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Review Article

Molecular Testing of *FLT3* Mutations in Hematolymphoid Malignancies in the Era of Next-generation Sequencing

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Abstract

Background and objectives: FMS-like tyrosine kinase 3 (*FLT3*) mutations are among the most common genetic alterations in acute myeloid leukemia (AML) and play a pivotal role in leukemogenesis. The two primary mutation types, internal tandem duplications (ITDs) and tyrosine kinase domain point mutations, serve as key prognostic markers and therapeutic targets. Advances in next-generation sequencing (NGS) have revolutionized *FLT3* mutation detection by providing precise insights into mutation architecture, enhancing risk stratification, and enabling personalized treatment strategies. Additionally, these advancements have facilitated molecular minimal residual disease (MRD) testing, which is instrumental in guiding post-remission management. This review summarizes the molecular characteristics, diagnostic approaches, and therapeutic implications of *FLT3* mutations in hematologic malignancies. ***Methods:*** A narrative review of the current literature on *FLT3* mutations was conducted, incorporating data from original research articles, clinical trials, and recent reviews. Relevant studies were identified through a PubMed literature search and manually curated. ***Results:*** *FLT3* mutations are detected in approximately 30% of AML cases and occur at lower frequencies in myelodysplastic syndromes, chronic myelomonocytic leukemia, acute lymphoblastic leukemia, and mixed phenotype acute leukemia. NGS enables comprehensive mutation profiling, revealing rare variants and subclonal complexity while supporting MRD detection with high analytic sensitivity. *FLT3*-ITD-based MRD positivity is strongly associated with relapse and poor survival in AML. Clinical trial data support FLT3 inhibitors, including midostaurin, gilteritinib, and quizartinib, in *FLT3*-mutated AML. Additionally, MRD-guided therapy and combination treatment strategies are promising approaches to overcoming resistance. ***Conclusions:*** *FLT3* mutations play a central role in the pathogenesis and treatment of AML and related malignancies. NGS-based testing and MRD monitoring transform clinical decision-making by refining risk stratification and enabling personalized therapeutic interventions. Establishing standardized testing protocols and the broader integration of FLT3-targeted therapies will be essential for optimizing patient outcomes.

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Introduction

FMS-like tyrosine kinase 3 (FLT3) is a critical regulator of hematopoietic cell survival, proliferation, and differentiation.[1,2] *FLT3* mutations, including internal tandem duplications (ITDs),[3] tyrosine kinase domain (TKD) mutations,[4] and other uncommon point mutations,[5–7] drive leukemogenesis by promoting excessive proliferation and impaired differentiation of early hematopoietic cells. These mutations are most frequently observed in acute myeloid leukemia (AML) and, to a lesser extent, in other hematolymphoid malignancies such as myelodysplastic syndromes (MDS) and acute lymphoblastic leukemia (ALL) (Table 1).[5,7–16]

A landmark study by Kiyoi *et al*.[17] demonstrated that *FLT3*-ITD mutations were the strongest independent prognostic factor for poor overall survival in AML patients younger than 60. Similarly, Rombouts *et al*.[18] reported that AML patients harboring *FLT3*-ITD mutations exhibited significantly lower complete remission rates, higher relapse rates, and worse event-free survival compared to those without the mutation, reinforcing its negative prognostic impact. Given this prognostic significance, *FLT3* mutation detection has become essential for risk stratification and guiding therapeutic decision-making. The development of targeted therapies, particularly FLT3 inhibitors, has further highlighted the clinical importance of identifying this genetic alteration.[10] Consequently, *FLT3* mutations have emerged as a key therapeutic target, revolutionizing AML management by integrating genetic testing and targeted therapies.[19,20] *FLT3* mutation testing is now indispensable to AML diagnosis and treatment planning.[21] Conventionally, *FLT3* mutation testing has relied on polymerase chain reaction (PCR)-based fragment length analysis, which has limited sensitivity and provides incomplete characterization of mutations.[22] The recent advent of next-generation sequencing (NGS) technologies has significantly improved the accuracy of *FLT3* mutation detection, enabling risk stratification and personalized treatment approaches.[23] NGS can reliably analyze DNA or RNA extracted

Keywords: *FLT3* mutation; *FLT3*-internal tandem duplication (ITD); Next generation sequencing (NGS); Acute myeloid leukemia (AML); Bioinformatics analysis; Measurable residual disease; Targeted therapy.

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Table 1. *FLT3* mutations documented in the literature

Mutation type	Exon location	Molecular mechanism	Disease association
FLT3-ITD	Exons 14–15	Disrupts JMD autoinhibition, causing constitutive activation of STAT5, MAPK, and PI3K/AKT pathways; promotes leukemogenesis.	AML: ~30%[8,12]; MDS: 0.7–3%[14]; CMML: 2.7%[14]; MPAL: 14%, T/myeloid subtypes[16]; T-ALL: 2.9%[15]
FLT3-TKD	Exons 16–20	Point mutations (e.g., D835) disrupt activation loop, causing ligand-independent activation and altered catalytic activity.	AML: 7–11%[10,13]; MDS: 0.2%[14]; CMML: 1.7%[14]; T-ALL: 1.4%[15]
FLT3-JMD	Exons 14–15	Rare point mutations (e.g., Y572C, V579A, V592A) disrupt regulatory dimerization; mechanistically similar to ITDs, causing constitutive activation.	AML: ~2%[5,11]
Others	Exon 5, 6, 10, 11, 14, 16, 20	Various mutations confer resistance (e.g., N676D/K, G697R) or sensitivity (e.g., Y572C, V592A/G) to FLT3 inhibitors.	AML[7]

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; FLT3, FMS-like tyrosine kinase 3; ITD, internal tandem duplication; JMD, juxtamembrane domain; MAPK, mitogen-activated protein kinase; MDS, myelodysplastic syndromes; MPAL, mixed phenotype acute leukemia; PI3K/AKT, phosphoinositide 3-kinase/protein kinase B; STAT5, signal transducer and activator of transcription 5; TKD, tyrosine kinase domain.

from both formalin-fixed and fresh-frozen tissues, providing equivalent diagnostic results.[24]

This review aimed to provide a comprehensive overview of *FLT3* mutations in acute leukemias, focusing on current approaches to *FLT3* mutation testing in clinical laboratories and future directions for integrating these insights into personalized AML care. It is intended to serve as a resource for pathologists and hematologists involved in diagnosing and managing AML and related malignancies, as well as researchers developing targeted therapies.

