

ORIGINAL ARTICLE

A Comparative Study on Next-Generation Sequencing for the Detection of FLT3 Mutations in Acute Myeloid Leukemia

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ABSTRACT

Background: FLT3 mutations are frequently observed in acute myeloid leukemia (AML) and serve as important prognostic markers. Next-generation sequencing (NGS), a recently adopted molecular diagnostic tool, enables comprehensive profiling of genetic alterations. This study investigated FLT3 mutations detected by targeted NGS in patients with AML and compared the findings with those obtained via fragment analysis or direct sequencing. **Methods:** A total of 185 patients were enrolled in the study, including 176 with newly diagnosed AML and 9 with relapsed AML. FLT3 mutations identified by targeted NGS were compared with the results from fragment analysis or direct sequencing.

Results: Among newly diagnosed AML patients, the overall FLT3 mutation positivity rate was 28.4% (50/176), comprising 21.6% (38/176) for FLT3-ITD, 8.5% (15/176) for FLT3-TKD, and 3.4% (6/176) for non-ITD/TKD FLT3 mutations. Most FLT3-ITDs (36/38) were detected by both NGS and fragment analysis, demonstrating high concordance. Out of the four FLT3-ITDs identified by NGS in relapsed AML, two with low allele frequencies (0.1 and 1.0) were not detected by fragment analysis. Among the 15 FLT3-TKD-positive cases identified by NGS, five with low allele frequencies (3.85 - 12.91) were not detected by direct sequencing. In newly diagnosed AML, multiple FLT3-ITD, multiple FLT3-TKD, and dual FLT3-ITD-TKD mutations were observed in 4.0% (7/176), 1.1% (2/176), and 1.1% (2/176) of patients, respectively.

Conclusions: NGS demonstrated high reliability and superior sensitivity compared to fragment analysis or direct sequencing for the detection of FLT3 mutations. Moreover, NGS provided additional genetic insights through its comprehensive mutational analysis.

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KEYWORDS

FLT3, FLT3-ITD, FLT3-TKD, acute myeloid leukemia, next-generation sequencing, fragment analysis, direct sequencing

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by a heterogeneous genetic landscape and complex clonal architecture [1]. Fms-like tyrosine kinase 3 (FLT3), a member of the receptor tyrosine kinase family, is commonly overexpressed in AML blasts [2]. FLT3 mutations represent the most prevalent genetic alteration in AML, occurring in approximately one-third of newly diagnosed patients

[2]. These mutations fall into two major categories: internal tandem duplications (ITDs) in the juxtamembrane domain, observed in approximately 20 - 25% of adult AML cases, and point mutations in the tyrosine kinase domain (TKD), most commonly involving codon D835, which occur in approximately 5 - 10% of patients [2,3]. The presence of FLT3-ITD is recognized as an independent adverse prognostic factor associated with increased relapse risk and reduced overall survival [2,3]. In contrast, the prognostic significance of FLT3-TKD mutations remains controversial and is not currently incorporated into standard AML risk stratification [2-4]. In recent years, next-generation sequencing (NGS) has been increasingly adopted in molecular diagnostic laboratories as a comprehensive platform for the detection of genetic abnormalities in myeloid malignancies, including AML. Nevertheless, the detection of large insertion/deletion variants, particularly FLT3-ITDs - which exhibit substantial size variability (ranging from 3 to 400 bp) - continues to pose technical challenges [5, 6]. As broader NGS panels have become more widely implemented, additional FLT3 mutations outside the canonical ITD and D835 regions have been identified. However, the frequency and landscape of these non-canonical (NC) FLT3 mutations remain difficult to characterize, as they are typically undetectable by conventional fragment analysis or direct sequencing methods. Furthermore, their clinical relevance has yet to be clearly established [2,6,7].

This study aimed to investigate FLT3 mutations detected by targeted NGS in patients with AML and compare these findings with those obtained through fragment analysis or direct sequencing.

MATERIALS AND METHODS

Patients and samples

This study included 185 patients with AML, comprising 176 newly diagnosed cases and 9 relapsed cases, who underwent NGS between November 2021 and May 2024. FLT3 mutation testing by fragment analysis and direct sequencing was also performed. Four of the newly diagnosed patients were re-evaluated at relapse and were included among the nine relapsed cases.

