



## Original Article

# Establishment of a cost-effective method to detect FLT-ITD and D835 mutations in acute myeloid leukemia patients in the Taiwanese population



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## ARTICLE INFO

## Article history:

Received 5 June 2014

Received in revised form

16 June 2014

Accepted 29 December 2014

Available online 11 March 2015

## Keywords:

Acute myeloid leukemia

FLT3-ITD

FLT3-D835

## ABSTRACT

**Objective:** The FMS-related tyrosine kinase 3 (*FLT-3*) gene is a hematopoietic growth factor receptor gene, an independent negative prognostic factor, which affects the proliferation and differentiation of stem cells or hematopoietic progenitor cells. Patients with *FLT-3* gene mutations have a worse prognosis and responsiveness to chemotherapy than those without these mutations. Our study aims to establish a conventional detection method for *FLT3-ITD* and D835 mutations in patients with acute myeloid leukemia (AML).

**Materials and methods:** In this study, we recruited 100 patients with AML. Primers were designed to distinguish between wild-type *FLT-3*, *FLT3-ITD*, and D835 variants. Methods using a polymerase chain reaction (PCR)-Agilent 2100 Bioanalyzer, PCR-ABI PRISM 3100 Genetic Analyzer, and PCR-agarose gel electrophoresis were compared.

**Results:** A high-accuracy, easily operated, low-cost technique to detect the *FLT-3* variation with 99.9% specificity was established in this study. The PCR platform, the Agilent 2100 Bioanalyzer (plus DNA 1000 LabChip kit) chip analysis platform, and the ABI PRISM 3100 Genetic Analyzer (plus GeneScan-500 size standard) short tandem repeat (STR) fluorescence analysis platform were used in different experimental comparisons. The ABI PRISM 3100 Genetic Analyzer (plus GeneScan-500 size standard) STR fluorescence analysis platform was the most suitable method to detect FLT3 variants. This method has a high degree of sensitivity, accuracy, and a specificity of 99.9%.

**Conclusion:** AML with the homozygous mutated *FLT-3* may have a worse cure rate than AML with heterozygous mutation. This mutation is not related to drug resistance, but is a factor in a high risk of relapse; it is also related to unfavorable overall survival. Our designed detection methods should provide key information to develop personalized medicine for AML patients.

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## 1. Introduction

In clinical and pathological terms, leukemia is subdivided into a variety of groups. The first division is between its acute and

chronic forms, and it can further be divided into nonlymphocytic (i.e., bone marrow) and lymphoblastic leukemia according to the source of leukemic cells [1]. Acute progression is extremely rapid and survival is approximately 1–3 months; in chronic progression, patients can survive for 3–4 years even without treatment (chronic myelogenous leukemia) or for > 5–10 years (chronic lymphatic leukemia) [2–5]. Acute leukemia is most often caused by premature hematopoietic stem cell proliferation, whereas chronic leukemia occurs with abnormal mature hematopoietic cells. Two kinds of chemotherapy are used in acute myeloid

Conflict of interest: none.

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<http://dx.doi.org/10.1016/j.tcmj.2014.12.005>

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leukemia (AML): high-dose injections of cytarabine combined with anthracycline and the traditional 3:7 therapy [6–9]. Remission-induction rates range from 60% to 85% and long-term survival rates are approximately 20–40% for patients undergoing consolidation and maintenance therapies after complete remission. Bone marrow transplantation and peripheral blood stem cell transplantation have resulted in higher survival rates in younger patients [10]. The cytogenetic study of leukemia is one of the important prognostic indicators, which can show the unique chromosomal rearrangement forms of AML that affect the outcome. Other factors, including age, therapy-induced secondary AML, a high white blood cell count, and genetic aberrations such as mutation of *FLT-3* and *NPM1*, etc. [11–14], carry different prognostic impacts. The recurrence rate is a very important issue. It has remained high after consolidation therapy, especially in elderly patients. According to the Formosa Cancer Foundation, the annual incidence of AML is approximately three to five/100,000 in the population of Taiwan. This estimate suggests that 600–1000 people will contract leukemia every year, with an annual mortality rate of about two to four/100,000 in the population. Disease statistics from 1979 to 2003 show that the overall annual incidence rate of AML is approximately one/100,000 in the Taiwan population [15]. In other areas, such as Nanjing, China, the overall average annual incidence rate of leukemia was 3.68/100,000 from 2003 to 2007 [16]. In the United States, the annual incidence is approximately 3.4/10,000 and about 12,000 people suffer from leukemia each year. Acute myelogenous leukemia has a high prevalence in developed countries. From 2002 to 2003 in the United States, a high mortality rate of about 2.7/10,000 was estimated after conventional therapy for AML compared with an 80% mortality rate after diagnosis [17–19].

