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Review

Mechanisms Underlying Resistance to FLT3 Inhibitors in Acute Myeloid Leukemia

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Received: 11 June 2020; Accepted: 16 July 2020; Published: 24 July 2020

Abstract: FLT3-ITD and FLT3-TKD mutations were observed in approximately 20 and 10% of acute myeloid leukemia (AML) cases, respectively. FLT3 inhibitors such as midostaurin, gilteritinib and quizartinib show excellent response rates in patients with FLT3-mutated AML, but its duration of response may not be sufficient yet. The majority of cases gain secondary resistance either by on-target and off-target abnormalities. On-target mutations (i.e., FLT3-TKD) such as D835Y keep the TK domain in its active form, abrogating pharmacodynamics of type II FLT3 inhibitors (e.g., midostaurin and quizartinib). Second generation type I inhibitors such as gilteritinib are consistently active against FLT3-TKD as well as FLT3-ITD. However, a "gatekeeper" mutation F691L shows universal resistance to all currently available FLT3 inhibitors. Off-target abnormalities are consisted with a variety of somatic mutations such as *NRAS*, *AXL* and *PIM1* that bypass or reinforce FLT3 signaling. Off-target mutations can occur just in the primary FLT3-mutated clone or be gained by the evolution of other clones. A small number of cases show primary resistance by an FL-dependent, FGF2-dependent, and stromal CYP3A4-mediated manner. To overcome these mechanisms, the development of novel agents such as covalently-coupling FLT3 inhibitor FF-10101 and the investigation of combination therapy with different class agents are now ongoing. Along with novel agents, gene sequencing may improve clinical approaches by detecting additional targetable mutations and determining individual patterns of clonal evolution.

Keywords: acute myeloid leukemia (AML); FMS-like tyrosine kinase 3 (FLT3); quizartinib; gilteritinib

1. Introduction

FMS-like tyrosine kinase 3 (FLT3) is classified as a type 3 receptor tyrosine kinase, along with KIT, FMS, and PDGFR [1–3]. FLT3 is composed of an extracellular region consisting of five immunoglobulin-like domains, and an intracellular region consisting of a juxtamembrane (JM) domain, two tyrosine kinase (TK) domains, and a C-terminal domain. FLT3 is expressed in normal hematopoietic stem cells and progenitor cells, and is dimerized upon binding with either membrane-bound or soluble FLT3 ligands (FLs) produced by bone marrow stromal cells, which subsequently causes the phosphorylation and activation of tyrosine residues in the activation-loop (A-loop) [4,5]. Phosphorylated FLT3 activates multiple intracellular signaling pathways involved in the survival, proliferation, and differentiation of hematopoietic stem cells, such as RAS/MAPK, PI3K/Akt/mTOR, and JAK/STAT5 [6–9]. Since FLT3 is frequently expressed in leukemic cells, FL stimulation induces proliferation and inhibits apoptosis in these cells [10,11]. In 1996, an internal tandem duplication in the JM domain-encoding region of *FLT3* (FLT3-ITD) was identified in acute myeloid leukemia (AML) cells [12]. Thereafter, several types of mutations, including point mutations, deletions, and insertions

have been detected around the D835 residue in the TK domain (FLT3-TKD) [13]. FLT3-ITD and FLT3-TKD mutations were observed in approximately 20 and 10% of AML cases, respectively [14–16]. Although both FLT3-ITD and FLT3-TKD are gain-of-function mutations, the upregulation of STAT5 was only observed in FLT3-ITD cell lines (32D/ITD) [17]. STAT5 positively regulated Pim-1, which eventually activated mTOR and Mcl-1, which consequently conferred resistance to Akt inhibition in FLT-ITD cell lines [18]. An experiment using transgenic mice with FLT3-ITD-positive hematopoietic stem cells revealed the clear promoting effects of nuclear factors in activated T-cells (NFATC1), a family of inflammatory transcriptional factors, on FLT3-ITD-driven precursor cell expansion and resistance to FLT3 inhibitors [19]. Recent studies suggest that circulating MYBL2, encoded by the cell-cycle checkpoint gene *MYBL2*, is detected in AML patients with FLT3-ITD mutations and is closely related to mutant FLT3 expression as well as to tumor cell activity [20]. Unlike FLT3-ITD consistently upregulating JAK/STAT signaling, FLT3-TKD enhance SHP1 and SHP2 activity that negatively regulate JAK signaling [21,22]. This may at least partially explain why FLT3-ITD showed more potent myeloproliferative advantages than those of FLT3-TKD in a mouse model [23,24]. The dual mutation of FLT3-ITD and -TKD (FLT3-ITD-TKD) has been found in a small population. A recent study showed that FLT3-ITD-TKD has the ability to activate STAT5, resulting in Bcl-x and RAD51 upregulation that accounts for drug resistance [25]. Since FLT3 mutations are frequently detected in AML and are associated with poor prognosis, this gene is considered a promising molecular target for AML [26,27]. It has been 20 years since abnormalities in the *FLT3* were first discovered, and the application of FLT3 inhibitors in clinical settings in Japan, Europe, and the United States has resulted in a paradigm shift in the treatment of FLT3-mutated AML. However, resistance to FLT3 inhibitors has also been reported concomitantly. Mechanisms of the resistance and strategies to overcome it have been vigorously studied and ever-reviewed [28–30]. Along with the comprehensive understanding of pathologic FLT3 signaling and the acquired alterations responsible for drug-resistance, non-FLT3 abnormalities that may be closely associated with leukemic clone evolution are revealing its importance, suggesting new approaches. In this review, we summarize our current understanding of resistance to FLT3 inhibitors and discuss the strategies for overcoming this issue.