Characteristics of *FLT3* gene and protein

FLT3 gene and protein structure

The *FLT3* gene [National Center for Biotechnology Information (NCBI) reference numbers: NC_000013.11 | NM_004119 | ENST00000241453 | CCDS31953] is located on chromosome 13q12.[25] The gene was first identified in 1991 by two independent groups and was originally named fetal liver kinase 2 (FLK-2) or stem cell kinase 1 (STK-1) due to its role in early hematopoietic cells.[26,27] The human *FLT3* gene consists of 24 exons,[28] encoding the extracellular domain (exons 1–12), the transmembrane domain (exon 13), the juxtamembrane domain (JMD; exons 14–15), and the tyrosine kinase domain (TKD; exons 15–23) of the transmembrane receptor, which belongs to the class III receptor tyrosine kinase family (Fig. 1).[29]

The extracellular domain of FLT3 consists of five immunoglobulin-like folds. The three most distal folds mediate high-affinity ligand binding, while the two proximal folds facilitate receptor dimerization. This highly glycosylated region ensures proper receptor folding and stability, which are essential for its localization to the cell surface and functional integrity.[30] Beneath this region, a single transmembrane α-helix anchors the receptor within the cell membrane, maintaining its orientation and structural stability.

Immediately downstream of the transmembrane region, the juxtamembrane domain plays a crucial role in stabilizing the receptor in its inactive conformation.[31,32] The intracellular portion of FLT3 contains the TKD, which is divided into two lobes: an N-terminal lobe and a C-terminal lobe. The N-terminal lobe, composed of a β-sheet and an α-helix, stabilizes the ATP-binding pocket, whereas the C-terminal lobe, consisting primarily of α-helices, contains the activation loop. In its inactive state, the activation loop adopts a closed conformation, preventing access to the catalytic cleft.

Biological function of FLT3

The FLT3 protein is predominantly expressed in early hematopoietic progenitor cells, particularly CD34-positive cells in the bone marrow, reflecting its essential role in early blood cell development.[33] While the receptor is also expressed in other tissues, its primary function is a key regulatory switch in hematopoiesis.[30,34] FLT3 ligand, which exists in both membrane-bound and soluble forms, is produced by cells in the bone marrow microenvironment.[35]

Under physiological conditions, ligand binding induces receptor dimerization and autophosphorylation, activating downstream signaling pathways such as PI3K/AKT, MAPK/ERK, and STAT.[34,36–38] These pathways regulate cell cycle progression, survival, and differentiation, ensuring the orderly production of functional blood cells. Beyond its role in hematopoiesis, FLT3 also contributes to immune system regulation by supporting the development of dendritic cells and natural killer cells.[39] The FLT3 ligand interacts with cytokines such as granulocyte-macrophage colony-stimulating factor and interleukin-4 to drive dendritic cell differentiation and expansion, showing its involvement in adaptive immunity.[40]

FLT3 mutations and their clinical significance

FLT3 mutations

The clinically significant mutations of *FLT3* include ITDs in the JMD and point mutations in the TKD, JMD, and other regions (Table 1 and Fig. 2a).[41,42] In its autoinhibited state, the JMD functions as a structural wedge, preventing the rotation of the N-terminal and C-terminal lobes of the TKD into an active configuration. ITDs within the JMD disrupt these interactions, destabilizing the inactive conformation and enabling ligand-independent activation of FLT3.

TKD mutations, such as substitutions at codon aspartate 835 (D835), interfere with this closed conformation, leading to constitutive ligand-independent receptor activation.[43] This aberrant activation results in persistent phosphorylation of the receptor and dysregulation of downstream signaling pathways, including STAT5, MAPK, and PI3K/AKT, which drive cell proliferation and leukemogenesis.

FLT3-JMD mutations occur in a region essential for regulating receptor dimerization and activation. Structural studies

