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Rapid detection of *FLT3*-ITD (exon14-15) gene mutations analysis in acute myelogenous leukemia patients by High Resolution Melting analysis

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ABSTRACT

Background: Molecular genetic characterization allows prognostic stratification and can potentially alter treatment choices in acute myeloid leukemia (AML). Activating mutations of the *Fsm-like tyrosine kinase*3 (*FLT3*) gene, especially *FLT3*-ITD mutations, have been associated with an adverse prognosis in AML. Therefore, *FLT3* mutation detection becomes essential for AML patients. High Resolution Melting (HRM) analysis is an alternative method for the rapid and affordable detection of gene mutations.

Objectives: To establish and evaluate a rapid and affordable method for detecting mutations of *FLT3*-ITD (exon14-15) gene in acute myeloid leukemia by High Resolution Melting analysis.

Materials and methods: Thirty-five patients with newly diagnosed AML from Maharaj Nakorn Chiang Mai Hospital were included in this study. *FLT3*-ITD mutation screening was performed by HRM analysis, and the results were compared with the data obtained using conventional PCR with gel electrophoresis and direct sequencing.

Results: Among the 35 AML patients studied, 6 patients were scored positively for *FLT3*-ITD mutation in the conventional PCR, whereas HRM analysis identified 7 out of 35 patients who were positive for *FLT3*-ITD mutation, which was concordant with direct sequencing results. Interestingly, one sample that was positive by HRM analysis was scored by conventional PCR as negative. Therefore, HRM analysis is more sensitive than conventional PCR.

Conclusion: HRM analysis is a rapid and promising screening method for *FLT3*-ITD mutation, enabling the real-time evaluation of AML progression, which is significant for decision-making regarding treatment. Our results showed that HRM analysis could be a useful clinical tool for the rapid and affordable screening of *FLT3*-ITD mutation in AML patients.

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Introduction

Acute myelogenous leukemia (AML), a hematological malignancy with a high mortality rate, is characterized by the overproduction and dysregulation of white blood cell proliferation.[1] In Thailand, AML is the major cause of death from hematological malignancy. According to current diagnostic criteria, the World Health Organization (WHO) classified AML by clinical features, morphology, immunophenotyping, cytogenetics, and molecular genetics.[2] The risk stratification into favorable, intermediate, and unfavorable is based on chromosomal abnormalities and genetic alterations such as *FLT3*, *KIT*, *NPM1*, and *CEPBPA*.[3] Among these, mutations of the *FMS-like tyrosine kinase* 3 (*FLT3*) are the most common genomic alteration in AML. *FLT3* mutations can be found in approximately 30% of AML patients and correlate with a poor prognosis. *FLT3* mutations can be subdivided into internal tandem duplicates (ITD), present in approximately 25% of patients, and point mutations in the tyrosine kinase domain (TKD), present in approximately 5%. Both *FLT3*-ITD and *FLT3*-TKD mutations are constitutively activating, leading to ligand-independent *FLT3* signaling and cellular proliferation. *FLT3*-ITD mutations result from a 3 bp repeated sequence varying in size from a 6 to 180 bp insertion in the juxtamembrane (JM) region (exons 14 to 15) of the *FLT3* wild-type gene. In wild-type (WT) *FLT3*, the *FLT3* JM domain inhibits receptor activation; the presence of ITDs disrupts this inhibitory effect, resulting in autophosphorylation. The constant activation of the *FLT3* receptor led to the uncontrolled proliferation of blast cells. *FLT3*-ITD mutations are the majority of *FLT3* mutations detected in AML patients.[4-8] Therefore, the identification of *FLT3* mutations by molecular analysis is of great importance for the prognostic information and the determination of appropriate therapeutic interventions in AML patients. Traditionally, *FLT3*-ITD mutations can be detected by using conventional polymerase chain reaction (PCR)-based methods followed by agarose gel electrophoresis, but they have limited to low sensitivity and also the inability to detect a small insertion of less than 20 bp. In response to the demand for rapid and sensitive methods to detect *FLT3* mutations, we established and evaluated a High Resolution Melting analysis for detecting mutations of *FLT3* gene in clinically sample from acute myeloid leukemia patients.