2. Prognostic Impact of FLT3 Mutations

FLT3-ITD mutation has been recognized as one of the major adverse prognostic factors with nearly twice the increase in hazard ratio [31]. As mentioned in the European LeukemiaNet (ELN) recommendations [27], high allelic burden (generally indicating 50% or more) of FLT3-ITD (FLT3-ITD^{high}) is consistently associated with worse prognosis [32–34]. On the other hand, the low allelic frequency of FLT3-ITD (FLT3-ITD^{low}) concomitant with NPM1 mutation possibly leads to favorable prognosis [35], though it has been fraught with controversy [36–38]. FLT3-ITD^{high} with wild type NPM1 and FLT3-ITD^{low} with mutated NPM1 are classified as intermediate-risk [27]. Unlike FLT3-ITD, the prognostic significance of FLT3-TKD has not been determined [32,39]. With the development of potent FLT3 inhibitors, better clinical outcomes would be expected, especially in patients with FLT3-ITD^{high}. Indeed, previously untreated FLT3-ITD^{high} patients who received intensive chemotherapy with sorafenib, a FLT3 inhibitor, showed no significant but seemingly better relapse-free and overall survival than those with FLT3-ITD^{low} AML [34]. It is not fully known if the FLT3 allelic burden affects the properties in acquiring resistance to FLT3 inhibitors. However, given a certain somatic mutation will belong to a single clone, a larger proportion of mutant FLT3 allele may link to less divergent leukemic clones and vice versa, which theoretically affect drug sensitivity, relapse rates and eventually survival rates. Zhang and his colleagues graphically displayed the clonal evolutions of two individual cases; one for a single clone with a high frequency of FLT3-TKD that later relapsed with an additional mutation within the same clone and the other for complex clones not associated with first-detected FLT3-ITD mutation with low frequency [40]. The prognostic impact of FLT3 mutations and its allele frequency possibly be changed in the era of FLT3 inhibitors.

3. Classification of FLT3 Inhibitors by Its Pharmacodynamics

As first-generation FLT3 inhibitors, existing TK inhibitors such as tandutinib (CT53518), lestaurtinib (CEP-701), sunitinib (SU11248), midostaurin (PKC412), and sorafenib (BAY 43-9006), which can effectively inhibit FLT3 kinase have been studied [41–45]. Thereafter, the compounds with higher selectivity and inhibitory activity were identified. Gilteritinib (ASP2215), quizartinib (AC220), and crenolanib (CP868596) were developed as second-generation FLT3 inhibitors [46–50]. These FLT3 inhibitors are roughly classified into two types (i.e., type I and type II) based on their binding mode to FLT3 molecules. The conformation of the three amino acid residues Asp–Phe–Gly (DFG) in the A-loop of the FLT3 molecule is altered in accordance with the phosphorylation status of the tyrosine residue, which leads to the formation of an active DFG-in conformation or an inactive DFG-out conformation [51–53]. Type I inhibitors bind to the ATP-binding site and its vicinity, and subsequently bind with molecules in both DFG-in and DFG-out conformations. Since the molecular homology of various TKs is high and the ATP-binding sites are highly conserved among kinases, type I inhibitors are often less selective. In contrast, type II inhibitors bind to the target kinase by utilizing the hydrophobic space that appears in the proximity of the ATP-binding site in the DFG-out conformation. Since the hydrophobic space in this structure varies significantly between various kinases, type II inhibitors are expected to be more selective than type I inhibitors and are unable to inhibit activated kinases in the DFG-in conformation. Midostaurin, gilteritinib, and crenolanib are type I inhibitors, while quizartinib and sorafenib are type II inhibitors [54]. FLT3-TKD maintains a constant DFG-in conformation owing to alterations in the TK domain, whereas FLT3-ITD can exist in both active DFG-in conformation and inactive DFG-out conformation. Therefore, while type I inhibitors inhibit both FLT3-TKD and FLT3-ITD, type II inhibitors only inhibit FLT-ITD owing to the differences in binding properties, with a few exceptions in first-generation agents (e.g., midosutaurin and sunitinib). For example, TK domain-altering D835 point mutations confer resistance to a type II second-generation inhibitor quizartinib, but not to type I gilteritinib and crenolanib [55]. However, a "gatekeeper" mutation F691L shows universal resistance to all the currently available FLT3 inhibitors [47,49,56–59]. The characteristics of the FLT3 inhibitors are summarized in Table 1.