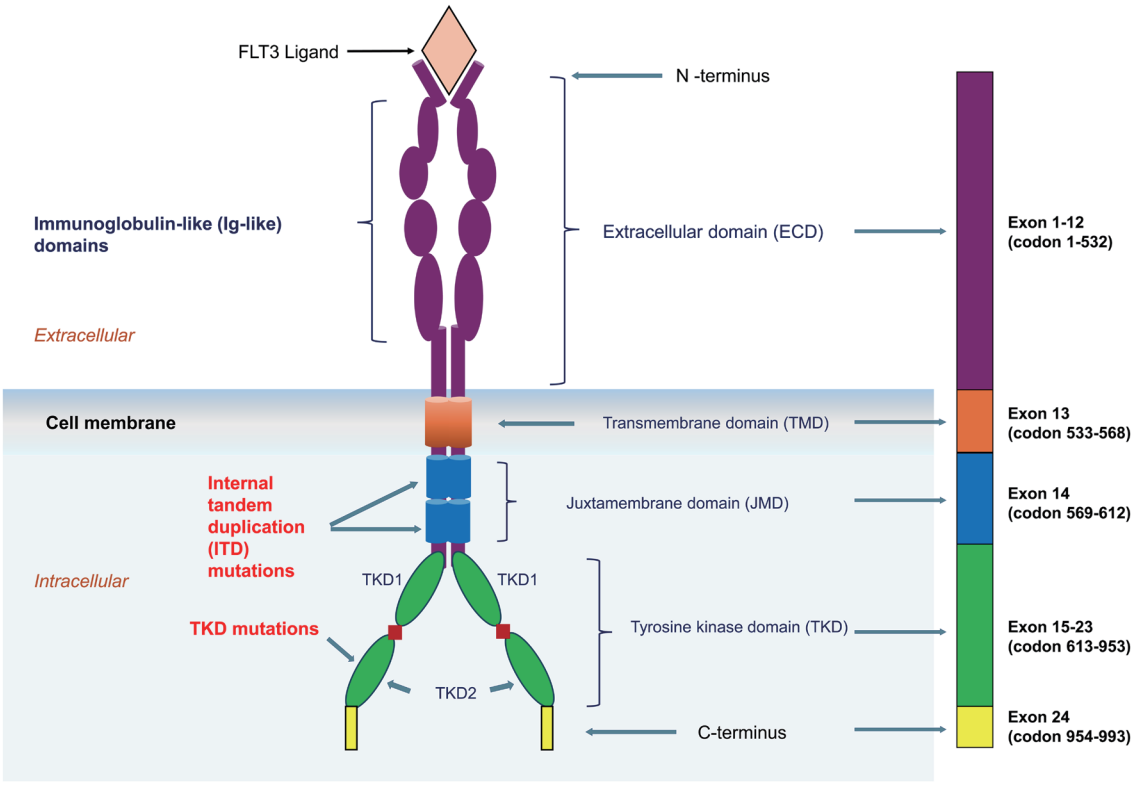

Fig. 1. *FLT3* gene and protein structure. The illustration of protein structure (domains) on the left side, and corresponding exons in the *FLT3* gene are displayed on the right side.

indicate that these mutations restrict domain motions, expand the kinase gate, and enhance drug-binding capacity.[5,11]

Aberrant FLT3 activity, whether due to mutations or dysregulated expression, disrupts the downstream signaling networks, promoting excessive proliferation and survival of immature blasts while impairing their differentiation. For instance, *FLT3*-ITD mutations are associated with STAT5 hyperactivation, which drives the transcription of pro-survival genes such as *BCL2L1* (BCL-XL) and *MYC* while suppressing transcription factors critical for myeloid differentiation, including PU.1 and C/EBPα. Additionally, *FLT3*-ITD enhances β-catenin activation via the WNT signaling pathway, further promoting leukemic cell proliferation.[6,44] These dysregulations are hallmarks of leukemogenesis, highlighting the critical role of FLT3 as a therapeutic target in AML.

Disease spectrum of FLT3 mutations in hematolymphoid neoplasms

Mutations in *FLT3* are most commonly observed in AML, where they are present in approximately 30% of newly diagnosed patients.[8,12] These mutations are predominantly ITDs in the JMD (~25%) or point mutations in the TKD (7–10%),[10,13] and they are rarely observed as *FLT3*-JMD (~2%) and other mutations.[5–7] *FLT3* mutations are also identified in other hematologic malignancies, including MDS, chronic myelomonocytic leukemia (CMML), ALL, and mixed phenotype acute leukemia (MPAL) (Table 1 and Fig. 2b). Their frequency and clinical impact vary across diseases, reflecting differences in underlying pathobiology and therapeutic approaches.

In MDS, *FLT3* mutations are rare, with reported frequencies ranging from 0.95% to 3%.[3,14,45] These mutations are more commonly associated with higher-risk subtypes, such as MDS with excess blasts, and may contribute to progression to AML.[14] In CMML, *FLT3* mutations occur at slightly higher frequencies, estimated at 4.3%.[14] In ALL, *FLT3* mutations are most frequently observed in specific subtypes, such as hyperdiploid B-cell ALL and CD117/KIT-positive T-cell ALL.[9,15] In hyperdiploid B-cell ALL, approximately 25% of cases harbor *FLT3* mutations, often involving novel in-frame deletions or point mutations in the receptor's JMD or activation loop regions.[9] In T-cell ALL, *FLT3* mutations are associated with the early T-cell precursor (ETP) immunophenotype, characterized by CD34 positivity, absent surface CD3 expression, and myeloid marker expression. *FLT3*-ITD mutations are also found in acute leukemia of ambiguous lineage (ALAL), reported in 14% of MPAL cases, particularly in the T/myeloid subtype, often co-occurring with abnormalities in *TP53*, *RUNX1*, *WT1*, and *NOTCH1*.[16,46]

Prognostic implications of FLT3 mutations

Among the most clinically significant genetic abnormalities in AML, FLT3 mutations are associated with higher blast counts, aggressive disease progression, and poorer survival outcomes.[8,17,18,12,47] A large United Kingdom Medical Research Council study involving 854 AML patients demonstrated that *FLT3*-ITD mutations were associated with significantly higher relapse rates (64% vs. 44% at five years) and lower overall survival (32% vs. 44% at five years) compared to patients without *FLT3* mutations.[8] Similarly, a recent meta-analysis of normal karyotype AML patients younger than 60 years demonstrated significantly poorer overall survival (hazard ratio [HR]: 1.86, 95% confidence interval [CI]: 1.57–2.20) and relapse-free survival (HR: 1.75, 95% CI: 1.54–2.18) associated with *FLT3*-ITD mutations.[47] Given these findings, rou-