Genomic DNA was extracted from bone marrow or peripheral blood samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) on the QIAcube automated platform, following the manufacturer's instructions. DNA quantification for targeted NGS was performed using the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a Qubit 4.0 Fluorometer.

Targeted NGS for FLT3 mutation

Targeted NGS was performed using the Oncomine Myeloid Research Assay (OMA; Thermo Fisher Scientific, Waltham, MA, USA), which is designed for the detection of mutations in myeloid malignancies. This assay

includes 526 amplicons targeting 40 genes, including FLT3 hotspot regions in exons 8, 11, 13, 14, 15, 16, 20, 23, and 25.

Target-specific amplification was conducted using the Ion Chef System (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. DNA libraries were sequenced on the Ion Torrent S5 XL platform (Thermo Fisher Scientific). Sequence alignment and base calling were carried out using Torrent Suite Software version 5.18, with the human genome build 19 as the reference. Variant annotation was conducted using Ion Reporter Software version 5.20 (Thermo Fisher Scientific). Variant filtering was performed using the SNV indel (5.20) and OncoPrint Variants (5.20) filter chains to identify clinically relevant alterations. Allele frequency (AF) was calculated as the proportion of sequencing reads containing the FLT3 mutation relative to the total read count at that position.

Fragment analysis for FLT3-ITD and direct sequencing for FLT3-TKD

Exons 14 and 15 of FLT3 were amplified by polymerase chain reaction (PCR) using fluorescently labeled primers. Fragment size analysis was performed via capillary electrophoresis on a 3500 Genetic Analyzer (Thermo Fisher Scientific), and data were interpreted using GeneMapper Software version 4.1 (Thermo Fisher Scientific) in accordance with the manufacturer's guidelines. The presence of FLT3-ITD mutations was determined by the appearance of peaks larger than the 329-bp wild-type product. The allele ratio (AR) was calculated as the ratio of the mutant peak area to that of the wild-type.

For detection of FLT3-TKD mutations, exon 20 of FLT3 was amplified by PCR and subjected to direct sequencing.

Statistical analysis

Pearson's correlation coefficient was calculated to assess the association between allelic ratios obtained by fragment analysis and allele frequencies obtained by NGS.

RESULTS

FLT3 mutations identified by NGS, fragment analysis, and direct sequencing according to AML subtype

Table 1 summarizes the FLT3 mutation positivity rates in newly diagnosed and relapsed AML cases. Among the 176 newly diagnosed patients, the overall FLT3 mutation positivity rate was 28.4% (50/176), with FLT3-ITD mutations detected in 21.6% (38/176) and FLT3-TKD mutations in 8.5% (15/176).

According to the WHO classification, AML with NPM1 mutation exhibited the highest positivity rates for both overall FLT3 (65.4%) and FLT3-ITD mutations (46.2%), except for AML with DEK::NUP214 fusion,

Table 1. Positive rates of FLT3 mutations by AML subtype in newly diagnosed and relapsed cases.

Diagnosis	Sample n	FLT3-ITD/TKD positive n (%)	FLT3-ITD positive n (%)	FLT3-TKD positive n (%)	Non-ITD/TKD FLT3 positive n (%)
Newly diagnosed AML	176	50 (28.4)	38 (21.6)	15 (8.5)	6 (3.4)
APL with PML::RARA fusion	25	8 (32.0)	8 (32.0)	1 (4.0)	2 (8.0)
AML with RUNX1::RUNX1T1 fusion	19	3 (15.8)	2 (10.5)	1 (5.3)	0
AML with CBFB::MYH11 fusion	5	3 (60.0)	1 (20.0)	2 (40.0)	0
AML with DEK::NUP214 fusion	1	1 (100.0)	1 (100.0)	0	0
AML with KMT2A rearrangement	6	1 (16.7)	1 (16.7)	0	0
AML with MECOM rearrangement	1	0	0	0	0
AML with NPM1 mutation	26	17 (65.4)	12 (46.2)	6 (23.0)	2 (7.7)
AML with CEBPA mutation	8	2 (25.0)	2 (25.0)	0	0
AML, myelodysplasia-related	58	4 (6.9)	2 (3.5)	2 (3.5)	0
AML defined by differentiation	26	11 (42.3)	9 (34.6)	3 (11.5)	1 (3.9)
AUL	1	0	0	0	1 (100)
Relapsed AML	9	4 (44.4)	4 (44.4)	1 (11.1)	1 (11.1)

AML: acute myeloid leukemia, APL: acute promyelocytic leukemia, AUL: acute undifferentiated leukemia, NGS: next-generation sequencing.