High Resolution Melting (HRM) analysis was first established in 2003, and has since been developed for the high-throughput and convenient genotyping of individual polymorphic loci. The key to this technology is the use of saturating fluorescence dyes, which intercalate into double-stranded DNA during amplification of the DNA without inhibiting the PCR reaction. The dye fluoresces strongly when intercalated into the double-stranded DNA, but as the temperature increases during the HRM analysis, so the DNA melts and the intercalating dyes are released without fluorescence. The changes in fluorescence are sequence specific and can be recorded and analyzed by the designed program.[9, 10] HRM is a closed-tube system that prevents contaminations and has increased sensitivity when compared to conventional PCR followed by agarose gel electrophoresis.

Therefore, the present study aimed to establish and evaluate a rapid and affordable method for detecting mutations of *FLT3* gene in acute myeloid leukemia using HRM analysis.

Materials and methods

Sample collection

A total of 35 blood samples were collected from patients newly diagnosed with acute myelogenous leukemia at Maharaj Nakorn Chiang Mai Hospital from January 2017 to December 2018. This research was approved by the Research Ethics Committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (AMSEC-61EM-006).

Cell line and culture

The MV4-11 cell line (*FLT3*-ITD) and KG-1a cell line (*FLT3*-wild type) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI1640, Gibco, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin streptomycin (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO_2 incubator.

DNA extraction

Blood samples and cell lines were extracted using DNA extraction kit (NucleoSpin® Blood, Macherey-Nagel). Briefly, whole blood 200 µL was mixed with 25 µL of proteinase K and 200 µL of buffer B3 and then vortex mixed vigorously for the lysis of cells. After that, the mixture was incubated at 70°C for 10-15 minutes and 210 µL of absolute ethanol was added to the mixture before being vortexed again. Then, the mixture was transferred into a Nucleospin Blood Column from the DNA extraction kit and centrifuged at 12,000 rpm for 2 minutes. After that, the silica membrane was washed 2 times and was dried by centrifuging the column at 12,000 rpm for 2 minutes. Then, 100 µL of elution buffer was added, and the column was incubated for 1 minute and centrifuged at 12,000 rpm for 2 minutes to elute pure DNA.

Conventional PCR assay

A primer pair was designed to specifically amplify both of *FLT3*-ITD mutant and *FLT3*-wild type at exon 14-15 (F 5'-GCAATTTAGGTATGAAAGCCAGC-3' and R 5'-CTTTCAG-CATTTTGACGGCAAC-3') yielding a 300 bp wild-type PCR product. Any patient with an additional higher molecular weight band was considered to be *FLT3*-ITD mutant. The conventional PCR for *FLT3* mutation was performed on the MyCycler Thermal Cycler machine (BIO-RAD). Positive and negative controls, as well as a blank control with distilled water, were included in each run of unknown samples. Here, 50 ng/µL of DNA samples were amplified in a total volume of 20 µL containing 0.2 µM of each primer and 10 µL of 2X Quick Taq Hs DyeMix (Toyobo, Japan). PCR was performed at 95°C for 3 minutes followed by 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension step at 72°C for 3 minutes. The PCR products were separated by electrophoresis through 3% agarose gel electrophoresis and the PCR product bands were viewed under UV illumination. Cases in which an additional higher molecular weight band was identified

were considered *FLT3*-ITD–positive.