The FMS-related tyrosine kinase 3 [*FLT-3*; also known as stem cell tyrosine kinase 1 (*STK1*)] gene is a hematopoietic growth factor receptor gene affecting the proliferation and differentiation of stem cells or hematopoietic progenitor cells and is an independent negative prognostic factor [20]. *FLT-3* is overexpressed in the majority of AML cases in adult patients, and two *FLT-3*-specific variants have been identified. Insertion of tandem duplication into exon 11 and exon 12 in the wild-type *FLT-3* produces internal tandem duplication (ITD), which results in constitutive activation of the negative regulation of the juxtamembrane domain; repeated lengths of the fragment contain 6–180 bases in the coding region [21,22]. The ITD mutation leads to sustained spontaneous phosphorylation of the *FLT-3* receptor and *FLT-3* oncogene expression. Activating the mutation of D835 within the activation loop of *FLT-3* in human hematologic malignancies leads to persistent activation of the *FLT-3* receptor [23].

It is still controversial whether aggressive treatment is capable of influencing the poor prognosis with these mutations. The *FLT-3* mutation does not affect complete remission rates that indicate the response to induction chemotherapy [24]. Our study aim was to establish a polymerase chain reaction (PCR)-based system to detect the *FLT3-ITD* and D835 mutations. Establishing the identification of these two variants from other subtypes of AML should provide important genetic information and improve appropriate treatment. In addition, we can also understand how these genetic markers influence responses to chemotherapy.

## 2. Materials and methods

### 2.1. Study patients

We recruited 100 AML patients at Wan Fang Hospital, Taipei, Taiwan. Informed consent was obtained from all participants. All

procedures were approved by the Institutional Review Board at Wan Fang Hospital.

### 2.2. PCR primers

To test the gene patterns of *FLT3-ITD* mutations and D835, primers designed to distinguish between the wild-type *FLT-3* gene and the mutant *FLT3-ITD* gene, and between the D835 mutants were distinguished by restriction enzyme digestion after PCR, as shown in Table 1. In a previous study, an *FLT3* wild-type fragment of approximately 329 bp was amplified using genomic DNA as a template in PCR; in addition, an *FLT3* fragment of 456 bp was amplified using reverse transcription-PCR [21]. The size of the ITD fragments ranged from 6 bp to 180 bp. The *FLT3-ITD* demonstrated different lengths of DNA fragments compared with the wild type.

### 2.3. PCR

The PCR conditions in this study were as follows: at 95°C for 12 minutes, 95°C for 30 seconds, 50°C for 45 seconds, and 72°C for 1 minute for 40 cycles, followed by 72°C for 10 minutes, and then stopped at 4°C.

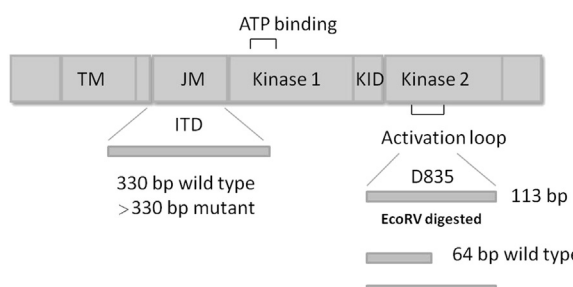
### 2.4. Restriction enzyme digestion

The D835 mutant type showed around 113 bp and the wild type could be digested by restriction enzyme at around 64 bp. Advanced *EcoRV* digestion was carried out at 37°C for 1 hour and 30 minutes, and then stopped at 4°C. The resulting short DNA fragments could be identified range from 20 bp to 200 bp.

### 2.5. The Agilent 2100 Bioanalyzer (plus DNA 1000 LabChip kit)

The Agilent 2100 Bioanalyzer plus DNA 1000 LabChip kit (Agilent Technologies, Santa Clara, CA, USA) was used for the analysis of DNA and RNA. The resulting DNA fragments (25 bp–1 kb) ranged from 5 bp to 100 bp.

