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FLT3 mutations in acute myeloid leukemia: Therapeutic paradigm beyond inhibitor development

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Abstract

FMS-like tyrosine kinase 3 (FLT3) is a type III receptor tyrosine kinase that plays an important role in hematopoietic cell survival, proliferation and differentiation. The most clinically important point is that mutation of the *FLT3* gene is the most frequent genetic alteration and a poor prognostic factor in acute myeloid leukemia (AML) patients. There are two major types of *FLT3* mutations: internal tandem duplication mutations in the juxtamembrane domain (*FLT3*-ITD) and point mutations or deletion in the tyrosine kinase domain (*FLT3*-TKD). Both mutant FLT3 molecules are activated through ligand-independent dimerization and trans-phosphorylation. Mutant FLT3 induces the activation of multiple intracellular signaling pathways, mainly STAT5, MAPK and AKT signals, leading to cell proliferation and anti–apoptosis. Because high-dose chemotherapy and allogeneic hematopoietic stem cell transplantation cannot sufficiently improve the prognosis, clinical development of FLT3 kinase inhibitors expected. Although several FLT3 inhibitors have been developed, it takes more than 20 years from the first identification of *FLT3* mutations until FLT3 inhibitors become clinically available for AML patients with *FLT3* mutations. To date, three FLT3 inhibitors have been clinically approved as monotherapy or combination therapy with conventional chemotherapeutic agents in Japan and/or Europe and United states. However, several mechanisms of resistance to FLT3 inhibitors have already become apparent during their clinical trials. The resistance mechanisms are complex and emerging resistant clones are heterogenous. Further basic and clinical studies are required to establish the best therapeutic strategy for AML patients with *FLT3* mutations.

KEYWORDS
acute myeloid leukemia, FMS-like tyrosine kinase, inhibitor, resistance, tyrosine kinase

1 | INTRODUCTION

FMS-like tyrosine kinase 3 (FLT3) is a type III receptor tyrosine kinase (RTK) together with KIT, FMS and PDGFR.[1-3] FLT3 consists of five immunoglobulin-like domains in the extracellular region, a juxtamembrane (JM) domain, a tyrosine kinase (TK) domain separated by a kinase insert domain, and a C-terminal domain in the intracellular region. FLT3 is expressed on normal hematopoietic stem/progenitor cells, and its ligand (FL) is expressed as a membrane-bound or soluble form by bone marrow stroma cells.[4,5] FLT3 is dimerized by the binding of FL to its extracellular domain, and tyrosine residues in the activation-loop (A-loop) are

subsequently trans-phosphorylated. Activated FLT3 induces multiple intracellular signaling pathways, leading to hematopoietic cell survival, proliferation and differentiation.[6] FLT3 is also expressed on most acute leukemia cells, and FL stimulation enhances proliferation and reduces apoptosis.[7,8] In 1996, an internal tandem duplication in the JM domain-coding sequence of the *FLT3* gene (*FLT3*-ITD) was first identified in acute myeloid leukemia (AML) cells.[9] Subsequently, we found a missense point mutation at the D835 residue and point mutations, deletions and insertions in the codons surrounding D835 within a TK domain of *FLT3* (*FLT3*-TKD).[10] *FLT3*-ITD and *FLT3*-TKD occur in approximately 20 and 10% of AML, respectively.[11-13] Because *FLT3* mutation is the most frequent gene mutation in the protein-coding regions and is associated with a poor prognosis, mutant FLT3 serves as a promising molecular target for the treatment of AML.[14,15] More than 20 years after the discovery of the *FLT3* gene mutation, FLT3 inhibitors have been approved for clinical use, leading to therapeutic paradigms for AML with *FLT3* mutations (Figure 1). In this review, we summarize the clinical and biological significance of *FLT3* mutations, and discuss future therapeutic strategies involving FLT3 inhibitors.

2 | CLINICAL SIGNIFICANCE OF FLT3 MUTATIONS

To date, genetic alterations in AML have been almost completely identified by the next generation sequencing. Several comprehensive genetic studies have revealed that mutations in *FLT3*, *NPM1* and *DNMT3A* genes are frequently identified in AML patients. Although their frequencies varied slightly between analyzed cohorts, *FLT3* mutation was identified in approximately 30% of patients with AML.[13,16,17] In Japanese adult AML patients registered to the Japan Adult Leukemia Study Group (JALSG) AML201 study, *FLT3* was the most frequently (25.4%) identified mutation (Figure 2A).[12] Many clinical studies have revealed clinical characteristics of AML with *FLT3* mutations.[11,18-20] *FLT3* mutations are mainly found in myeloid neoplasms such as AML and myelodysplastic syndromes (MDS). In MDS, both *FLT3*-ITD and *FLT3*-TKD are infrequent at a level of approximately 3%, while the frequency increases in advanced-stage MDS; approximately 15% of patients with AML developing from MDS harbor *FLT3* mutations. *FLT3*-ITD is far less common in patients with acute lymphocytic leukemia (ALL), while *FLT3*-TKD is recurrently found in patients with a *KMT2A* gene rearrangement or hyperdiploidy.[21,22] *FLT3* mutations may be associated with the age of patients with AML. *FLT3*-ITD is found in approximately 25% of adult patients but in more than 30% of patients over 55 years of age.[23,24] In contrast, it is found in only approximately 10% of pediatric patients and in less than 5% of infant AML patients under 1 year of age.[25]