PCR and HRM analysis

PCR and HRM for *FLT3* mutation were performed on a Corbett Rotor-Gene 6000 HRM Real-Time PCR Machine, a real-time PCR machine with HRM capability. All samples were tested in triplicate. The PCR reaction mixture contained 50 ng/µL of DNA, 0.3 µM of each primer (F 5'-GCAATTTAG-GTATGAAAGCCAGC-3' and R 5'- CTTTCAGCATTTTGACGG-CAAC-3'), and 10 µL of THUNDERBIRD SYBR® qPCR Mix (Toyobo, Japan). Reaction conditions consisted of an activation step at 95°C for 3 minutes followed by 40 cycles amplification of 5 seconds at 95°C and 30 seconds at 60°C. Subsequently, the products were heated to 95°C and then cooled to 4°C. HRM was performed from 75°C to 95°C, increasing by 2°C/second with 25 acquisitions per degree. Upon completion of the run, analysis was performed using the software supplied with the Corbett Rotor-Gene 6000 HRM Real-Time PCR Machine. Melting curves were generated, normalized and temperature-shifted to allow samples to be directly compared by Rotor Gene 6000 Series Software 17 (Qiagen). The HRM analysis was validated by direct sequencing.

DNA sequencing

The PCR products of the samples were further submitted for sequencing in both directions using fluorescent dye-terminator sequencing on the ABI3730xl DNA Sequencer.

By using HRM analysis to detect *FLT3*-ITD mutations in 35 AML patients, 7 cases were positive for *FLT3*-ITD mutation (Tm 78.8±1.1°C) and the rest of the samples were negative (Tm 80.4±0.2°C). HRM analysis was represented as a well-differentiated normalized melting curve with two clear separate clusters corresponding to the different nucleotide sequences from wild-type, while selecting only a sample from the same cluster to compare to a wild-type control for analysis, which guaranteed a clear sample clustering (Figure 2). All PCR products were confirmed by direct sequencing in both directions (Figure 3). The results were 100% concordant with HRM analysis. The mutations were inserted by repeated sequence size ranging from 24 to 138 bp in exons 14-15 of the *FLT3* gene (Table 1).

Results

Thirty-five AML patients were included in this study. The MV4-11 cell line and KG-1a cell line were homoduplex mutated (positive) control and wild-type (negative) controls, respectively. Six patients were *FLT3*-ITD mutated, and 29 patients were wild-type, as determined by conventional PCR followed by 3% agarose gel electrophoresis. Patients with an additional higher molecular weight band were identified as *FLT3*-ITD–positive. As shown in Figure 1, mutated PCR products showed two distinct bands on agarose gel electrophoresis, probably based on heteroduplex formation. In contrast, mutated PCR products showed an upper band product of 300 bp (Figure1, lane 6), which represents homoduplex formation.

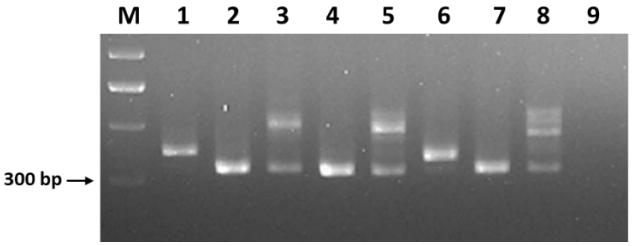

Figure 1. *Conventional PCR products on a 3% agarose gel. Double band and upper product of 300 bp: heteroduplex and homoduplex mutation, Single band at 300 bp: wildtype sequence, Lane 1: mutated control MV4-11 cell line, Lane 2: wild-type control KG-1a cell line, Lane 3, 5, 6, and 8: samples with mutated FLT3-ITD, Lane 4 and 7: samples with wild-type FLT3, Lane 9: no template control.*