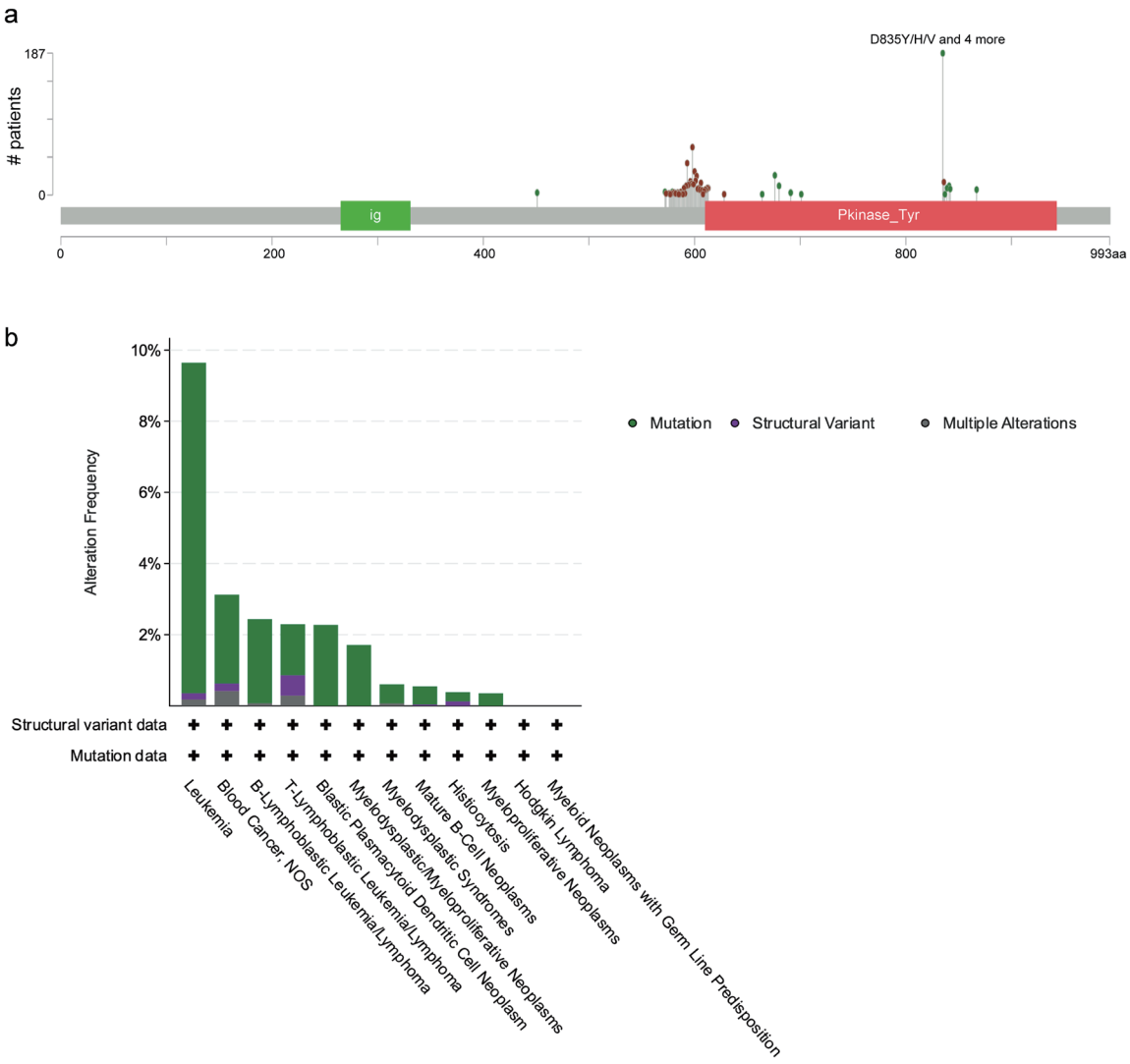

Fig. 2. *FLT3* mutations in hematolymphoid neoplasms. (a) Location and types of mutations in *FLT3*. Only the 999 mutations designated as "Driver" are displayed. Color code: Green: missense mutation (362), black: truncation (3), brown: inframe insertions or deletions (632), and purple: fusion (2). The mutations are clustered in the juxtamembrane domain (JMD) and the tyrosine kinase domain (red box, Pkinase_Tyr). (b) Disease spectrum of *FLT3* mutations. Data from The American Association for Cancer Research (AACR) Project GENIE (Genomics Evidence Neoplasia Information Exchange) public database v17 and figure created by cBioportal.[41,42] Data link https://genie.cbioportal.org/study?id=6793a338854f636a386473a0 or https://bit.ly/3PM31n5 (Accessed January 24, 2025). Total 27,910 samples from 17,134 patients were included. Although not specifically provided, most cases in the "Leukemia" category are likely acute myeloid leukemia, not otherwise specified.

tine *FLT3* mutation testing has become essential in clinical practice for diagnosis, prognosis, and treatment strategies, including the use of allogeneic stem cell transplantation and targeted therapies.[8,48,49]

While some studies suggest that shorter ITD lengths are associated with better outcomes and longer lengths with worse prognoses,[50,51] recent analyses have found no significant impact of ITD length on overall or relapse-free survival using established thresholds.[51] Instead, the mutant allelic burden appears to be a stronger predictor of prognosis.[52]

The prognostic impact of *FLT3*-ITD mutations is further influenced by co-occurring genetic alterations. Favorable-risk mutations, such as *NPM1* or biallelic *CEBPA* mutations, can mitigate the adverse effects of *FLT3*-ITD, leading to improved overall and disease-free survival.[53–55] In contrast, adverse-risk markers, including *DNMT3A* mutations and complex karyotypes, do not consistently worsen outcomes.[56] For instance, a study of 103 AML patients found that *FLT3*-ITD in combination with favorable-risk mutations was associated with significantly better survival compared to *FLT3*-ITD alone, whereas co-occurrence with adverse-risk mutations did not further exacerbate prognosis.[57]

TKD mutations are commonly associated with a normal karyotype and elevated leukocyte counts.[10,13] They frequently co-occur with *NPM1* (8.8%), *CEBPA* (7.9%), and *NRAS* (7.7%) mutations, while their combination with ITD (2.9%) or *KIT* codon D816 mutations (2.3%) is rare.[13] Unlike ITDs, TKD mutations generally do not worsen survival[10]; however, prognosis depends on co-mutations.[13] Unfavorable outcomes have been observed in cases with t(15;17); *PML-RARA*, ITD/TKD double mutants, and *MLL*-PTD/TKD double mutants. Conversely, TKD mutations co-occurring with *NPM1* or *CEBPA* mutations have been associated with favorable event-free survival.[13] A meta-analysis of 20 studies involving 10,970

Table 2. FDA-approved FLT3 inhibitors

Drug	Generation/Type*	Target	FDA-approved indications	Toxicities
Midostaurin[66]	First/Type I	FLT3, KIT, VEGFR, PDGFR	Combination with chemotherapy for newly diagnosed AML	Febrile neutropenia, nausea, vomiting
Gilteritinib[67]	Second/Type I	FLT3, AXL, LTK, ALK	Monotherapy for relapsed/refractory AML	Peripheral edema, pancreatitis, diarrhea
Quizartinib[68]	Second/Type II	FLT3, KIT	Combination with chemotherapy for newly diagnosed AML	QT prolongation, myelosuppression
Crenolanib[69]	Second/Type I	FLT3, PDGFR	Under investigation in combination therapy	Peripheral edema, differentiation syndrome

*See text for the two types of inhibition mechanisms. ALK, anaplastic lymphoma kinase; AML, acute myeloid leukemia; AXL, AXL receptor tyrosine kinase; FDA, United States Food and Drug Administration; FLT3, FMS-like tyrosine kinase 3; KIT, stem cell factor receptor; LTK, leukocyte tyrosine kinase; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

AML patients highlighted ethnic differences in the prognosis of TKD mutations. While TKD mutations had no significant impact on disease-free survival (HR = 1.12) or overall survival (HR = 0.98), they were associated with improved survival in Asian patients (disease-free survival HR = 0.56, overall survival HR = 0.63) but worse disease-free survival in Caucasians (HR = 1.34).[58]

Beyond the well-characterized ITD and D835 mutations, rare *FLT3* mutations have been identified across multiple domains, including the extracellular, juxtamembrane, and kinase regions (Fig. 2a). These mutations, often uncovered through advanced sequencing techniques, contribute to the genetic complexity of AML. *FLT3*-JMD mutations represent a small but biologically and clinically distinct subset of *FLT3* alterations.[11,59] They frequently co-occur with *FLT3*-ITD or TKD mutations in separate clonal populations.