FLT3 mutations are associated with specific cytogenetics or other genetic mutations. *FLT3* mutations are frequently found in cytogenetically normal AML (CN-AML) but are infrequent in AML with altered karyotypes; however, AML with *DEK-NUP214* and *PML-RARA* (acute promyelocytic leukemia, APL) frequently harbors *FLT3* mutations.[11] *FLT3*-ITD is particularly associated with a variant morphology of APL and the presence of the short breakpoint cluster region 3 *PML-RARA* isoforms.[26,27] In addition, *FLT3* mutations are infrequent in core-binding factor AML (CBF-AML) consisting of AML with *RUNX1-RUNX1T1* and *CBFB-MYH11*, although *FLT3*-TKD is frequently found in AML with *CBFB-MYH11*.[28,29] *FLT3* mutations frequently overlap with *NPM1*, *DNMT3A* and *KMT2A* partial tandem duplication (*KMT2A*-PTD) mutations but are mutually exclusive with *KIT*, *K/NRAS* and *CEBPA*-double (*CEBPA*-D) mutations

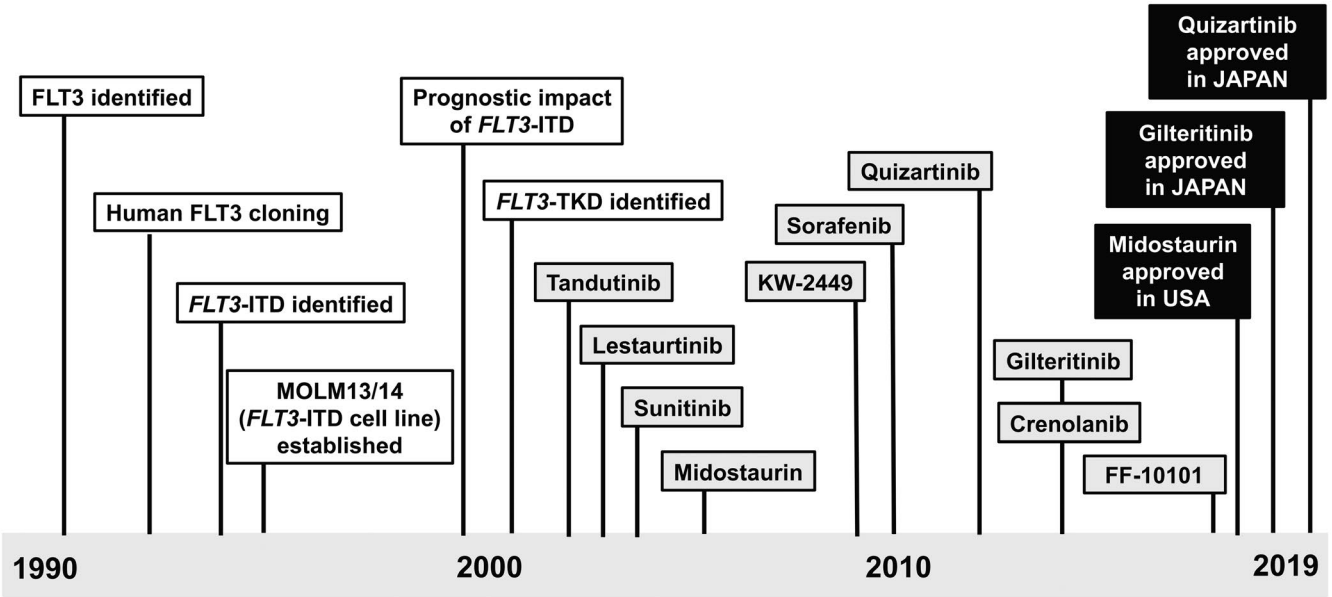

FIGURE 1 History to practical use of FLT3 inhibitors. The main historical events up to the practical use of FLT3 inhibitors are demonstrated. Indicated points of FLT3 inhibitors are the start times of clinical trials

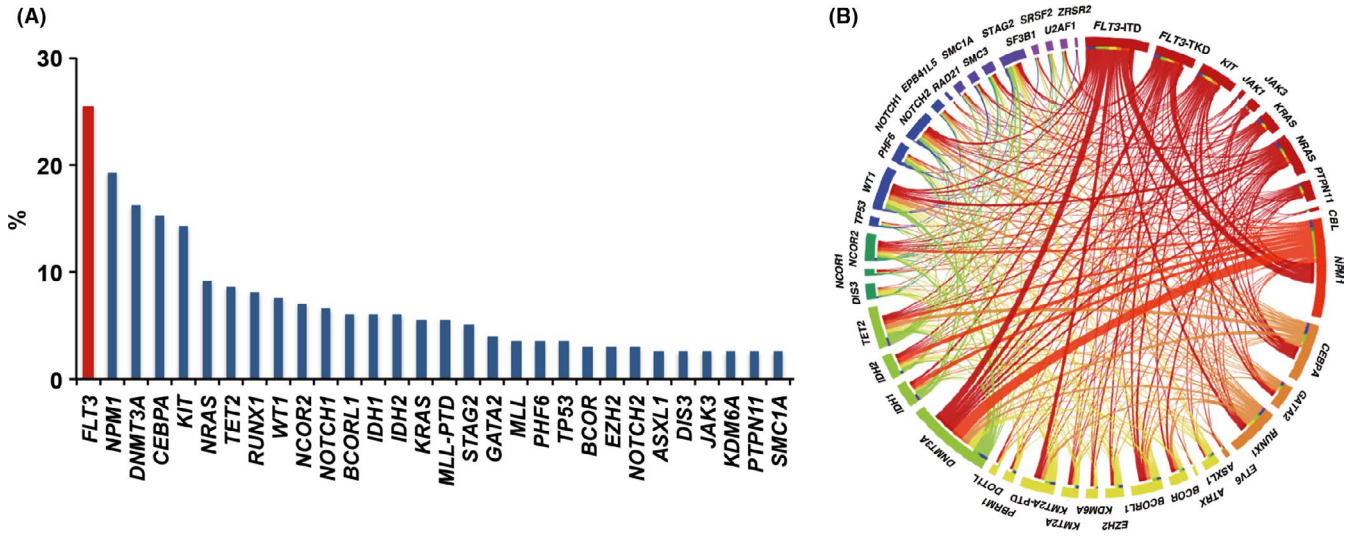

FIGURE 2 The frequency of *FLT3* mutations and co–occurring mutations in acute myeloid leukemia (AML) patients. The frequency of *FLT3* mutations and co–occurring mutations in 199 AML patients who were registered in the Japan Adult Leukemia Study Group (JALSG) AML201 study. *FLT3* mutation is the most frequently identified in AML patients (A), and frequently co–occurs with *NPM1*, *DNMT3A*, *IDH1/2*, *TET2*, *GATA2* and *KMT2A*-partial tandem duplication mutations (B). Figures are adopted from the reference 12