Table 1. FLT3 inhibitors.

Agent		Generation	Type	Selectivity	IC50 (nM)	Drug Sensitivity			
						ITD	D835Y	ITD-D835Y	F691L
Midostaurin	(PKC412)	First	I	Low	139	S	S	R	R
Sunitinib	(SU11248)	First	I	Low	250	S	R	R	R
Lestaurtinib	(CEP701)	First	I	Low	5	S	Int	S	–
Gilteritinib	(ASP2215)	Second	I	Moderate	1.6	S	S	Int	R
Crenolanib	(CP868596)	Second	I	Moderate	2	S	S	Int	R
Sorafenib	(BAY43-9006)	First	II	Moderate	58	S	R	R	R
Tandutinib	(CT53518)	First	II	High	100	S	R	–	–
Quizartinib	(AC220)	Second	II	High	<1.0	S	R	R	R

S (sensitive) means the IC50 is less than or equal to that of FLT3-ITD. R (resistant) means more than two folds increase in IC50. Int (intermediate) remains a 1.0–2.0-folds increase. Here is the reference of selectivity [60–62], IC50 for FLT3-ITD [47,49,63–68] and drug sensitivity [47,55,69–72].

4. Current Clinical Role of FLT3 Inhibitors

Among a number of tyrosine kinase inhibitors active against pathologic FLT3 signaling, gilteritinib and midostaurin are now available for the treatment of FLT3-mutated AML in most developed countries. Quizartinib is currently available only in Japan. Stone and his colleagues reported a randomized phase 3 trail, RATIFY, where midostaurin or placebo were added to standard therapy in patients with newly diagnosed FLT3-mutated AML [73]. The midosutaurin group showed longer survival (hazards ration (HR) 0.78) and improved event-free interval (HR 0.78) than the counterpart. Recently, the combination of midostaurin and standard therapy followed by midostaurin maintenance also showed better outcomes compared with historical controls (HR 0.58 in event-free survival) [74]. Efficacy

of single-agent gilteritinib for relapsed/refractory FLT3-mutated AML was proved in a randomized phase 3 trial, ADMIRAL [75]. The median overall survival was significantly longer in the gilteritinib group than the conventional chemotherapy group (9.3 months vs. 5.6 months), with a higher percentage of patients who underwent allo-stem cell transplantation (SCT) (26% vs. 15%). However, the median event-free interval was less than 3 months. Similarly, the phase 3 QuANTUM-R trial showed the superiority of single-agent quizartinib over salvage chemotherapy in the same situation (HR 0.76 in overall survival) [76]. Quizartinib has also been tested in the first-line setting and showed activity in a phase 1 trial [77]. In addition to the approved drugs mentioned above, other FLT3 inhibitors also have displayed clinical benefits. Published trials and their primary results are summarized in Table 2. Sorafenib, already approved for renal cell cancer, thyroid cancer and hepatocellular carcinoma, were evaluated in either in a first-line and salvage situation combined with chemotherapy and HMAs (hypomethylating agents), showing promising results [78–84]. A novel second-generation FLT3 inhibitor crenolanib has shown possible benefits in combination with conventional chemotherapy, in either first-line and salvage treatment [85–87]. Lestaurtinib, however, failed to display clinical benefit when administered as maintenance therapy following standard treatment [88,89].

Table 2. Clinical results of FLT3 inhibitors.