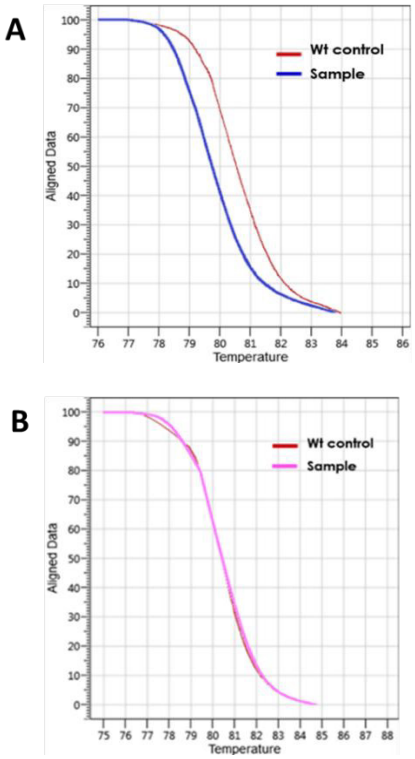

Figure 2. *HRM analysis presented on normalized melting curve plot. A: mutation cluster compared to the wild-type control, B: wild-type cluster compared to the wild-type control, Red indicate wild-type FLT3-ITD controls, Blue indicates a sample with mutated FLT3-ITD, while Pink indicates a sample with wild-type FLT3-ITD.*

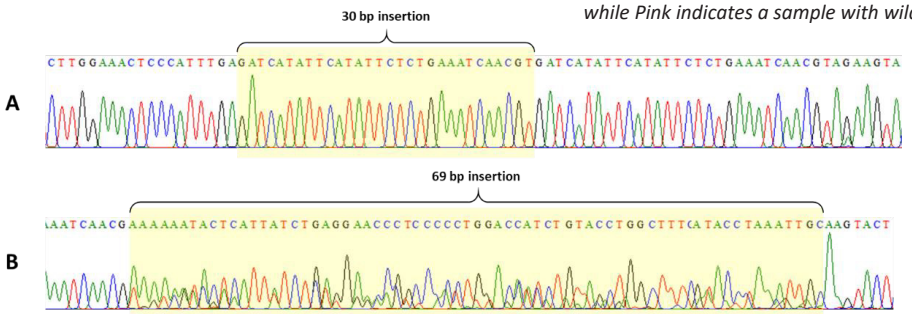

Figure 3. *Direct sequencing in the reverse direction. A: FLT3-ITD–positive sample, B: FLT3-ITD–positive sample with a discrepant result between conventional PCR and HRM analysis.*

Discussion

Genomic investigations of acute myeloid leukemia (AML) have demonstrated that the mutation of several genes is involved in AML development, leading to new genomic classifications, predictive biomarkers, therapeutic targets and individual therapeutic optimization. Mutations of the *FLT3* gene have been found in approximately 30% of all AML cases. The most common type present in AML patients is *FLT3*-ITD mutations.[8, 11] Although *FLT3*-ITD mutation has been associated with a poor prognosis in AML patients, the identification of *FLT3*-ITD has become an important indicator for determining treatment modalities, especially first-generation *FLT3* inhibitors.[7] Since *FLT3*-ITD mutations result from base pair insertion, it was shown that *FLT3*-ITD could be analyzed by traditional PCR with agarose gel electrophoresis.[12]

Table 1 The results of *FLT3*-ITD detections in patient samples.