(Figure 2B).[12,13] The co–occurrences of *FLT3* mutations and other cytogenetic and genetic alterations reflect the concept that AML is the consequence of two broad complementation classes of mutations: those that confer a proliferative and/or survival advantage to hematopoietic progenitors including activating mutations in tyrosine kinases, such as *KIT* and *FLT3* or their downstream effectors such as *RAS*, and those that impair hematopoietic differentiation and confer properties of self-renewal, including *PML-RARA*, *RUNX1-RUNX1T1* and *CBFB-MYH11* or *NPM1* and *DNMT3A* mutations.[30-32]

FLT3 mutation is strongly associated with leukocytosis and an increased percentage of blast cells in the peripheral blood and bone marrow of AML patients.[10,33] Several large-scale studies demonstrated that *FLT3*-ITD is an independent poor prognostic factor for overall survival (OS), relapse-free survival (RFS) and event-free survival (EFS) in patients with AML excluding APL. Although the clinical impact of *FLT3*-TKD on the long-term outcome is controversial, our meta-analysis of four published studies including 1160 adult patients with AML indicated its adverse effects.[34]

Because *FLT3* mutations are closely associated with a poor prognosis in patients with AML, the WHO classification and the guidelines of the European LeukemiaNet (ELN) and National Comprehensive Cancer Network (NCCN) recommend that *FLT3* mutations should be analyzed for stratifying patients into distinct risk groups at the diagnosis of AML.[15] The ELN first recommended a risk classification system based on the cytogenetic and genetic status in 2010 (ELN-2010).[35] In this system, CN-AML patients with mutated *NPM1* and wild-type *FLT3* were categorized into a favorable risk group, and those with *FLT3*-ITD were classified into an intermediate-II group. Recently, ELN revised the risk stratification system for AML (ELN-2017), in which the allelic ratio (AR) of *FLT3*-ITD is considered for risk stratification because several groups demonstrated that high *FLT3*-ITD AR (≥0.5) (*FLT3*-ITD^{high}) is associated with a poor prognosis but not low *FLT3*-ITD AR (<0.5) (*FLT3*-ITD^{low}).[15] Therefore, the ELN-2017 system includes CN-AML patients with mutated *NPM1* and *FLT3*-ITD^{low} in addition to those with mutated *NPM1* and wild-type *FLT3*. However, we and others reported that *FLT3*-ITD^{low} has an impact on the long-term prognosis, as for *FLT3*-ITD^{high}, in Japanese AML patients.[36,37]

Although it has been reported that high-dose daunorubicin and gemtuzumab ozogamicin might reduce the adverse effect of *FLT3* mutations, a retrospective analysis showed that allogeneic hematopoietic stem cell transplantation (allo-HSCT) did not improve the poor prognosis of patients with *FLT3* mutations.[38,39] Therefore, novel treatment paradigms including FLT3 inhibitors for patients with *FLT3* mutations should be further evaluated.

3 | BIOLOGICAL EFFECTS OF FLT3 MUTATIONS ON ACUTE MYELOID LEUKEMIA

Wild-type (Wt)-FLT3 is activated through ligand-dependent dimerization and trans-phosphorylation. Activated Wt-FLT3 induces activations of multiple intracellular signaling pathways, mainly MAPK and AKT signals, leading to cell proliferation and anti–apoptosis. Mutant FLT3 ligand independently forms a dimer, resulting in constitutive activation.[40,41] Notably, mutant FLT3 activates STAT5 in addition to MAPK and AKT signals.[40,42] In vitro, it was demonstrated that the constitutively active mutant FLT3 kinase induces autonomous cell proliferation to cytokine-dependent cell lines, such as Ba/F3, FDCP1 and 32D cells.[23] Importantly, mice transplanted with mutant FLT3-transfected hematopoietic stem cells develop oligoclonal myeloproliferative disorder (MPD) but not clonal AML.[43] However, when mutant FLT3-transfected *PML-RARA*-expressing or *dnmt3a*-null hematopoietic stem cells are transplanted, the mice rapidly develop AML.[31,32] These results

collectively indicate that mutant FLT3 is sufficient to induce MPD, but additional mutations that impair hematopoietic differentiation and/or proliferation are necessary for the development of clonal AML.

4 | FIRST-GENERATION FLT3 INHIBITORS

Because *FLT3* mutation is the most frequent genetic alteration associated with a poor prognosis in AML patients, mutant FLT3 serves as a promising molecular target for the treatment of AML patients with *FLT3* mutations. At first, tyrosine kinase inhibitors (TKI), which have a potency to inhibit FLT3 kinase, were subjected to clinical trials for evaluating efficacy and safety; these TKI are called first-generation FLT3 inhibitors, including tandutinib, sunitinib, midostaurin, lestaurtinib and sorafenib (Table 1).[44-48] However, the clinical efficacy as a monotherapy of the first-generation FLT3 inhibitors for AML was unimpressive in early phase studies because of low clinical efficacy and adverse events.[14] Furthermore, these studies revealed that maintaining an effective plasma concentration of inhibitors is essential for achieving clinical efficacy.