SORAFENIB (BAY 43-9006)										
Authors and Jounals	**Trial Name**	**Objectives**	**Disease Status**	**Agents * Controls Not Shown**	**Phase/Design**	**Response Rate**		**Median PFS**	**Median OS**	**Sequential allo-SCT**
Rolling, et al. Lancet Oncol 2015	SORAML	AML (age < 60)	Newly diagnosed	Sorafenib + Standard therapy	II	CR	60% (81/134)	21 mo. [9–32]	Not Reached (3-yr OS 63%)	31% (42/132)
Uy, et al. Blood Advances 2016	CALGB 11001	AML (age > 60) with FLT3-ITD and/or TKD	Newly diagnosed	Sorafenib + Standard therapy	II	CR	74% (40/54)	8.8 mo. (FLT3-ITD) 7.8 mo. (FLT3-TKD)	15.0 mo. (FLT3-ITD) 16.2 mo. (FLT3-TKD)	53% (22/54)
Ohanian, et al. Am J Hematol 2018	–	AML (age > 60) with FLT3-ITD	Newly diagnosed	Sorafenib + Azacitidine	I/II	CR/Cri PR	70% (19/27) 7% (2/27)	7.1 mo. (only in responders)	8.3 mo. (in all participants)	11% (3/27)
Sasaki, et al. Cancer 2019	–	AML with FLT3-ITD	Newly diagnosed	Soragenib + Standard therapy	Retrospective	CR/CRi	99% (78/79)	31 mo. [5.7–56.8]	17 mo. [11.1–22.4]	67% (53/79)
Muppidi, et al. Clinical Lymphoma Myeloma and Leukemia 2015	–	AML with FLT3-ITD	Newly diagnosed or relapsed	Sorafenib + Decitabine	Case Series	CR/CRi	83% (5/6)	Not Reported	5.1 mo. [1.9–14.5]	33% (2/6)
Ravandi, et al. Blood 2013	–	AML with FLT3-ITD	Relapsed or refractory (including prior allo-SCT)	Sorafenib + Azacitidine	II	CR/Cri PR	43% (16/37) 3% (1/37)	3.8 mo. [1.0–16.4]	6.2 mo.	16% (6/37)
Bazarbachi, et al. Heamatologica 2019	–	AML with FLT3-ITD	Relapsed ater allo-SCT	Sorafenib as part of/after salvage	Retrospective	CR	39% (10/26)	Not Reported	(2-yr. OS 38%)	13% (3/26)
MIDOSTAURIN (PKC412)										
Authorsand Jounals	**Trial Name**	**Objectives**	**Disease Status**	**Agents * Controls Not Shown**	**Phase/Design**	**Response Rate**		**Median PFS**	**Median OS**	**Sequential allo-SCT**
Stone, et al. N Engl J Med 2017	RATIFY	AML with FLT3-ITD and/or TKD	Newly diagnosed	Midostaurin + Standard induction/consolidation	III	CR	70% (504/717)	8.2 mo. [5.4–10.7]	74.7 mo. [31.5–inf.]	57% (287/504)
Schlenk, et al. Blood 2019	AMLSG 16-10	AML with FLT3-ITD and/or TKD	Newly diagnosed	Midostaurin + Standard induction/consolidation f/b Midostaurin maitenance	II	CR/CRi	76% (217/292)	13.2 mo. [10.0–18.3]	26.0 mo. [18.9–37.0]	62% (134/217)
Fischer, et al. J Clin Oncol 2010	–	AML or high-risk MDS	Relapsed or refractory or ineligible to standard therapy	Midostaurin	IIB	PR HI Blast	1% (1/97) 46% (16/35 *) 71% (25/35 *) * only in FLT3-mt	Not Reported	4.3 mo. [3.5–5.2]	31% (42/132)
Strati, et al. Am J Hematol 2015	–	AML or high-risk MDS	Relapsed or refractory or ineligible to standard therapy	Midostaurin + Azacitidine	I/II	CR/Cri PR/MLFS	15% (8/54) 13% (7/54)	4.6 mo. [2.3–6.9] * Duration of Response	5.1 mo. [3.5–6.7]	0% (0/8)
Walker, et al. Leukemia & Lymphoma 2016	–	AML	Relapsed or refractory (including prior allo-SCT)	Midostaurin + Bortezomib + Chemotherapy(MEC)	I	CR/CRi	83% (19/23)	Not Reported	10.8 mo.	63% (12/19)
Maziarz, et al. Blood 2018	RADIUS	AML with FLT3-ITD	in 1st CR after allo-SCT	Midostaurin + Standard of care	II	Not Applicable		(18mo.-PFS 89%)	Not Reported	Not Applicable
GILTERITINIB (ASP2215)										
Authors and Jounals	**Trial Name**	**Objectives**	**Disease Status**	**Agents * Controls Not Shown**	**Phase/Design**	**Response Rate**		**Median PFS**	**Median OS**	**Sequential allo-SCT**
Perl, et al. N Engl J Med 2019	ADMIRAL	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Gilteritinib	III	CR/Cri PR	54% (134/247) 13% (33/247)	2.8 mo. [1.4–3.7]	9.3 mo. [7.7–10.7]	26% (63/247)
Perl, et al. Lancet Oncol 2017	–	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Gilteritinib	I/II	CR/Cri PR	41% (69/169) 11% (19/169)	4.6 mo. * Duration of Response	7.1 mo.	22% (37/169)
Usuki, et al. Cancer Science 2018	–	AML	Relapsed or refractory	Gilteritinib	I	CR/Cri PR	60% (3/5 *) 20% (1/5 *) * only in FLT3-mt.	Not Reported	Not Reported	Not Reported