Pediatric AML studies report a *FLT3*-JMD mutation prevalence of 7.6%, with mutations such as E598D, L576R, and Y599C often coexisting with ITDs.[7,60] In adults, rare driver mutations, including S451F and V592A, have also been reported.[61] Clinically, *FLT3*-JMD mutations are associated with higher relapse rates and shorter disease-free survival than TKD mutations, with outcomes similar to those observed in *FLT3*-ITD-positive cases.[11] Importantly, *FLT3*-JMD mutations exhibit increased sensitivity to FLT3 inhibitors, such as gilteritinib and sorafenib, compared to ITD.[11] N676K and V592G respond to sorafenib,[62] while N676K also confers resistance to midostaurin.[63] More data to correlate molecular findings with clinical outcomes will likely further clarify the biological relevance of these drivers versus passenger mutations.

The clinical significance of *FLT3* mutations in hematolymphoid malignancies beyond AML remains less well understood. Serial analyses suggest that *FLT3*-ITD mutations are frequently acquired during the transformation of MDS to AML, implicating them in disease progression. However, *FLT3* mutations do not appear to predict overall survival in MDS, and their prognostic significance remains uncertain. Similarly, while *FLT3*-ITD and TKD variants have not significantly impacted overall survival in CMML, they may be associated with disease transformation in select cases.[14]

Preliminary findings in ALL suggest that *FLT3* mutations are more prevalent in relapsed cases, supporting the exploration of *FLT3* inhibitors in refractory settings. Activating *FLT3* mutations likely plays a role in the leukemogenesis of ETP-ALL, with FLT3 inhibitors showing potential therapeutic applications in relapsed or refractory disease.[9,15] Recent case studies have also highlighted the benefits of FLT3 inhibitors in *FLT3*-mutated ALAL and MPAL patients.[46,64]

FLT3 as a therapeutic target

FLT3 mutations play a key role in AML pathogenesis, making them a critical target for molecular therapies.[65] Early studies and preclinical models showed that FLT3 inhibition could reduce leukemic burden and enhance chemotherapy.[19] These insights have led to clinical trials evaluating FLT3 inhibitors. Over time, these inhibitors have evolved from first-generation agents exhibiting broad off-target effects to second-generation compounds with enhanced specificity, potency, and tolerability. FLT3 inhibitors are further classified based on their mechanism of action.

Type I inhibitors bind to the ATP-binding site of the receptor in its active conformation (DFG-in), effectively inhibiting both ITD and TKD mutations. In contrast, type II inhibitors bind to the inactive conformation (DFG-out), demonstrating potent activity against ITD mutations but reduced efficacy against common TKD alterations, such as D835 (Table 2).[66–69]

Based on these findings, midostaurin became the first FLT3 inhibitor to receive United States Food and Drug Administration (US FDA) approval for use in combination with chemotherapy in *FLT3*-mutated AML. Midostaurin remains the only agent to gain FDA approval among first-generation inhibitors, supported by data from the phase 3 RATIFY trial, a double-blind, placebo-controlled study involving 717 patients.[66] The study demonstrated a significant improvement in survival with midostaurin compared to placebo (hazard ratio: 0.77; $P = 0.016$). This benefit was observed across multiple *FLT3* mutation subtypes, including ITD and TKD variants. Sorafenib is a type II inhibitor with activity against FLT3, as well as RAF, vascular endothelial growth factor receptors, platelet-derived growth factor receptor (PDGFR), KIT, and RET. Initially approved for solid tumors, sorafenib added to standard chemotherapy significantly improved event-free survival in the SORAML trial but increased toxicities such as fever and hand-foot syndrome.[70] Sorafenib maintenance therapy after allogeneic hematopoietic stem cell transplantation (HSCT) reduced relapse and death risks.[71] However, the ALLG trial found no significant improvement in event-free survival when sorafenib was combined with intensive chemotherapy in newly diagnosed patients.

Second-generation FLT3 inhibitor gilteritinib received FDA approval in 2018 based on the phase 3 ADMIRAL trial, which demonstrated significant benefits over salvage chemotherapy in treating relapsed or refractory FLT3-mutated AML.[67] While gilteritinib has not yet been approved for treating newly diagnosed AML, studies have highlighted the potential of gilteritinib in diverse clinical settings.[72,73] Quizartinib, a type II FLT3 inhibitor, received FDA approval in 2023 for use in

newly diagnosed *FLT3*-ITD-positive AML based on the phase 3 QuANTUM-First trial, which demonstrated a significant improvement in overall survival with quizartinib compared to placebo.[68] More recently, crenolanib, a second-generation type I FLT3 inhibitor, has shown promising results when combined with intensive chemotherapy in newly diagnosed *FLT3*-mutant AML.[69]

The success of second-generation agents confirms the viability of FLT3 inhibition as a therapeutic strategy and encourages ongoing efforts to integrate these agents earlier in the treatment paradigm. However, resistance to FLT3 inhibitors poses a significant challenge.[74–76] Secondary resistance develops under the selective pressure of therapy, driven by clonal evolution and genetic alterations.[77] Common mechanisms include *FLT3* TKD mutations, activation of parallel survival pathways (e.g., RAS/MAPK and PI3K/AKT), and modifications in the tumor microenvironment.[76,78] To overcome resistance, exploring rational combinations with other targeted therapies (e.g., BCL-2 or MEK inhibitors) or hypomethylating agents such as decitabine has demonstrated synergistic effects and improved outcomes in preclinical and early-phase clinical trials.[79–81] As molecular profiling and adaptive treatment strategies advance, these inhibitors will likely remain integral to personalized AML therapy.