Sample No.	Traditional PCR with agarose gel electrophoresis	PCR with HRM analysis	Sequencing
1	*FLT3*-ITD mutation	*FLT3*-ITD mutation	Mutation with 30 bp insertion
2	Wild-type	Wild-type	Wild-type
3	Wild-type	Wild-type	Wild-type
4	Wild-type	Wild-type	Wild-type
5	Wild-type	Wild-type	Wild-type
6	Wild-type	Wild-type	Wild-type
7	Wild-type	Wild-type	Wild-type
8	Wild-type	Wild-type	Wild-type
9	*FLT3*-ITD mutation	*FLT3*-ITD mutation	Mutation with 138 bp insertion
10	Wild-type	Wild-type	Wild-type
11	Wild-type	Wild-type	Wild-type
12	-ITD mutation	*FLT3*-ITD mutation	Mutation with 84 bp insertion
13	Wild-type	Wild-type	Wild-type
14	*FLT3*-ITD mutation	*FLT3*-ITD mutation	Mutation with 78 bp insertion
15	*FLT3*-ITD mutation	*FLT3*-ITD mutation	Mutation with 24 bp insertion
16	Wild-type	*FLT3*-ITD mutation	Mutation with 69 bp insertion
17	Wild-type	Wild-type	Wild-type
18	Wild-type	Wild-type	Wild-type
19	Wild-type	Wild-type	Wild-type
20	Wild-type	Wild-type	Wild-type
21	Wild-type	Wild-type	Wild-type
22	Wild-type	Wild-type	Wild-type
23	Wild-type	Wild-type	Wild-type
24	Wild-type	Wild-type	Wild-type
25	Wild-type	Wild-type	Wild-type
26	Wild-type	Wild-type	Wild-type
27	Wild-type	Wild-type	Wild-type
28	Wild-type	Wild-type	Wild-type
29	Wild-type	Wild-type	Wild-type
30	Wild-type	Wild-type	Wild-type
31	Wild-type	Wild-type	Wild-type
32	Wild-type	Wild-type	Wild-type
33	Wild-type	Wild-type	Wild-type
34	Wild-type	Wild-type	Wild-type
35	*FLT3*-ITD mutation	*FLT3*-ITD mutation	Mutation with 51 bp insertion

However, It was unable to detect mutated DNA at levels lower than 6.7% in a mutant- wild-type mixture.[7] Moreover, gel electrophoresis will not be able to clearly differentiate mutant from wild-type when inserted fragments are shorter than 20 bp such as in the case of *FLT3*-ITD with a 3 bp insertion.[13]

Real-time PCR with HRM analysis is easy to set up and the turnaround time is about 2-3 hours; several studies have employed the HRM assay to develop a molecular approach for the detection of gene mutation in various diseases including leukemia.[14-17] The major advantage of HRM is preventing contamination due to the closed tube system. HRM is a simple method: after PCR, carried out in the presence of a suitable dye, the product is heated while the level of fluorescence is measured. As the temperature rises and the duplex passes through its melting transition, dye is released, and fluorescence intensity is reduced. Although several instruments capable of performing the fluorescence acquisition exist, they vary in performance, with those designed for HRM giving a more satisfactory outcome. In a previous study, it was shown that the HRM method is capable of detecting up to 1% of mutated DNA which clearly differed from the wild-type template.[18]

Our analysis included 35 patients with AML. We found that 6 of the 35 AML patients were positive for the *FLT3*-ITD mutation in the conventional PCR, whereas HRM analysis could detect 7 out of 35 patients who were positive for the *FLT3*-ITD mutation. The mutations involved an inserted sequence with a size ranging from 24 to 138 bp. Interestingly, one patient (sample no. 16) who was positive by HRM analysis was scored as negative by conventional PCR, probably due to the low percentage of mutant allele in the *FLT3* wild-type background, as this case is lower than the limitations of detection using conventional PCR with agarose gel electrophoresis. It could not be detected by conventional PCR with agarose gel electrophoresis. Therefore, HRM analysis is more sensitive than conventional PCR and is a suitable method for the daily routine of a molecular laboratory. Once a mutation is detected, DNA sequencing could be used for confirmation if necessary. Overall, it can be considered a rapid and cost-effective method.

HRM analysis is a rapid, inexpensive method that does not involve opening the PCR tube, which has the advantage of preventing contamination. Furthermore, HRM analysis improves sensitivity when screening for *FLT3*-ITD mutation and reduces the complications of post-PCR methods such as agarose gel electrophoresis.

Conclusion

HRM analysis is a rapid, promising screening method for *FLT3*-ITD mutation and enables the real-time evaluation of AML progression, which is of great importance for decision-making regarding treatment. It is more sensitive than conventional PCR, is inexpensive and can be integrated into the routine molecular diagnosis of AML.

Conflict of interest

There are no conflicts of interest associated with this publication.

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