**Table 1**  
PCR primers and probes labeled with dye for detecting *FLT3-ITD* and D835 mutations.<sup>a</sup>



D835	F-FAM R-HEX	CCGCCAGGAACGTGCTTG GCAGCCTCACATTGCCCC
	F R	CCGCCAGGAACGTGCTTG GCAGCCTCACATTGCCCC
ITD	11F 12R 12R-FAM 11F-HEX	GCAATTTAGGTATGAAAGCCAGC CTTTCAGCATTTTGACGGCAACC CTTTCAGCATTTTGACGGCAACC GCAATTTAGGTATGAAAGCCAGC

ATP = ATP binding domain; F = forward primer; JM = juxtamembrane domain; KID = kinase insert domain; Kinase 2 = tyrosine-kinase domain 2; PCR = polymerase chain reaction; R = reverse primer; TM = transmembrane domain.

<sup>a</sup> The internal tandem duplication (ITD) mutation is located at the JM domain. The missense D835 is usually located in the TK2.

**Table 2**  
Comparison of FLT mutation detection methods developed in this study.

Method	Sizing resolution	Samples per run	Run time	Dye	Calling	Price (NT) per sample	Equipment cost
2% Agarose electrophoresis	20–200 bp	1–24	2 h <sup>a</sup>	No	Eye vision	1.6 (24 samples) <sup>b</sup>	Very low
Agilent 2100 bioanalyzer (plus DNA 1000 LabChip kit)	10% gel (from 25 -100 bp) 5% gel (from 100 -500 bp) 2% gel ( from 500 -1000 bp)	1–12	30 min <sup>a</sup>	A dye concentrate is mixed with the gel	Analyzer software	79 (12 samples) <sup>c</sup>	Medium
ABI PRISM 3100 Genetic Analyzer (plus GeneScan-500 size standard)	1–500 bp	1–16	1.5 h <sup>a</sup>	Fluorescein-labeled primer	Analyzer software	200 (16 samples) <sup>d</sup>	High

<sup>a</sup> Estimation of time required is less than or equal to the maximum number of samples.

<sup>b</sup> Estimation of the price for a single sample batch of 24 samples.

<sup>c</sup> Estimation of the price for a single sample batch of 12 samples.

<sup>d</sup> Estimation of the price for a single sample batch of 16 samples.

### 2.6. PCR-ABI PRISM 3100 Genetic Analyzer (plus GeneScan-500 size standard)

The PCR-ABI PRISM 3100 Genetic Analyzer [Applied Biosystems (ABI), Forest City, CA, USA] was used for DNA analysis. In this method, we used a blue fluorescent tag (carboxyfluorescein) in the inverted primer (reverse primer) as shown in Table 2 and capillary electrophoresis. The resulting DNA fragments (35–500 bp) ranged from 1 bp to 500 bp [24].

## 3. Results

### 3.1. Concentration and sensitivity tests for ITD and D835 primers using PCR–agarose electrophoresis

The series dilution of DNA for PCR demonstrated the limitations of the ITD/D835 primers. The DNA concentration limitation was 5 fg and the primer dimer was demonstrated to be low DNA-concentration dependent (Fig. 1). The DNA sample was mixed in different ratios of the ITD wild type (RS4-11; 100 ng) to the mutant form (MV4-11; 25 ng) or RS4-11(25 ng) with MV4-11(100 ng) for PCR. Only a high concentration of DNA was detected (data not showed) in the series dilution of the DNA concentration.

### 3.2. Concentration and sensitivity test for ITD primer using the Agilent 2100 Bioanalyzer (plus DNA 1000 LabChip kit)

The results were the same as the PCR results, and the primer dimer was also demonstrated to be low DNA-concentration dependent (data not shown). The DNA ratio test showed the same results as the PCR. In the DNA ratio of the mutant form (25 ng

or 2.5 ng) with the ITD wild type (100 ng) and the ratio of the ITD wild type (2.5 ng) with the mutant form (100 ng), the DNA fragment was detected only in high concentrations in the series dilution of the DNA concentration (Fig. 2). The DNA concentration limitation was 5 fg (data not shown).