Table 2. Detection of FLT3-ITD mutations by next-generation sequencing and fragment analysis.

Diagnosis	Sample n	NGS		Fragment analysis		Size (bp), mean (range)	Multiple ITDs n (%)
		FLT3-ITD positive n (%)	Allele frequency mean (range)	FLT3-ITD positive n (%)	Allele ratio, mean (range)		
Newly diagnosed AML	176	36 (20.5)	18.90 (0.40 - 71.50)	38 (21.6)	0.59 (0.004 - 3.54)	43.76 (6 - 162)	7 (4.0)
APL with PML::RARA fusion	25	7 (28.0)	19.02 (4.00 - 40.23)	8 (32.0)	0.44 (0.09 - 0.85)	50.25 (21 - 162)	0
AML with RUNX1::RUNX1T1 fusion	19	2 (10.5)	45.65 (33.00 - 58.30)	2 (10.5)	0.99 (0.66 - 1.32)	25.50 (15 - 36)	0
AML with CBFB::MYH11 fusion	5	1 (20.0)	6.70	1 (20.0)	0.99	108	0
AML with DEK::NUP214 fusion	1	1 (100.0)	5.10	1 (100.0)	0.74	99	0
AML with KMT2A rearrangement	6	1 (16.7)	37.58	1 (16.7)	0.81	21	0
AML with NPM1 mutation	26	12 (46.2)	22.27 (1.20 - 71.50)	12 (46.2)	1.05 (0.02 - 3.54)	39.71 (6 - 96)	5 (19.2)
AML with CEBPA mutation	8	2 (25.0)	1.80 (0.60 - 3.70)	2 (25.0)	0.03 (0.01 - 0.04)	39.00 (21 - 57)	1 (12.5)
AML, myelodysplasia-related	58	2 (3.5)	10.65 (10.50 - 10.80)	2 (3.5)	0.26 (0.05 - 0.46)	59.50 (51 - 69)	0
AML defined by differentiation	26	8 (32.0)	18.93 (0.40 - 39.00)	9 (34.6)	0.47 (0.004 - 0.99)	38.33 (27 - 63)	1 (3.9)
Relapsed AML	9	4 (44.4)	20.49 (0.10 - 65.63)	2 (22.2)	1.06 (0.23 - 2.26)	35.40 (30 - 45)	1 (11.1)

Allele frequencies are presented as percentages.

AML: acute myeloid leukemia, APL: acute promyelocytic leukemia, ITD: internal tandem duplication, NGS: next-generation sequencing.

Table 3. Detection of FLT3-TKD mutations by next-generation sequencing and direct sequencing.

Diagnosis	Sample n	NGS		Direct sequencing	Variant sequence	Exon	Multiple TKDs n (%)	FLT3-ITD-TKD dual positive n (%)
		FLT3-TKD positive n (%)	Allele frequency mean (range)	FLT3-TKD positive n (%)				
Newly diagnosed AML	176	15 (8.5)	24.04 (2.80 - 84.13)	10 (5.7)			2 (1.1)	2 (1.1)
APL with PML::RARA fusion	25	1 (4.0)	7.32	0	D835V	20	0	0
AML with RUNX1::RUNX1T1 fusion	19	1 (5.3)	44.93	1 (5.3)	D835Y	20	0	0
AML with CBFB::MYH11 fusion	5	2 (40.0)	14.60 (3.85 - 25.34)	1 (20.0)	N676K D835H	16 20	0	0
AML with NPM1 mutation	26	6 (23.0)	22.34 (5.10 - 46.04)	4 (15.4)	D835F/H/ V/Y N841T	20 20	1 (3.9)	1 (3.9)
AML, myelodysplasia-related	58	2 (3.5)	63.84 (43.55 - 84.13)	2 (3.5)	D835Y N841T	20 20	0	0
AML defined by differentiation	26	3 (11.5)	10.78 (2.80 - 22.82)	2 (7.7)	N676K D835Y N841I	16 20 20	1 (3.9)	1 (3.9)
Relapsed AML	9	1 (11.1)	12.91	0	D835V	20	0	0

Allele frequencies are presented as percentages.

AML: acute myeloid leukemia, APL: acute promyelocytic leukemia, ITD: internal tandem duplication, TKD: tyrosine kinase domain, NGS: next-generation sequencing.