Blood concentration is widely used as a target indicator of the pharmacological effects of drugs. However, it does not reflect the clinical efficacy of FLT3 inhibitors because most of the inhibitors are bound to plasma proteins, resulting in a decrease of free compounds that actually exert a pharmacological action. As shown in the Table 1, GI_{50} values of FLT3 inhibitors in human plasma are higher than in culture medium. In particular, GI_{50} values of lestaurtinib and midostaurin in human plasma were more than 300 times higher than those in culture medium.

The plasma inhibitory activity (PIA) assay has been established as a novel index for evaluating the clinical efficacy of FLT3 inhibitors, replacing the blood concentration, and has been incorporated into the evaluation criteria in clinical trials.[49] In the PIA assay, mutant FLT3-expressing cells are cultured with plasma obtained from a drug-administered patient, and then the phosphorylation status of the mutant FLT3 is evaluated by western blot analysis. In general, the phosphorylation statuses are compared between the culture with the plasma before drug administration and the trough plasma after administration. Previous clinical trials showed that clinical efficacies of FLT3 inhibitors were achieved when the phosphorylation of mutant FLT3 was completely suppressed even in the trough plasma by the PIA assay. The PIA assay clearly showed that the first-generation FLT3 inhibitors did not sufficiently suppress the phosphorylation of mutant FLT3 even at the target blood concentration. In particular, because lestaurtinib and midostaurin consist of an indolocarbazole molecule, which is known to be tightly bound to acid--glycoprotein (AGP) in human plasma, the PIA assay demonstrated that the plasma concentration of these compounds could not reach a biologically effective level.[49,50]

Due to the pharmacological and clinical disadvantages, the clinical studies of first-generation FLT3 inhibitors as monotherapy for AML were discontinued. Midostaurin, lestaurtinib and sorafenib were, therefore, evaluated in combination with chemotherapies for AML with FLT3 mutations. Although the addition of lestaurtinib or sorafenib to conventional chemotherapy did not show benefits in clinical trials, a randomized phase 3 study (RATIFY study) demonstrated the superiority of midostaurin in addition to the conventional induction and consolidation chemotherapies for overall survival (OS).[50-52] Based on the results of this study, midostaurin was approved as a combination agent with standard chemotherapy by the US Food and Drug Administration (FDA) in 2017. Of interest

TABLE 1 FLT3 inhibitors in clinical development

	Tandutinib	Lestaurtinib	Sorafenib	Midostaurin	Quizartinib	Gilteritinib	Crenolanib	FF-10101
FLT3 kinase inhibition IC_{50} (nmol/L)	220	3	58	6.3	1.6	0.29	1.3	0.2
Plasma/Medium ratios of GI_{50} values in *FLT3*-ITD-expressing-cells	Unknown	350	88.3	300	18	22.8	20.5	26.2
Growth inhibition to Wt-*FLT3* and ITD-*FLT3*-co–expressing-32D GI_{50} (nmol/L)								
FL (0 ng/mL)	Unknown	9.7	5.3	20.3	1.4	8.9	23.4	1.4
FL (10 ng/mL)	Unknown	9.2	16.0	22.7	6.5	36.4	43.5	7.5
Inhibitory type	Type II	Type I	Type II	Type I	Type II	Type I	Type I	Type I (Covalent)
Other targets	PDGFR KIT	TRKA PKC KDR PDGFR	RAF VEGFR PDGFR KIT	PKC KDR KIT PDGFR	KIT	AXL LTK ALK	KIT PDGFR RET	FMS
Development stage for AML	Withdrawn	Phase 2	Phase 3	Approved as combination with chemo for AML	Approved as single agent for R/R AML	Approved as single agent for R/R AML	Phase 3	Phase 1/2a

is the fact that sorafenib maintenance therapy significantly improved RFS and OS after allo-HSCT in AML patients with *FLT3*-ITD in CR (SORMAIN study).[53] Notably, this result is supported by a report that sorafenib may induce graft versus host disease (GVHD) as a result of increased IL-15 and donor cytotoxic T-cell activity.[54]

5 | SECOND-GENERATION FLT3 INHIBITORS

Because first-generation FLT3 inhibitors were not originally screened for sensitivity and selectivity against activated FLT3 kinase, the development of second-generation FLT3 inhibitors, which have more selective and potent inhibitory activities, continues (Table 1).

Gilteritinib is a highly selective and potent FLT3 inhibitor, and it also shows inhibitory activity against the receptor tyrosine kinase AXL.[55,56] AXL was shown to be upregulated and activated in malignant cells, including AML, and its high expression was reported to be associated with a poor prognosis.[57] Furthermore, it was reported that FLT3 inhibitors induce AXL activation, reducing their growth inhibitory effects in vitro.[58] In a phase 1/2 trial of 252 patients with relapsed or refractory (R/R) AML over 18 years of age, safety and efficacy were examined at doses from 20 to 450 mg/day.[59] The PIA assay revealed that >90% suppression of FLT3 phosphorylation was achieved at >80mg/day. In an interim analysis of a phase 3 trial for R/R AML with FLT3 mutations (ADMIRAL study), gilteritinib showed superior complete remission (CR) and CR with partial recovery of blood cells (CRh) than conventional chemotherapy. Furthermore, the subsequent analysis for the primary endpoint showed that the median OS of gilteritinib-treated patients (9.3 months) was significantly longer than that of conventional chemotherapy-treated patients (5.6 months) ($P < .001$).[60] Gilteritinib was approved for R/R AML patients with FLT3 mutations as the first single agent FLT3 inhibitor in Japan in 2018.