### 3.3. Concentration and sensitivity test for ITD primer using the ABI PRISM 3100 Genetic Analyzer (plus GeneScan-500 size standard) short tandem repeat

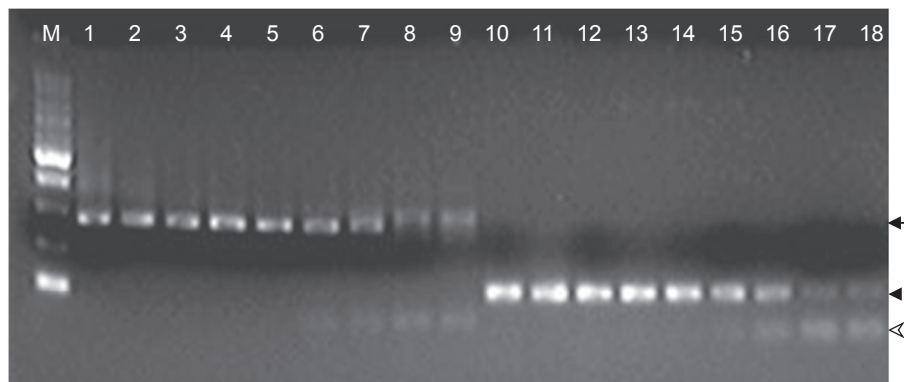
In the concentration tests, the ITD wild type and mutant form were detected in a ratio of wild type (2.5 ng) with the mutant form (2.5 ng). The obtained result was the same as that obtained earlier using the PCR–agarose electrophoresis and the Agilent 2100 Bioanalyzer. Only high concentrations of DNA could be detected in the series dilution ratio. In the sensitivity test for the ITD and D835 primers, short tandem repeat (STR) analysis could detect 5 fg of the DNA concentration (Fig. 3).

### 3.4. Technical analysis and comparison

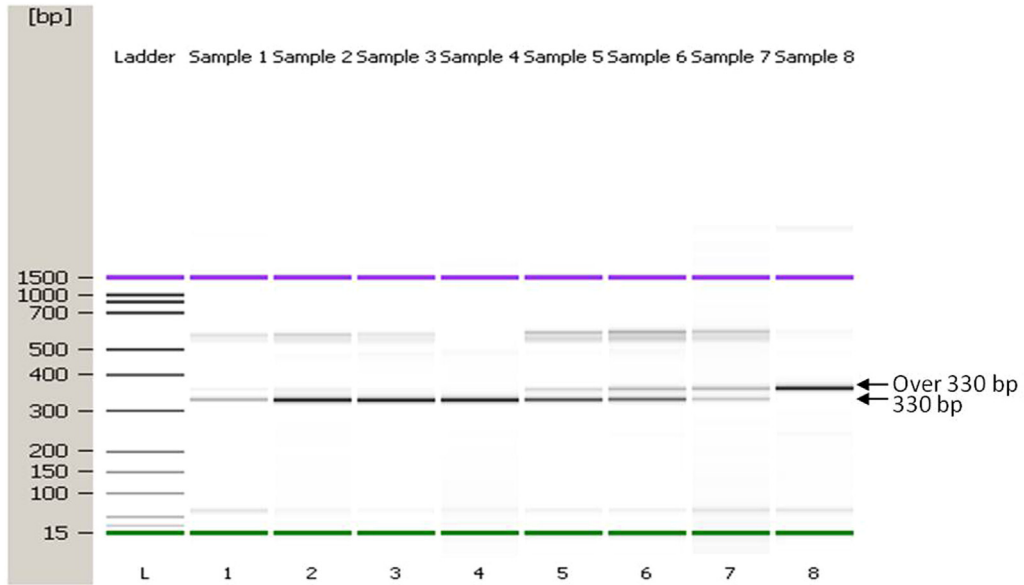
In Table 2, the three techniques are compared, and the detection limitations and the required running times for each method are presented. The minimal resolutions of detection were from 5 bp to 20 bp and the running times ranged from 30 minutes to 2 hours.

