *FLT3* mutation vis-à-vis the survival rate and determination of the prognostic impact.

In conclusion, This is the first study on the frequency and pattern of *FLT3*-ITD mutations in Northeast Thailand. The frequency of the mutation and their patterns are similar to reports of people in other regions. The simple PCR method for detection the *FLT3*-ITD mutation was simple and accurate.

Acknowledgements

The authors thank Mr. Bryan Roderick Hamman for assistance with the English-language presentation of the manuscript.

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