Quizartinib was screened for selectivity and affinity against FLT3 kinase using the KinomeScan technique.[61] Quizartinib shows high selectivity for and strong inhibitory activity against FLT3-ITD but not FLT3-TKD.[62] In phase 1 and 2 clinical trials involving R/R AML patients, quizartinib showed a >50% response rate for *FLT3*-ITD-positive AML patients; however, many CR with incomplete hematologic recovery (CRi) cases were observed in the patients who responded, and QTc prolongation was demonstrated to have a marked toxicity.[63,64] It was suggested that poor blood cell recovery after treatment with quizartinib is caused by its inhibitory activity against KIT.[65] However, in a phase 3 trial for R/R *FLT3*-ITD-positive AML (QuANTUM-R study), in which an amendment of the administration dose reduced the frequency of QTc prolongation, the median OS of quizartinib-treated patients (6.2 months) was significantly longer than that of conventional chemotherapy-treated patients (4.7 months) ($P = .02$).[66] Quizartinib was approved for R/R AML patients with *FLT3*-ITD in Japan in 2019.

Crenolanib has inhibitory activity both against FLT3-ITD and FLT3-TKD. It also has inhibitory activity against PDGFR.[67,68] A single agent phase 2 trial involving *FLT3* mutation-positive R/R AML patients showed a response rate of 47%, but digestive toxicity was revealed.[69] A phase 3 study of crenolanib versus midostaurin administered following induction chemotherapy and consolidation therapy in newly diagnosed AML patients with *FLT3* mutation is currently ongoing.

6 | PRIMARY RESISTANCE MECHANISMS OF FLT3 INHIBITORS

Although three FLT3 inhibitors have been approved for clinical use in Japan and/or Europe and United states, several resistance mechanisms of FLT3 inhibitors have become apparent in clinical studies. These resistance mechanisms are classified into primary and secondary resistance mechanisms. Primary resistance includes different inhibitory activity against types of *FLT3* mutations (*FLT3*-ITD and *FLT3*-TKD), FL-dependent impedance, FGF2-promoting resistance, bone marrow stromal CYP3A-mediated resistance and other activating signals (Figure 3).[70-74]

6.1 | Type I and type II inhibitors

Different inhibitory activities between FLT3-ITD and FLT3-TKD are caused by the binding affinity of FLT3 inhibitors. FLT3 has an active or inactive conformation according to the orientation of three amino acid residues, Asp-Phe-Gly, in the activation loop (A-loop). FLT3 inhibitors can be divided into two major types (type I and type II) according to the mode of binding to FLT3. The type I inhibitor binds to both active and inactive conformations of FLT3, but the type II inhibitor can bind to only an inactive conformation (Figure 4).[75] Midostaurin, gilteritinib and crenolanib are type I inhibitors, and are designed to have a high affinity for the ATP-binding region of the active conformation of FLT3. Because the protein structure of the ATP-binding region is similar between kinases, many type I inhibitors show potency against multiple kinases. Quizartinib and sorafenib are type II inhibitors. They fit into the back pocket of the ATP-binding region maintained in the inactive conformation and interact with the side chains of related amino acid residues, improving inhibitory activity and selectivity. However, they lose binding affinity to an active conformation of FLT3 because of the use of the back pocket in addition to the ATP-binding region for the interaction with FLT3.

6.2 | FL-dependent resistance

FL-dependent impedance of an FLT3 inhibitor is a clinically important resistance mechanism. It was reported that an increased plasma FL concentration after chemotherapy impeded the efficacies of FLT3 inhibitors.[50,71] We focused on evidence that most FLT3-mutated AML cells co–express Wt-FLT3, and demonstrated that FL-dependent Wt-FLT3 activation reduced inhibitory effects of FLT3 inhibitors.[76] We evaluated the growth-inhibitory effects