Table 4. Detection of non-ITD/TKD FLT3 mutations by next-generation sequencing.

Diagnosis	Sample n	Non-ITD/TKD FLT3 positive n (%)	Allele frequency mean (range)	Variant sequence	Exon	Multiple non-ITD/TKD FLT3s n (%)	Co-occurred FLT3-ITD/TKD n (%)
Newly diagnosed AML	176	6 (3.4)	29.09 (16.43 - 49.85)			1 (0.6)	3 (0.02)
APL with PML::RARA fusion	25	2 (8.0)	23.21 (21.96 - 24.45)	D600del L576P	14 14	0	1 (4.0)
AML with NPM1 mutation	26	2 (7.7)	31.58 (16.43 - 49.85)	P336L Y572C L576Q	8 14 14	1 (3.9)	1 (3.9)
AML defined by differentiation	26	1 (3.9)	27.35	L576R	14	0	1 (3.9)
AUL	1	1 (100.0)	35.17	Y572S	14	0	0
Relapsed AML	9	1 (11.1)	81.38	P336L	8	0	1 (11.1)

Allele frequencies are presented as percentages.

AML: acute myeloid leukemia, APL: acute promyelocytic leukemia, AUL: acute undifferentiated leukemia, ITD: internal tandem duplication, TKD: tyrosine kinase domain.

which was FLT3-ITD positive in the single case identified. Other subtypes with notable FLT3-ITD mutation frequencies included AML with CBFB::MYH11 fusion,

AML defined by differentiation, APL with PML::RARA fusion, and AML with CEBPA mutation. FLT3-TKD mutations were most frequently observed in AML

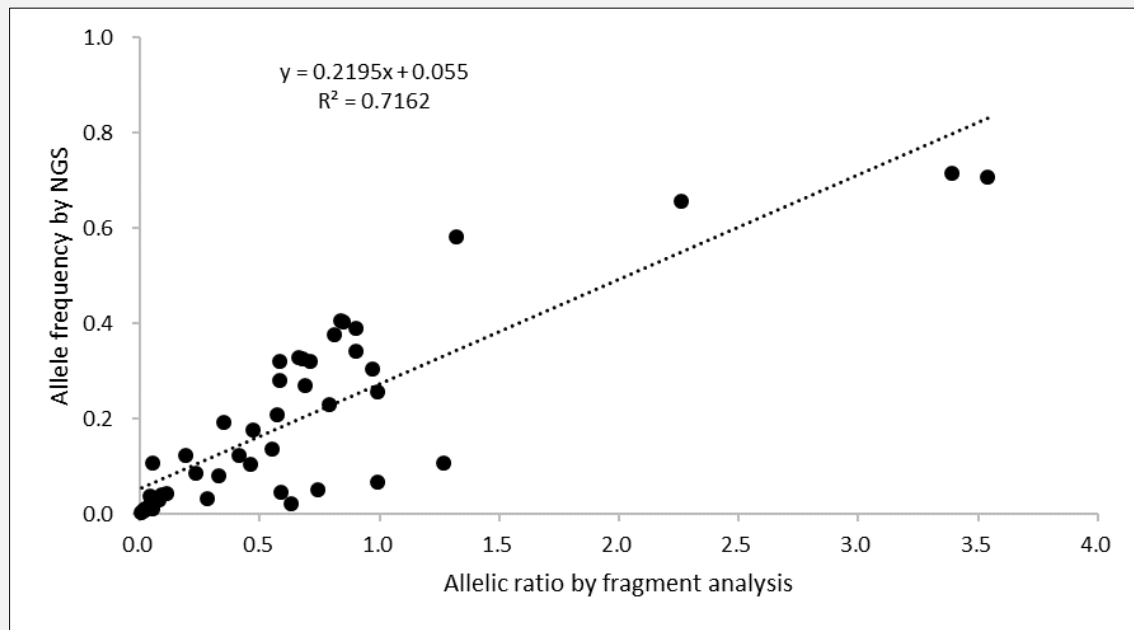


Figure 1. Correlation between allele ratio determined by fragment analysis and allele frequency determined by next-generation sequencing.

NGS: next-generation sequencing.

with CBFβ::MYH11 fusion (40.0%), followed by AML with NPM1 mutation (23.0%) and AML defined by differentiation (11.5%).