Detection of *FLT3* mutations in clinical laboratories

Given the prognostic and therapeutic significance of *FLT3* mutations in acute leukemias, accurate mutational analysis is crucial following the preliminary diagnosis of high-grade myeloid neoplasms to facilitate the timely initiation of targeted therapeutic interventions.[48,49] Over the past two decades, *FLT3* mutation detection has evolved significantly, transitioning from traditional molecular techniques to high-throughput sequencing technologies alongside an expanding understanding of AML biology. The molecular methods currently employed in clinical laboratories for *FLT3* mutation detection include fluorescence-labeled PCR with fragment length analysis (PCR-FLA) and targeted gene panel NGS.

Traditional PCR with fragment length analysis

In 2003, Murphy *et al*.[22] described a molecular approach capable of detecting both *FLT3*-ITD and *FLT3* codon D835 mutations using a single multiplex PCR followed by EcoRV restriction endonuclease digestion and capillary electrophoresis (CE) separation. In this PCR-based assay, the genomic regions harboring both *FLT3*-ITD (encoded by exon 14 and exon 15) and D835 (encoded by exon 20) mutations are amplified simultaneously using two pairs of fluorescent-labeled primers. Following amplification, EcoRV, a restriction endonuclease from *Escherichia coli*, digests the PCR products. The *FLT3* D835 mutations disrupt an EcoRV recognition site (GATATC) in the wild-type sequence, preventing digestion. Consequently, the assay produces fluorescent-labeled wild-type PCR fragments measuring 80 base pairs (bp) and mutant fragments measuring 129 bp. An undigested control product measuring 150 bp is used to confirm enzymatic activity (Fig. 3). For *FLT3*-ITD detection, the wild-type sequence generates an amplicon of 330 bp. Mutations involving insertions in exon 14 and exon 15 result in longer fragments. CE visualization enables quantification of the *FLT3*-ITD allelic ratio (AR) by comparing the area under the curve of the mutant and wild-type amplicon peaks.

The multiplex PCR-FLA approach remains widely utilized in clinical laboratories and is recommended at the initial diagnosis of acute leukemias. This is due to its cost-effective, technically straightforward setup utilizing traditional molecular biology tools such as PCR, enzymatic digestion, and CE, and its rapid turnaround time, with results available within 1–2 days from DNA extraction.[48] However, this method has significant limitations:

- *Limited Detection Range*: The assay is designed to detect only a few hotspot *FLT3*-TKD point mutations unless the PCR products are further sequenced.[82]
- *Lack of Insertion Details*: *FLT3*-ITD fragment analysis does not provide specific information about the inserted sequences or their precise locations, which may have prognostic implications.[83]
- *Limited sensitivity to detect multiple ITDs*: Multiple *FLT3*-ITDs can result in overlapping mutation peaks, leading to inaccurate quantification of mutation numbers.
- *PCR Bias*: The assay shows reduced efficiency in detecting long ITD mutations.
- *Low Analytic Sensitivity*: With an analytic sensitivity of 1–5%, PCR-FLA is unsuitable for measurable residual disease (MRD) testing.[84]

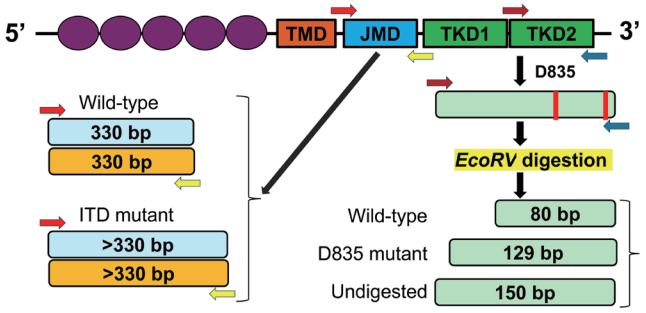

Fig. 3. *FLT3* mutation test by polymerase chain reaction and fragment length analysis (PCR-FLA). The primer sites are shown on the illustrated protein sequence with domains labeled, and the amplicon sizes for ITD and TKD mutations are displayed. The EcoRV enzyme digestion sites are marked with red color bars; the reverse primer for TKD2 is designed to add an EcoRV site at the 3'-end of the amplicon to provide a control for enzyme activity. ITD, internal tandem duplication; JMD, Juxtamembrane domain; TKD, tyrosine kinase domain; TMD, transmembrane domain.

NGS-based FLT3 mutation testing

Over the past two decades, NGS, particularly multiplex-targeted gene panel NGS, has become an indispensable tool in molecular laboratory testing, particularly for diagnosing genetic disorders and cancers. Although most clinical guidelines regarding *FLT3*-ITD are based on data derived from PCR-FLA, NGS-based mutation profiling has become routine in many clinical diagnostic laboratories. NGS platforms, such as Illumina and Ion Torrent, enable high-throughput sequencing of *FLT3* alongside other clinically significant genes. Even when PCR-FLA is employed at the initial diagnosis of acute leukemias to facilitate early treatment decisions, targeted gene panel NGS for myeloid neoplasms, including *FLT3*, is typically performed to complete the initial workup.