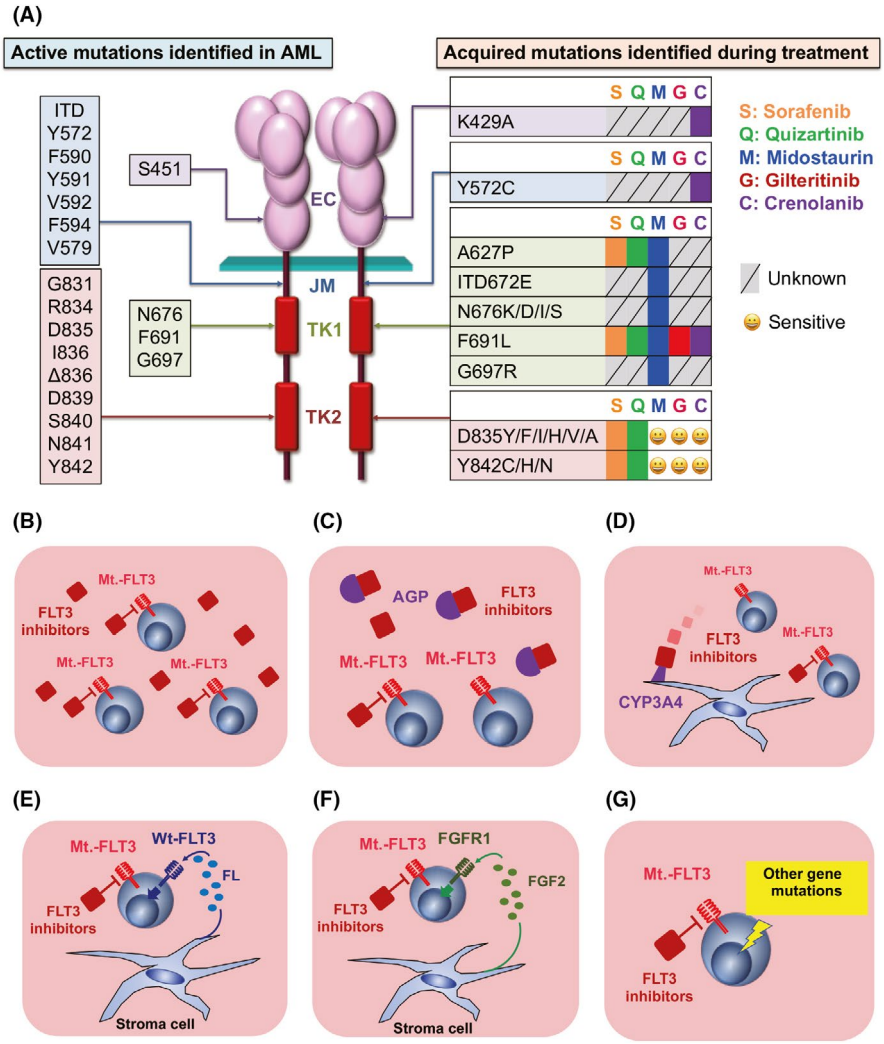

FIGURE 3 Resistant mechanism of FLT3 inhibitors. A, On–target resistance of FLT3 inhibitors. To date, several activating *FLT3* mutations have been identified in acute myeloid leukemia (AML) cells. During the treatment with FLT3 inhibitors, additional mutations in the *FLT3* gene are acquired. Although the potencies of FLT3 inhibitors against each acquired mutation are different, those against the gatekeeper mutation, F691L, are low. B, In the culture medium, most FLT3 inhibitors exist as free-forms, and inhibit the proliferation of mutant FLT3-expressing cells. C, Binding to plasma proteins, such as AGP, reduce the free-inhibitor concentration in blood. The bone marrow microenvironment is associated with a primary resistant mechanism. D, FL reduces the inhibitory activity of FLT3 inhibitors through the activation of Wt-FLT3. E, FGF2 reduces the inhibitory activity of FLT3 inhibitors through the activation of FGFR1. F, CYP3A4 expressed in bone marrow stromal cells metabolizes FLT3 inhibitors. G, Other gene mutations, particularly RAS/MAPK pathway gene mutations, confer resistance during treatment with FLT3 inhibitors. AGP, acid-α-glycoprotein; BM, bone marrow; EC, extra-cellular; FL, FLT3 ligand; JM, juxtamembrane; PB, peripheral blood; TK, tyrosine kinase

of FLT3 inhibitors in the presence or absence of exogenous FL using sole FLT3-ITD-FLT3-expressing and Wt-FLT3 and ITD-FLT3 co–expressing 32D cells. FL stimulation significantly increased GI_{50} values of all FLT3 inhibitors in Wt-FLT3 and ITD-FLT3 co–expressing cells compared to sole ITD-FLT3 cells. In sole ITD-FLT3 cells, quizartinib inhibited the phosphorylation of ITD-FLT3 and its downstream molecules, STAT5, AKT and MAPK. Although ITD-FLT3 was re–phosphorylated by the addition of FL, downstream molecules were not affected by FL. In contrast, FL activated Wt-FLT3, but not ITD-FLT3, in Wt-FLT3 and ITD-FLT3 co–expressing cells in the presence of quizartinib. In parallel with the activation of Wt-FLT3, AKT and MAPK, but not STAT5, were re–phosphorylated by FL. These results indicate that FL activated Wt-FLT3, but not ITD-FLT3, in the co–expressing cells. In kinase-dead (KD) FLT3 (K644A) and ITD-FLT3 co–expressing cells, FL did not activate MAPK nor affect the growth-inhibitory effect of FLT3 inhibitors. These results indicate that FL activated Wt-FLT3, but not ITD-FLT3, in the co–expressing cells. Furthermore, the addition of a MEK inhibitor blocked the reduced effect of FL on the growth inhibitory effect of FLT3 inhibitors, indicating that FL-dependent activation of the Wt-FLT3-MAPK axis is a key mechanism in FL-induced resistance of FLT3 inhibitors. Interestingly, the addition of FL more strongly reduces inhibitory activities of gilteritinib, quizartinib, and sorafenib on Wt- and ITD-FLT3 co–expressing cells than those of midostaurin and lestaurtinib (Table 1). Although midostaurin and lestaurtinib have similar inhibitory activities