FLT3 mutations were also identified in 4 of the 9 patients with relapsed AML, all of whom harbored FLT3-ITD mutations. Among these patients, one also had an FLT3-TKD mutation and another had a non-ITD/TKD FLT3 mutation.

FLT3-ITD positive cases identified by NGS and fragment analysis

Table 2 presents the comparison of FLT3-ITD mutation detection by NGS and fragment analysis. Among the 176 newly diagnosed AML cases, FLT3-ITD mutations were detected in 20.5% (36/176) by NGS and in 21.6% (38/176) by fragment analysis. The detected FLT3-ITDs ranged from 6 to 162 base pairs (bp) in length. AFs determined by NGS ranged from 0.40 to 71.50, while ARs determined by fragment analysis ranged from 0.004 to 3.54. All FLT3-ITD mutations identified by NGS were also detected by fragment analysis. However, two additional cases were detected only by fragment analysis and not by NGS. One of these was an APL case with a long insertion (162 bp) and an AR of 0.25, and the other showed a low AR of 0.02 for a 63 bp insertion. Multiple FLT3-ITD variants were identified in 4.0% (7/176) of

newly diagnosed patients: six patients harbored two variants and one patient harbored three. In one case where fragment analysis revealed two peaks, NGS detected only one variant (AR 3.54, 21 bp), whereas the smaller variant (AR 0.05, 6 bp) was not identified by NGS. Notably, five of the seven cases with multiple ITDs occurred in AML with NPM1 mutation. Among the four relapsed AML patients with FLT3-ITD mutations, all variants were detected by NGS. However, two variants with low variant allele frequencies (VAFs of 0.1 and 1.0) were not detected by fragment analysis. In one relapsed case, two distinct FLT3-ITD mutations were identified.

AF values determined by NGS demonstrated a strong positive correlation with AR values obtained from fragment analysis, yielding a Pearson correlation coefficient of 0.85 (Figure 1).

FLT3-TKD-positive cases identified by NGS and direct sequencing

Table 3 presents the FLT3-TKD mutation findings as identified by NGS and direct sequencing. Among the 176 newly diagnosed AML patients, the FLT3-TKD mutation positivity rate was 8.5% (15/176) by NGS and 5.7% (10/176) by direct sequencing. The AF of FLT3-TKD mutations identified by NGS ranged from 2.80%

to 84.13%. All mutations detected by direct sequencing were also identified by NGS. However, five FLT3-TKD mutations involving codons 835 and 841 were detected only by NGS, with AFs ranging from 2.80% to 12.91%. In contrast, all mutations with AFs $\geq 13.37\%$ were detected by both methods. Multiple FLT3-TKD mutations were identified by NGS in 1.1% (2/176) of newly diagnosed cases. Each of these two cases harbored two distinct TKD mutations: one in AML with NPM1 mutation and one in AML defined by differentiation. In total, 17 FLT3-TKD mutations were identified, the majority of which were located in exon 20. Notably, two mutations (N676K) were located in exon 16.

Among the 9 relapsed AML patients, one case harbored a FLT3-TKD mutation (VAF 12.91) detected by NGS but not by direct sequencing.

Non-ITD/TKD FLT3-positive cases identified by NGS

Table 4 details the FLT3 mutations outside the canonical ITD and TKD regions as detected by NGS. Among the 176 newly diagnosed AML patients, 3.4% (6/176) were positive for non-ITD/TKD FLT3 mutations. These mutations were detected only in specific AML subtypes, including APL with PML::RARA fusion, AML with NPM1 mutation, AML defined by differentiation, and acute undifferentiated leukemia (AUL). The AF of these variants ranged from 16.43% to 49.85%. Multiple non-ITD/TKD FLT3 mutations were observed in 1 out of the 6 positive cases, which harbored two distinct variants.

A total of seven non-ITD/TKD mutations were identified, out of which six were located in exon 14 and one (P336L) in exon 8. Co-occurrence with FLT3-ITD or FLT3-TKD mutations was observed in three cases: one APL case also carried a TKD mutation (D835V); one AML with NPM1 mutation case also carried a FLT3-ITD; and one case harbored both a FLT3-ITD and two TKD mutations (N841I and N676K). Among the relapsed AML cases, one patient was found to have a non-ITD/TKD FLT3 mutation that co-occurred with a FLT3-ITD.