Compared with PCR-FLA-based *FLT3* mutation testing, NGS can detect *FLT3* mutations beyond hotspot regions, including gain-of-function activating mutations in the N-lobe and activation loop, which may influence response to *FLT3* inhibitors or confer treatment resistance. Additionally, NGS enables the identification of treatment resistance-associated mutations as well as the simultaneous detection of clinically significant co-mutations in *NPM1*, *CEBPA*, and *IDH1/2*, allowing for refined risk stratification and better treatment decisions.[11,59,85] Although NGS theoretically identifies all types of mutations, its ability to detect large insertions or deletions

(indels) is limited due to challenges in sequence alignment. Depending on the target enrichment method and the data analysis tools used, routine NGS is not always reliable for identifying insertions and deletions longer than 15 bp.[86]

Significant progress has been made in the bioinformatics analysis of NGS data to identify *FLT3*-ITD (reviewed by Yuan *et al*.)[86] Detection tools are typically categorized into alignment-based and assembly-based approaches. Alignment-based tools, such as Pindel,[87] ITDseek,[88] getITD,[89] ScanITD,[90] and FLT3_ITD_ext,[91] align raw reads to the reference sequence and extract discordant reads to detect *FLT3*-ITD. Assembly-based tools, including BreaKmer, ITDetector, and ITD assembler,[92–94] reconstruct misaligned short reads using specialized algorithms before realigning the assembled contigs to the reference genome.[86] Comparative studies have shown that FLT3_ITD_ext performs best for both qualitative and quantitative analysis of simulated *FLT3*-ITD with an average insertion length of 200 bp (±20 bp) and biological samples.[86] These tools, developed in programming languages such as C++, Python, and Perl, require fine-tuning, and their integration into the NGS workflow necessitates bioinformatics expertise. Clinical laboratories that adopt proprietary NGS library preparation kits often utilize packaged tools designed specifically for those kits, integrating both wet-lab and bioinformatics validations to optimize performance in detecting various genetic alterations.

Targeted gene panel NGS is the preferred method in clinical laboratories for tumor sample sequencing, as it achieves high read depth while conserving sequencing resources. Two common approaches for enriching target genes during library preparation are hybrid capture-based and amplification-based methods. Hybrid capture-based enrichment employs sequence-specific capture probes that are complementary to regions of interest, whereas amplification-based methods use multiplex PCR to enrich target sequences while simultaneously tagging them with patient-specific indexes and sequencing platform adaptors.

Although hybridization-based methods are technically more demanding, they enable the capture of larger fragments and are less affected by mismatches and allele dropout. In contrast, amplicon-based methods require less hands-on time but are more susceptible to PCR bias and significantly influenced by primer design.[91,95]

The *FLT3*-ITD AR, reflecting the mutant clone burden, has historically been an important prognostic factor.[65] However, the 2022 European LeukemiaNet (ELN) recommendations for AML diagnosis and management no longer consider the arbitrary cutoff of 0.5 for *FLT3*-ITD AR in risk classification, citing challenges in standardizing measurements, the impact of *FLT3* inhibitor-based treatments, and the increasing use of MRD testing to guide treatment decisions.[49] Nonetheless, assessing *FLT3*-ITD allelic burden remains relevant in research and clinical management.[48] The AR may still be meaningful when reporting NGS results for molecular diagnostic laboratories. Recent studies have demonstrated that by optimizing bioinformatic analyses, hybrid capture-based targeted panel NGS can achieve sufficient sequencing coverage of *FLT3* to accurately detect ITDs beyond 200 bp and calculate AR that correlates well with PCR-FLA results.[23,85,96] The AR is calculated using variant allele frequency (VAF) from NGS results as AR = *FLT3*-ITD VAF ÷ (1 − ITD VAF). A VAF of 0.33 aligns with the PCR-FLA AR cutoff of 0.5. However, this correlation is likely method-dependent and influenced by bioinformatics strategies.[96,97] Targeted NGS assays with amplicon-based enrichment, particularly those using anchored multiplex PCR, which employs redundant gene-specific primers to cover *FLT3* exons 14 and 15,[91] have shown excellent sensitivity in detecting large ITDs up to 300 bp.[91,98] Given the potential for large ITDs and mutations at primer sites that result in allele dropout, multiple primers are necessary to detect all ITDs. Larger insertions may be underestimated compared to PCR-FLA due to reduced PCR amplification efficiency and alignment challenges with long fragments during bioinformatics analysis.[99] Refining informatics pipelines can improve AR calculations.[91]

By generating nucleotide reads, NGS provides precise location and the molecular architecture of *FLT3*-ITD.[98] ITDs may start in exon 14 or intron 14–15 and extend into exon 15.[85,91,98] Notably, external sequences up to 27 bp may also be inserted at ITD junctions. Among 105 ITDs studied by Ding *et al*.,[98] only 42% were pure tandem duplications. ITDs extending into intronic regions can affect RNA splicing, meaning DNA sequencing alone may not accurately predict amino acid sequences.[98] A comparative analysis of DNA versus cDNA sequencing revealed higher detection sensitivity and higher ARs with cDNA-based detection. The higher AR from cDNA also correlated with poor clinical outcomes when combined with longer ITDs (>48 bp),[100] although the clinical outcomes were study-dependent. However, the lengths of ITD from DNA and RNA were identical. It appears that ITDs extending into the intronic region would not remove or add any splice sites. Given that the intronic sequence is inframe with exons 14 and 15 and there is no stop codon in the intronic region, the ITD sequence generated from DNA would be identical to the RNA sequence, and the nomenclature created from DNA sequencing would be correct.[98]

NGS methods also identify different variants harbored in subpopulations of neoplastic cells. Multiple mutations of up to seven different ITD variants have been documented,[91] indicating clonal heterogeneity within the neoplastic cell population.[98,99] More subclones may be detected in one patient when deep sequencing with better analytic sensitivity is achieved.[101] Interestingly, multiple variants of *FLT3*-ITD also appear frequently in ALAL.[46] Although the clinical significance of these findings has yet to be characterized,[101,102] these results provide a deeper understanding of clonal architecture and allow for accurate follow-up of the evolution of different clones in the disease course.[83] In addition to identifying *FLT3*-ITD using standard informatics pipelines, accurately naming *FLT3*-ITD variants using standardized HGVS nomenclature is challenging but crucial for inter-laboratory comparisons and tracking disease evolution.[98,99] To address this, we have developed a Python script-based web application that standardizes *FLT3*-ITD nomenclature using assembled sequencing reads as input.[98]

As sequencing costs decline, some clinical laboratories have explored whole-exome sequencing and whole-genome sequencing for cancer mutation profiling, including *FLT3*-ITD detection.[73] Emerging long-read sequencing technologies, such as Nanopore, show promise in efficient variant phasing, the analysis of GC-rich or repetitive regions, the characterization of genomic structural variants, and the identification of full-length transcripts and isoforms.[103] As these technologies continue to advance, they may become mainstream clinical methods in the future.[103,104]