### 3.5. The FLT3-ITD and D835 mutation frequencies in AML

In our cohort, we showed the genotype/allele frequency in patients: the heterozygous variation occurred in 7.7% and homozygous variation in 1.1% of patients with FLT3-ITD; the heterozygous



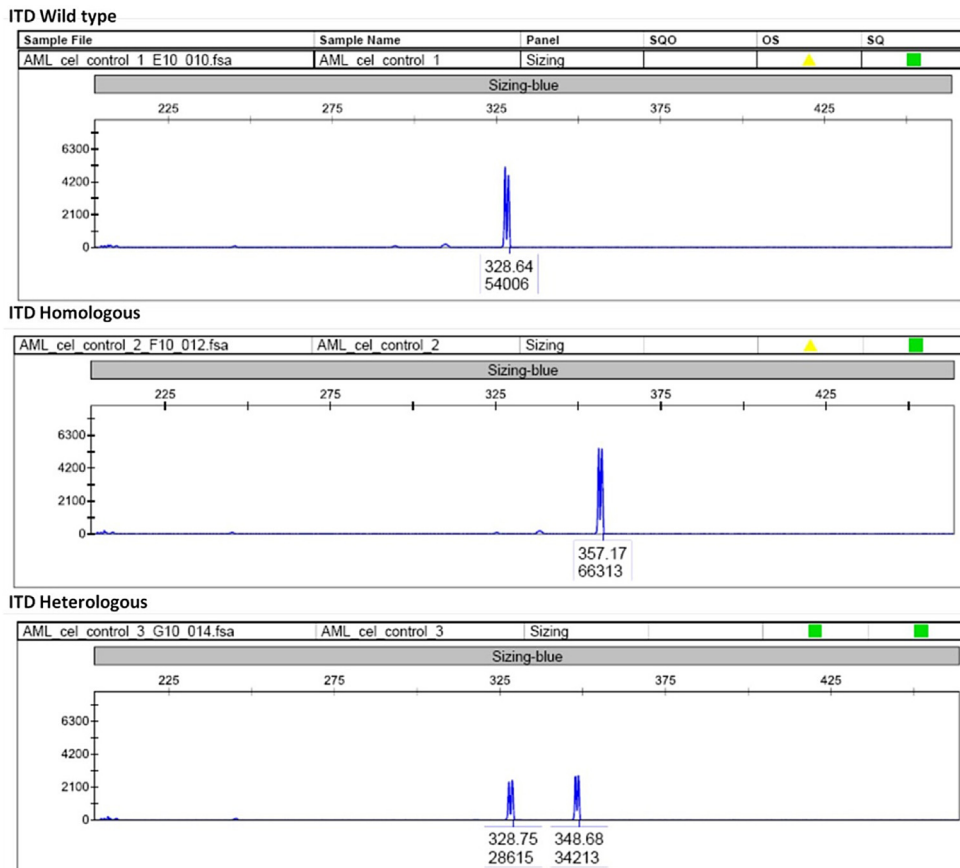
**Fig. 1.** Testing for sensitivity and detection limitations of the internal tandem duplication (ITD) and D835 primers. 1–9 = ITD 330 bp (100 ng, 50 ng, 5 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, and 5 fg); 10–18 = D835 100 bp (100 ng, 50 ng, 5 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg, and 5 fg); M = 100-bp marker. The arrow indicates 330 bp. The closed arrowhead indicates 113 bp. The open arrowhead indicates primer dimers.



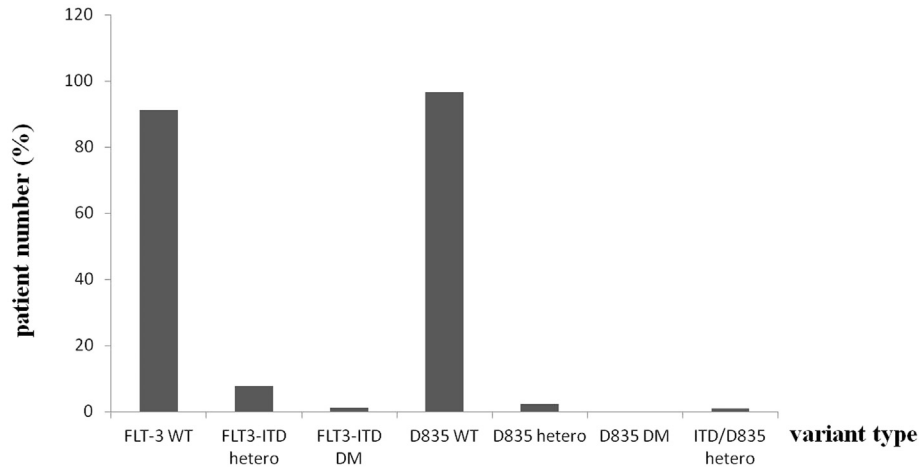
**Fig. 2.** The concentration ratio test of the internal tandem duplication wild type and mutant form. Samples 1–4 = RS4-11, 100 ng/various concentrations of MV4-11 (100 ng, 50 ng, 25 ng, and 2.5 ng); Samples 5–8 = MV4-11, 100 ng/various concentrations of RS4-11 (100 ng, 50 ng, 25 ng, and 2.5 ng). The arrows indicate 330 bp and > 330 bp.