DISCUSSION

In the present study, the overall FLT3 mutation positivity rate among newly diagnosed AML patients was 28.4%, with FLT3-ITD and FLT3-TKD mutation rates of 21.6% and 8.5%, respectively. These findings align with previously reported data indicating FLT3 mutation rates of approximately 25 - 30%, including FLT3-ITD in 20 - 25% and FLT3-TKD in 5 - 10% of AML cases [2,3]. FLT3-mutant AML encompasses a heterogeneous group of disease entities, frequently exhibiting additional genetic and cytogenetic alterations. The most common co-occurring mutation is in NPM1, reported in approximately 50 - 60% of FLT3-mutated AML cases [3, 8]. FLT3 mutations are also associated with PML::

RARA fusion, core-binding factor AML, and DEK::NUP214 fusion, the latter showing FLT3-ITD in up to 90% of cases [9]. These associations are consistent with the current findings, which demonstrated high FLT3 mutation rates in AML with DEK::NUP214 fusion, AML with NPM1 mutation, AML with CBFB::MYH11 fusion, and AML defined by differentiation.

FLT3-ITD mutations were identified in 38 out of the 176 newly diagnosed patients. As shown in this study, FLT3-ITD mutations are heterogeneous with respect to their genomic location, insertion length, and number of duplications. A high degree of concordance was observed between NGS and fragment analysis, with 36 out of 38 cases detected by both methods. Furthermore, AF values from NGS positively correlated with AR values obtained from fragment analysis, yielding a Pearson correlation coefficient of 0.85. Previous studies have similarly reported strong agreement between these methods, with concordance rates of 99.6 - 100% for FLT3-ITD detection and correlation coefficients of 0.757 (Spearman) and 0.85 (Pearson) between AF and AR values [5,6]. In two cases, FLT3-ITDs were identified by fragment analysis but not by NGS. One of these involved an APL case with a large insertion (162 bp, AR 0.25), which may have evaded NGS detection due to limitations associated with short-read sequencing. The other case, with a small peak at 63 bp (AR 0.02), may have reflected either a low-level variant below NGS detection thresholds or a potential false positive result interpretation in fragment analysis. Among relapsed AML patients, FLT3-ITD mutations were detected in 4 out of 9 cases. Two of these were only detected by NGS, with VAFs of 0.1 and 1.0, while those with VAFs ≥ 8.7 were detected by both methods. These observations underscore the superior sensitivity of NGS in detecting low-frequency mutations. Given that the detection threshold for fragment analysis is generally estimated at 1 - 5% mutant allele burden, while NGS can reliably detect variants below 1% AF, low-level disease may escape detection by fragment analysis but remain detectable by NGS [5,10,11].

FLT3-TKD mutations were identified in 15 out of the 176 newly diagnosed AML cases. All TKD mutations with AF values exceeding 13.37% were detected by both NGS and direct sequencing. In contrast, five mutations with lower AFs (ranging from 2.80% to 12.91%) were detected exclusively by NGS, likely reflecting the higher analytical sensitivity of NGS compared with Sanger sequencing. The detection limit of Sanger sequencing has been reported to range between 15 - 20%, whereas NGS is capable of detecting variants with AFs below 1% [12,13]. The majority of TKD variants were located at codons 835 and 841 within exon 20; however, two N676K mutations were identified in exon 16 - a region not typically targeted by conventional PCR-based or direct sequencing assays but accessible through comprehensive NGS panels.

In the present study, multiple FLT3-ITD mutations were identified in 4.0% (7/176) of newly diagnosed AML pa-