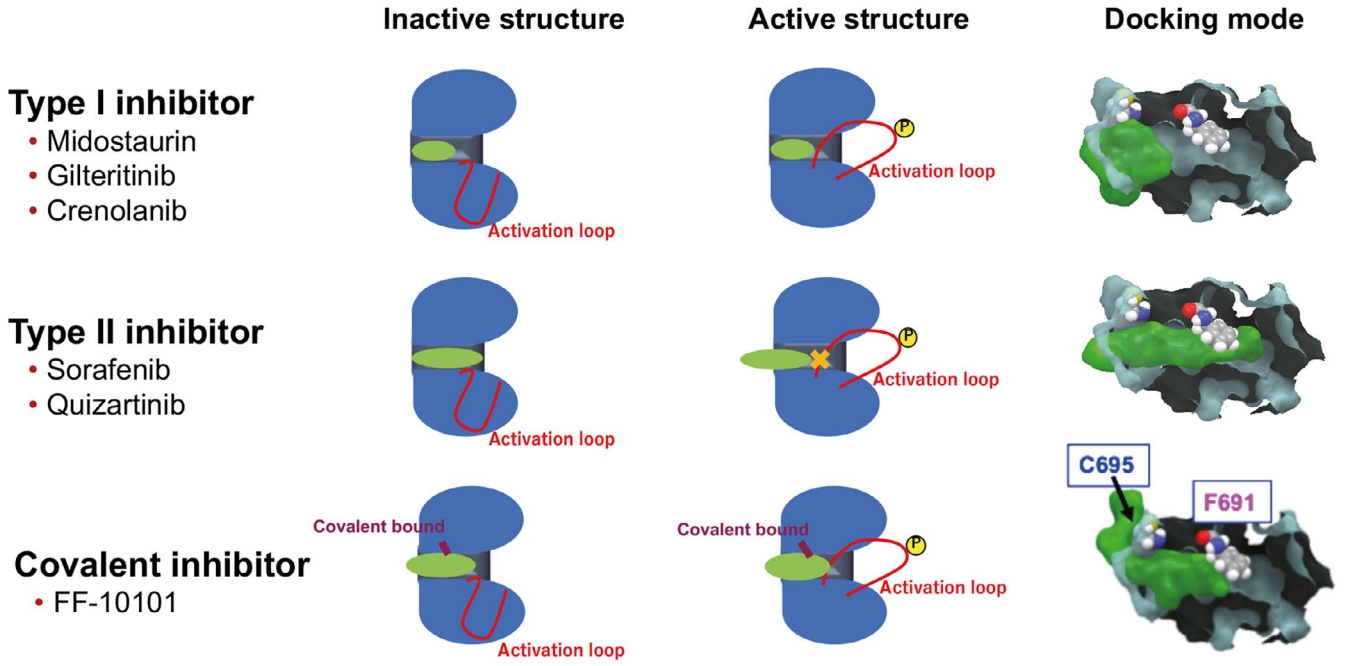

FIGURE 4 Binding modes of FLT3 inhibitors to FLT3 molecule. Schematic diagrams of FLT3 kinase inhibition are shown. FLT3 inhibitors are classified into Type I and Type II inhibitors according to the mode of binding to FLT3. The Type I inhibitors bind to only the ATP-binding site, enabling binding to both active and inactive conformations of FLT3. In contrast, because the type II inhibitors are designed to favorably bind to the back pocket of the inactive conformation of FLT3 to increase the affinity for the ATP-binding site, they can bind to only the inactive conformation. FF-10101 is designed to form a covalent binding between the C695 residue of FLT3. This covalent bond formation of FF-10101 maintains the ability to bind to both the active and inactive conformations of FLT3

against both ITD-FLT3 and Wt-FLT3, gilteritinib, quizartinib and sorafenib show a lower potency against Wt-FLT3 than ITD-FLT3. These results indicate that different potencies against ITD-FLT3 and Wt-FLT3 are associated with the FL-dependent impendence of growth-inhibitory effects on Wt-FLT3 and ITD-FLT3 co–expressing cells.

6.3 | Another primary resistance mechanisms

In addition to FL, other cytokines, growth factors and soluble proteins from the bone marrow microenvironment were evaluated for their resistance effects against quizartinib. Among them, fibroblast growth factor 2 (FGF2) promoted resistance through activation of FGFR1 and downstream MAPK effectors. Notably, FGF2 expression in marrow stromal cells was increased in *FLT3*-ITD-positive AML patients treated with quizartinib, and the expression level peaked prior to overt clinical relapse and the detection of resistant mutations (Figure 3).[72] Furthermore, expression of CYP3A4 in bone marrow stromal cells was reported to inhibit the activity of three different FLT3 inhibitors (sorafenib, quizartinib and gilteritinib) against *FLT3*-ITD positive AML (Figure 3).[73] It is well known that CYP3A4 inactivates many chemotherapeutic agents for TKI. In particular, hepatic CYP3A4 has been shown to inactivate essentially all TKI, including FLT3 inhibitors. These results collectively suggested that AML cells in BM might escape inhibitor exposure at an effective concentration through a bone marrow microenvironment-mediated mechanism, resulting in the persistence of AML cells, which will acquire resistance.

7 | SECONDARY RESISTANCE MECHANISMS OF FLT3 INHIBITORS

The accumulation of resistant cases in clinical trials of FLT3 inhibitors has revealed their secondary resistance mechanisms. Secondary resistance mechanisms against FLT3 inhibitors were classified into on-target and off-target mechanisms, in which leukemia cells become dependent on other signaling pathways.

7.1 | On-target resistance

In on-target resistance, leukemia cells retain dependency on FLT3 signals but show resistance to FLT3 inhibitors by acquiring mutations in the FLT3 gene (Figure 3). Secondary resistance mutations were first identified in patients with *FLT3*-ITD who relapsed following quizartinib treatment.[74] In those patients, secondary mutations at the D835 residue, Y842 residue, or the gatekeeper residue F691 in the kinase domain were identified, and *FLT3*-ITD + D835Y, +D835V, +Y842C, +Y842H,or +F691L-expressing Ba/F3 cells showed resistance to growth-inhibitory and dephosphorylation activities of quizartinib. These resistance mutations in the A-loop were also identified in patients who were treated with the other

type II inhibitor sorafenib. Such acquired resistance reflects the lower binding affinity of type II inhibitors to the activated form of FLT3, which is mediated by TK domain mutations. Notably, the variant allele frequency (VAF) of the acquired mutation on the *FLT3*-ITD allele was less than 50% in patients who were clinically resistant to the type II inhibitor quizartinib, suggesting the existence of another resistance mechanism. Subsequent analysis demonstrated that 4 of the 8 quizartinib-resistant patients harbored more than one resistance mutation in the TK domain.[77] Furthermore, 7 of the 8 resistant patients harbored the TK domain mutation in the native *FLT3* allele in addition to the *FLT3*-ITD allele. Importantly, there were patients in whom the mutation type and mutation frequency were different between the native and ITD *FLT3* alleles. In this study, it was also identified that AML cells from 1 quizartinib-resistant patient acquired no resistance mutation either in native or ITD *FLT3* alleles. Although no gene mutation other than *FLT3* was identified, an off-target resistance mechanism might be involved in this patient. These findings collectively indicate that polyclonal mechanisms occurred in most AML patients who relapsed following quizartinib treatment.