variation of D835 was found in 2.2% of AML patients and the ITD/D835 heterozygous variation in 1% of AML patients (Fig. 4, Table 3). The number of base pairs in AML patients was < 335 bp for the wild-type allele, and ranged from 350 bp to > 475 bp for the mutant

allele in *FLT3-ITD* patients. The average number of base pairs for *FLT3-ITD* was 328.73 bp and the average fragment length of D835 was around 63.64 bp for the wild-type allele. Most patients had the heterologous allele; the homologous allele was detected in only one



**Fig. 3.** Different types of internal tandem duplication (ITD) fragments on short tandem repeat analysis. AML = acute myeloid leukemia.



**Fig. 4.** The percentage of FLT-3 internal tandem duplication (ITD) and D835 genotypes in acute myeloid leukemia (AML) patients. DM = double mutant allele; Hetero = heterozygous allele; WT = wild-type allele.

**Table 3**  
Genotypes of FLT-ITD and D835 in the enrolled AML patients.

ID	ITD	Genotype	D835	Genotype
1	NA	NA	NA	NA
2	329.01	WT	63.61	WT
3	328.82	WT	63.66	WT
4	328.82	WT	63.7	WT
5	329.05	WT	63.62	WT
6	328.86/482.49	Heterozygous	63.69	WT
7	328.78	WT	63.65	WT
8	328.99	WT	63.64	WT
9	328.51	WT	63.48	WT
10	328.87	WT	63.74	WT
11	328.9/416.52	Heterozygous	63.63	WT
12	348.94	Homozygous	63.64	WT
13	NA	NA	NA	NA
14	328.78	WT	63.77	WT
15	NA	NA	NA	NA
16	NA	NA	NA	NA
17	328.75	WT	63.73	WT
18	328.77	WT	63.74	WT
19	328.74	WT	63.75	WT
20	328.85	WT	63.63/111.84	Heterozygous
21	328.72	WT	63.66	WT
22	NA	NA	NA	NA
23	328.84	WT	63.68	WT
24	329.01	WT	63.64	WT
25	328.52/351.47	Heterozygous	63.68	WT
26	328.58/374.71	Heterozygous	63.7	WT
27	328.54	WT	63.66	WT
28	328.56	WT	63.72	WT
29	328.56	WT	63.66	WT
30	328.53/380.69	Heterozygous	63.57	WT
31	328.51	WT	63.69	WT
32	328.6	WT	63.7	WT
33	NA	NA	NA	NA
34	328.94	WT	63.57	WT
35	328.32	WT	63.45	WT
36	329.18	WT	63.64	WT
37	329.07	WT	63.58	WT
38	328.54	WT	63.74	WT
39	328.53	WT	63.67	WT
40	328.52	WT	63.68	WT
41	329	WT	63.64	WT
42	328.56	WT	63.71	WT
43	328.55	WT	63.66	WT
44	328.65	WT	63.67	WT
45	328.56	WT	63.69	WT
46	329.02	WT	63.52	WT
47	328.41	WT	63.45	WT
48	NA	NA	NA	NA