FLT3-ITD based measurable residual disease testing

MRD, which refers to leukemia cells present below the detection threshold of conventional microscopy (morphologic remission), is critical for assessing relapse risk and guiding therapy in AML.[105] MRD detected through advanced techniques such as multiparameter flow cytometry, quantitative PCR, or NGS after treatment is strongly associated with

higher relapse rates and poorer survival,[105] highlighting its importance in guiding post-remission strategies such as allogeneic stem cell transplantation or targeted maintenance therapies. MRD monitoring has become essential to AML clinical management, providing crucial insights into treatment response, relapse risk, and long-term outcomes.[105]

The superior sensitivity of NGS in detecting low allelic burden of *FLT3*-ITD and *FLT3*-TKD mutations makes it a better approach than other methods for MRD testing in *FLT3* mutated AML patients.[96,106] Traditionally, *FLT3*-ITD mutations were considered suboptimal for MRD assessment due to their late occurrence in leukemogenesis and unstable levels during the clinical course.[107] However, recent studies have shown that detecting residual *FLT3*-ITD is a strong predictor of relapse in AML. Mutations in signaling pathway genes (*FLT3*, *KIT*, *RAS*, and others) most likely represent residual AML when detected.[105] NGS-based detection of *FLT3*-ITD MRD in complete remission identifies AML patients at high risk of relapse and poor survival. Notably, *FLT3*-ITD MRD provides independent prognostic value, outperforming established markers like *FLT3*-ITD allelic ratio, mutant *NPM1*, and multiparameter flow cytometry, highlighting its utility for dynamic risk assessment and preemptive intervention.[108] More recently, Rücker *et al*.[109] demonstrated that achieving MRD negativity after two cycles of chemotherapy significantly reduced 4-year relapse risk and improved overall survival in patients receiving intensive chemotherapy plus midostaurin. Conversely, MRD conversion from negative to positive during follow-up was associated with a high risk of relapse and death.[101,109] A positive pre- or post-transplant *FLT3*-ITD MRD was also confirmed to be associated with relapse and overall survival,[110,111] with a dose-dependent correlation in patients who received HSCT.[110] Levis *et al*.[102] studied the benefit of gilteritinib maintenance therapy post-HSCT in 356 adult patients who had *FLT3*-ITD-mutated AML and found that gilteritinib maintenance only benefited patients with detectable peri-HCT MRD, confirmed the conclusion from an earlier study. Higher levels of detectable MRD and multiple *FLT3*-ITD clones detected as MRD were associated with poorer survival. While the MRD-positive participants in the placebo group relapsed rapidly post-HCT, those in the gilteritinib group relapsed through the progression of *FLT3* wild-type clones, persistent MRD after stopping gilteritinib, or multiclonal disease progression.[102] These findings establish *FLT3*-ITD MRD as a reliable prognostic tool and support its routine use in post-therapy and post-HCT assessments to refine risk stratification and guide treatment decisions. The recent Canadian consensus on the clinical utility of *FLT3* mutation testing in acute leukemia recommended that laboratories should plan to offer this analysis.[48]

To achieve the high analytic sensitivity of *FLT3*-ITD detection by NGS, deep sequencing of amplicon-based library preparation capturing the exon 14 to 15 region of *FLT3* is used.[89,101,112,113] The target amplicon is either generated from DNA or cDNA template.[109,113,114] The primers contained gene-specific regions, sequencing adaptors, and sample-specific barcodes, allowing for generating a library in one PCR step.[102] A second run of PCR can also add the adaptors and sample barcodes.[109] In addition to sequencing depth, sufficient template input for library complexity is also important to increase the analytic sensitivity. The ELN MRD Working Party recommended using 5 mL of bone marrow aspirate from the first pull for molecular MRD assessment. If peripheral blood is used, at least 10 mL is needed, depending on the white blood cell count.[105] Based on the calculation that 100,000 cell equivalents of genomic DNA correspond to 660 ng, the NGS MRD method reported by Levis *et al*.[113] utilized a template DNA input of 700 ng to ensure sufficient sensitivity for detecting ≥1/10,000 *FLT3*-ITD–containing cells (10^{-4}, 0.01%). Deep sequencing with unique molecular identifiers may enhance hybrid capture-based NGS, enabling the analytic sensitivity required for *FLT3* MRD detection.[110] Informatics analysis pipelines should be optimized to identify low levels of *FLT3*-ITD.[89,113] The reported analytic sensitivities for *FLT3* MRD range from 0.1% to 0.001% (10^{-3} to 10^{-5}). Pooled *FLT3*-ITD positive DNA samples at different allelic ratios can serve as analytic sensitivity controls for NGS-based *FLT3*-ITD quantitation and MRD testing.[106] The amplicon-based NGS MRD for *FLT3*-ITD at analytic sensitivity <0.01%, together with MRD detection by *NPM1* mutations, including integrated proprietary bioinformatic software, are now commercially available,[102,110] opening the door for clinical diagnostic laboratories to establish these tests with a well-established protocol.

However, standardized guidelines regarding template DNA input, testing protocols, analytic sensitivity requirements, and bioinformatics optimization have yet to be developed. Future studies should prioritize multicenter collaborations and consensus discussions to address these critical gaps and establish standardized laboratory practices. Further research is warranted to evaluate the clinical utility and prognostic significance of different *FLT3* architectural variants identified through advanced NGS methods, thereby integrating molecular findings more precisely into therapeutic decision-making.

Conclusions

Accurate detection of *FLT3* mutations is critical for effective clinical decision-making in AML, particularly with the advent of targeted therapies utilizing FLT3 inhibitors. Recent advances in molecular testing, particularly NGS-based approaches, have significantly enhanced our understanding of *FLT3* mutation pathobiology, facilitated precise risk stratification, and enabled personalized treatment strategies.

Moreover, deep sequencing with high analytic sensitivity has driven substantial progress in *FLT3* mutation-based MRD testing, providing valuable guidance for post-remission management. As our understanding of *FLT3* molecular heterogeneity continues to evolve and next-generation FLT3 inhibitors are integrated into combination regimens, the clinical relevance of specific *FLT3* mutation variants may become increasingly apparent. These advancements can potentially refine precision oncology approaches, ultimately improving outcomes and prognoses for patients with *FLT3*-mutated hematologic malignancies.

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