7.2 | Off-target resistance

Because quizartinib and sorafenib are type II inhibitors, it is understandable that resistant clones acquired mutations in the TK domain of the *FLT3* gene. In contrast, resistant clones after treatment with the type I inhibitors gilteritinib and crenolanib, which have a potency against FLT3-TKD, showed different resistance features from those after type II inhibitor treatment. Comparable genetic analysis between baseline and progression samples from patients treated with gilteritinib demonstrated several distinct patterns of clonal selection during the gilteritinib treatment.[78] In 5 of the 41 gilteritinib-resistant patients (12.2%), FLT3 mutations were not identified in AML cells after gilteritinib treatment; however, RAS/MAPK pathway mutations were identified in all of them, indicating that FLT3 mutation-negative clones newly acquired RAS/MAPK pathway mutations and expanded as a resistant clone. In the other 36 patients, resistant clones persisted in the original FLT3 mutations. In 5 of them, FLT3-F691L mutation was additionally acquired in resistant cells harboring the original FLT3 mutation. Although FLT3-M837K and FLT3-C35S mutations were identified in one resistant patient each, both mutations were confirmed to be silent because mutant FLT3 cannot induce autonomous proliferation to Ba/F3 cells. In contrast to type II inhibitors such as quizartinib and sorafenib, the low frequency of additional *FLT3* mutation might reflect the potency of gilteritinib against FLT3-TKD. However, even if the additional *FLT3* mutations identified after treatment did not have transforming activity, we should consider that resistance mutation in the *FLT3* gene would be acquired during the gilteritinib treatment. Of note is the fact that RAS/MAPK pathway mutations including *NRAS*, *KRAS*, *PTPN11*, *CBL* and *BRAF* mutations were acquired in 10 of the 36 patients whose leukemia cells showed persistent *FLT3* mutations (Table 2). Importantly, the *FLT3*-F691L mutation and RAS/MAPK pathway mutations were mutually exclusive. These results collectively indicate that the off-target resistance mechanism is more common with gilteritinib than type II inhibitors.

TABLE 2 Off–target gene mutations identified in patients during treatments with gilteritinib and crenolanib

	Gilteritinib	Crenolanib
RAS/MAPK pathway genes	*NRAS*, *KRAS*, *PTPN11* *CBL*, *BRAF*	*NRAS*, *KRAS*, *PTPN11*
Epigenetic-modifying genes	*IDH2*	*IDH1*, *IDH2* *TET2*, *DNMT3A* *ASXL1*, *BCOR*
Myeloid transcription factor genes	*CEBPA* *RUNX1*	*CEBPA* *RUNX1*
Spliceosome-complex genes		*U2AF1* *SF3B1*
Cohesion complex genes		*STAG2*
Others	*WT1* *TBL1XR1* *BCR-ABL*	

Characteristics of resistant clones after treatment with another type I inhibitor crenolanib were also different from those after type II inhibitors.[79] In crenolanib-treated patients, acquired mutations on the *FLT3* gene were infrequent in residual AML cells after treatment. In 50 resistant patients treated with crenolanib, 5 FLT3 mutations (D200N, K429F, Y572C, L601F and F691L) were identified after treatment in 6 individuals, while D200N and L601F mutations did not lead to transforming activity. In contrast, several gene mutations, which are classified into epigenetic regulators, myeloid transcription factors and the cohesin complex, were identified in crenolanib poor responders. Notably, *NRAS*, *STAG2*, *CEBPA*, *ASXL1* and *IDH2* mutations arose mostly in *FLT3* mutation-independent clones, indicating that these clones escaped and expanded during crenolanib treatment. However, *TET2*, *IDH1* and *TP53* mutations predominantly co–occur in *FLT3*-mutated clones during crenolanib treatment (Table 2).

8 | COVALENT-BINDING TYPE FLT3 INHIBITOR

Previous studies collectively suggested that on–target resistance frequently occurs in patients after treatment with type II inhibitors, but off–target resistance is frequent in those after treatment with type I inhibitors. Because these studies were mainly conducted using the samples from patients who were treated in clinical trials, the accumulation of resistance features derived from patients treated in daily clinical practice is necessary to fully understand the resistance mechanism of each FLT3 inhibitor. However, the further development of novel FLT3 inhibitors is required to overcome the mutation of the gatekeeper residue (F691) in the *FLT3* gene. Many

type II inhibitors are designed to favorably bind to the back pocket of the inactive conformation of FLT3 to increase affinity to the ATP-binding site. In contrast, because many type I inhibitors are designed to bind to only the ATP-binding site, they can bind to the active conformation of FLT3; however, they have more broad-kinase inhibitory activities than type II inhibitors, due to the similarity of binding sites among kinases. To resolve these problems, we developed a novel FLT3 inhibitor, FF-10101, in collaboration with FUJIFILM (Kanagawa, Japan), which was designed to form a covalent binding between the C695 residue of FLT3 (Figure 4).yyyy[80] This covalent bond formation of FF-10101 induces irreversible inhibition of FLT3 and potent and selective inhibitory activity, and maintains the binding ability both to the active and inactive conformations of FLT3. The unique binding of FF-10101 also provides broad and potent inhibitory effects on various FLT3 mutations, including the gatekeeper mutation F691L. We are now conducting a phase 1/2a dose escalation and dose ranging study of FF-10101 in subjects with relapsed or refractory acute myeloid leukemia to determine the safety, tolerability, PK and preliminary efficacy (NCT03194685).

9 | CONCLUSION

It takes more than 20 years after the first identification of *FLT3* mutations until FLT3 inhibitors become clinically available for AML patients with *FLT3* mutations. Although many issues regarding the clinical use of FLT3 inhibitors have been resolved during the long development period, further efforts are required to establish the most suitable therapeutic strategy for each inhibitor and for each patient. Because AML is a genetically heterogenous disease and *FLT3* mutation is a late event during leukemogenesis, mono-therapy of FLT3 inhibitor has limitations for curing AML patients with *FLT3* mutations. Although the RATIFY study demonstrated that the combination of chemotherapy and midostaurin improved the prognosis of AML patients with *FLT3* mutations, many patients underwent allo-HSCT during the treatment. In addition, the extent to which combination therapies of other FLT3 inhibitors with chemotherapy or other targeting agents improves the prognosis remains unclear. Further basic and clinical studies are required to establish biomarkers for selecting the best therapeutic strategy according to the characteristics of AML cells and patients' conditions.

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CONFLICT OF INTEREST

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