tients and in 18.4% (7/38) of those harboring FLT3-ITD mutations. Out of these, six patients exhibited two distinct ITD mutations, while one patient harbored three. FLT3-ITDs vary considerably in both length and genomic location within the juxtamembrane domain (JMD) of the FLT3 receptor, with some extending into the tyrosine kinase domain 1 (TKD1). Prior studies have reported that up to one-third of patients with FLT3-ITD-positive AML may exhibit multiple ITDs [14]. For instance, in one study of newly diagnosed FLT3-ITD-mutated AML patients, 38.9% (35/90) had multiple ITD mutations and were associated with significantly lower complete remission rates compared to patients with a single ITD [15]. Another study reported a similar prevalence of multiple FLT3-ITDs at 35.2% (139/395), identifying two mutations in 66 patients and three mutations in 73 patients [16]. However, this latter study found no significant differences in overall survival or relapse-free survival between patients with single versus multiple ITD mutations, highlighting variability in prognostic implications across studies. Multiple FLT3-TKD mutations were also observed in two newly diagnosed AML patients: one case exhibited concurrent D835Y and D835V mutations, and the other harbored N841I and N676K mutations. Additionally, FLT3-ITD-TKD dual mutations were detected in two cases - one involving a single FLT3-ITD and a single TKD mutation, and the other involving one FLT3-ITD along with two TKD mutations (N841I and N676K). Dual FLT3-ITD-TKD mutations have been reported in prior literature, with an estimated prevalence of approximately 2% [17]. A previous NGS-based study of newly diagnosed and relapsed/refractory AML reported FLT3 mutations in 28.7% (49/171) of patients, including two cases with dual mutations: one with FLT3-ITD and FLT3-TKD, and another with FLT3-ITD and FLT3-F691L [18]. Notably, among nine patients who developed resistance to FLT3 inhibitors in that study, one was found to have acquired a novel FLT3 mutation that replaced the pre-existing one. Consistent with these findings, in the present study, two relapsed patients acquired new FLT3-ITD mutations not observed at initial diagnosis. In one of these cases, the patient developed two additional ITD mutations, while one of the previously detected FLT3-TKD mutations was no longer present. These observations suggest the presence of dynamic clonal evolution and genetic heterogeneity in FLT3-mutant AML. Clonal evolution is particularly relevant in FLT3-mutated AML, as FLT3 mutations may emerge during disease progression or in response to targeted therapy. The acquisition of new FLT3-ITD mutations during treatment has been associated with poor clinical outcomes and is recognized as an independent adverse prognostic factor [2,19,20].

With the advancement and broader implementation of sequencing technologies, FLT3 mutations outside the canonical ITD and D835 regions - referred to as NC FLT3 mutations - have increasingly been identified. However, these NC mutations remain insufficiently

characterized, and their prevalence, prognostic implications, and potential influence on sensitivity or resistance to FLT3 inhibitors remain uncertain and controversial. NC FLT3 mutations are most commonly located in exon 14 of the juxtamembrane domain (JMD), the primary site for ITDs, or within the TKD, but they have also been identified in other regions, including the extracellular domain (ECD). These mutations predominantly manifest as point mutations, although insertions and deletions have also been observed [2,7]. In a recent high-throughput sequencing study of FLT3-mutated AML patients, NC FLT3 mutations were detected in 5.5% (26/475) of cases, including 14 mutations within exon 16 of the TKD (codons 663 - 680), some of which co-occurred with canonical FLT3-ITD or TKD mutations [8]. Another targeted sequencing study of 1,540 adult AML patients identified four NC FLT3 variants, including two in the ECD (S451F and V591L) and two in the JMD (V592A and E487D) [21]. Similarly, a whole-genome sequencing analysis of pediatric AML reported a 7.6% (61/799) prevalence of NC FLT3 variants, including nine JMD mutations, with 14% (18/128) of FLT3-ITD-positive patients also harboring secondary FLT3 point mutations [22]. In the present study, non-ITD/TKD FLT3 mutations were identified in 3.4% (6/176) of newly diagnosed AML patients, and in 4.5% (8/176) when including two TKD mutations in exon 16 (N676K). Three of the six NC mutation-positive cases exhibited co-occurrence with FLT3-ITD or TKD mutations. Consistent with previous findings, six of the seven NC FLT3 mutations in this cohort were located in exon 14 of the JMD, while one was found in exon 8 of the ECD.

In summary, this study demonstrates that NGS offers reliable and superior sensitivity over conventional fragment analysis or direct sequencing for the detection of FLT3 mutations, which are commonly encountered and prognostically significant in AML. Nevertheless, the detection of large insertion mutations by NGS remains technically challenging. Importantly, NGS provided additional insights, including the identification of multiple FLT3-ITD or FLT3-TKD mutations, FLT3-ITD-TKD dual mutations, and especially NC FLT3 mutations, which exhibited considerable heterogeneity across patients. The frequency, clinical significance, prognostic value, and therapeutic implications - particularly regarding FLT3 inhibitor sensitivity or resistance - of these NC mutations remain to be fully elucidated and warrant further investigation.

Ethical Approval:

The protocol of this study was reviewed and approved by the Institutional Review Board of Kyungpook National University Hospital (approval no. 2025-04-032).

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The author declares no competing interests.

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