**Table 3 (continued)**

ID	ITD	Genotype	D835	Genotype
49	NA	NA	NA	NA
50	329	WT	63.62	WT
51	328.38	WT	63.67	WT
52	328.38	WT	63.74	WT
53	328.35	WT	63.75	WT
54	328.39	WT	63.74	WT
55	328.44	WT	63.7	WT
56	NA	NA	NA	NA
57	328.46	WT	63.76	WT
58	328.39	WT	63.76	WT
59	328.42	WT	63.42	WT
60	328.51	WT	63.39	WT
61	328.44	WT	63.76	WT
62	328.39	WT	63.74	WT
63	328.43	WT	63.75	WT
64	329.05	WT	63.58	WT
65	328.4	WT	63.65	WT
66	329.05	WT	63.62	WT
67	328.44	WT	63.65	WT
68	329.01	WT	63.67	WT
69	328.5	WT	63.66	WT
70	328.54	WT	63.63	WT
71	329.09	WT	63.65	WT
72	329.18	WT	63.51	WT
73	329.26	WT	63.62	WT
74	328.88	WT	63.61	WT
75	329.18/392.59	Heterozygous	63.63	WT
76	329.22/380.56	Heterozygous	63.69/111.89	Heterozygous
96	328.84	WT	63.63	WT
99	328.99	WT	63.62	WT
100	329.87	WT	62.63	WT

AML = acute myeloid leukemia; ITD = internal tandem duplication; NA = not applicable; WT = wild type.

patient with the *FLT3-ITD* mutation. This patient demonstrated the 348.94-bp mutant genotype.

#### 4. Discussion

The overall frequency of *FLT3* mutation was 17.5% [25] and the frequency of *FLT3-ITD* in the adult group of patients was 18.8%, which was lower than in other ethnic populations, such as Germans (32% and 21%, respectively) [26–28] and Japanese (23% and 31%, respectively) [29]. One report summed up previous data and showed an approximate rate of 17.7% [30]. Another report found an incidence of 24% in elderly ( $\geq 60$  years) patients and 22.8% in young



patients. The report also mentioned that these mutations could have a prognostic impact in elderly patients [31]. Some previous studies concluded that it is possible DNA banks were used, which likely overestimated the real incidence of *FLT3-ITD* mutations in the population [32,33]. A previous study also agreed that the frequency of *FLT3-ITD* is higher (18.8%) in adults compared with young AML patients [34]. In the Taiwanese population, a previous study found that the frequency of *FLT3-ITD* was 15.4% (14/91 pediatric patients) [35]. Other studies reported that *FLT3-ITD* occurred in 12.6% of AML patients in Taiwan [36–38]. In this study, the *FLT3-ITD* mutation was detected in 8% (8/100) of adults in Taiwan and the D835 mutation was detected in 2% of adults (1 patient had both mutations). The frequencies of the *FLT3-ITD* and D835 mutation were lower in our study than in previous studies in AML patients in Asian populations [35–38]. Because our sample size was small, the results only reflect the status of the patients recruited and do not present the real frequency of the *FLT3* mutation in Taiwan. The data demonstrated risk variations in patients with ITD mutations of over 330-bp and D835 mutations of around 110-bp fragments in the Taiwan population. AML patients with the homozygous mutated *FLT-3* may have a worse cure rate than those with the heterozygous mutation. This mutation is not related to drug resistance, but rather is a factor in a high risk of relapse; it is also related to unfavorable overall survival (OS). Some small molecular drugs use *FLT3-ITD* as the test marker. Some treatment regimens have greater efficacy in this poor-risk subset of patients with AML. The STR platform analysis to detect *FLT3-ITD* could analyze mutant differences in many cases, aiding prediction of outcomes and the development of possible novel therapies [39]. Disease-free survival (DFS) is also worse for patients with *FLT3-ITD* than those with the wild type. It is still not clear whether patients with D835 mutations have a worse OS and DFS compared with those with the wild type [40–42]. Identification of additional targets is needed to improve therapy.

We established three detection methods for *FLT3-ITD* and D835 in AML patients and compared their sensitivity and specificity in this study. A technique to detect *FLT-3* variation with high accuracy, easy operation, and low cost was developed. A comparison of the three methods showed that the ABI PRISM 3100 Genetic Analyzer (plus GeneScan-500 size standard) fragment analysis platform had a higher sensitivity and resolution than the PCR electrophoresis analysis platform and the Agilent 2100 Bioanalyzer (plus DNA 1000 LabChip kit) chip analysis platform for 16 samples processed at the same time. According to the 100 cases provided by Wan Fang Hospital for validation, the results demonstrated high accuracy and 99.9% specificity. The results should provide a high-quality diagnostic assay for personalized medicine for AML patients.

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