Table 2. *Cont.*

QUIZARTINIB (AC220)										
Authors and Jounals	**Trial Name**	**Objectives**	**Disease Status**	**Agents * Controls Not Shown**	**Phase/Design**	**Response Rate**		**Median PFS**	**Median OS**	**Sequential allo-SCT**
Altman, et al. Blood 2018	–	AML	Newly diagnosed	Quizartinib + Standard induction/consolidation f/b Quizartinib maitenance	I	CR/CRi	74% (14/19)	(Maximum 16.3 mo.)	Not Reported	47% (9/19)
Cortes, et al. Blood 2019	QuANTUM-R	AML with FLT3-ITD	Relapsed or refractory	Quizartinib	III	CR/Cri	48% (118/245)	1.4 mo. [0.0–1.9]	6.2 mo. [5.3–7.2]	32% (78/245)
Cortes, et al. Blood 2018	–	AML with FLT3-ITD	Relapsed or refractory	Quizartinib	IIB	CR/Cri PR	47% (36/76) 18% (14/76)	12.3 mo. [9.7–16.1]	22.6 mo. [19.9–28.3]	37% (28/76)
Cortes, et al. Lancet Oncol 2018	–	AML	Relapsed or refractory	Quizartinib	II	CR/Cri PR	50% (125/248) 25% (62/248) * only in ITD-mt.	2.8 mo. [1.4–3.6] * duration of CR, only in ITD-mt.	5.8 mo. [4.9–6.8] * only in ITD-mt.	35% (61/176)
Sandmaier, et al. Am J Hematol 2017	–	AML with FLT3-ITD	in 1st CR after allo-SCT	Quizartinib maintenance	I	Not Applicable		(0.4–22.8 mo.) * duration of maitenance	(3.0–32.7 mo.)	Not Applicable
CRENOLANIB (CP868596)										
Authors and Jounals	**Trial Name**	**Objectives**	**Disease Status**	**Agents * Controls Not Shown**	**Phase/Design**	**Response Rate**		**Median PFS**	**Median OS**	**Sequential allo-SCT**
Wang, et al. Blood 2016	–	AML with FLT3-ITD and/or TKD	Newly diagnosed	Crenolanib + Standard induction/consolidation f/b Crenolanib maintenance	II	CR/CRi	96% (24/25)	Not Reported	Not Reached (6 mo. OS 85%)	50% (12/24)
Randhawa, et al. Blood 2014	–	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Crenolanib	II	CR/Cri MLFS HI	23% (3/13) 8% (1/13) 31% (4/13) * only in TKI-naïve	3.0 mo. * only in TKI-naïve	12.7 mo. * only in TKI-naïve	26% (9/34)
Ohanian, et al. Blood 2016	–	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Crenolanib + Salvage chemotherapy (IDA/AraC)	I	CR/CRi	36% (4/11)	Not Reported	8.5 mo.	75% (3/4)
Iyer, et al. Blood 2016	–	AML	Relapsed or refractory	Crenolanib + Chemotherapy (HAM)	I	CR/CRi	67% (4/6)	Not Reported	Not Repoted	25% (1/4)
LESTAURTINIB (CEP701)										
Authors and Jounals	**Trial Name**	**Objectives**	**Disease Status**	**Agents * Controls Not Shown**	**Phase/Design**	**Response Rate**		**Median PFS**	**Median OS**	**Sequential allo-SCT**
Levis, et al. Blood 2017	–	AML with FLT3-ITD and/or TKD	Relapsed	Slavage chemotherapy (MEC) f/b Lestaurtinib maintenance	II	CR/CRi	26% (29/112)	Not Reported	5.2 mo.	20% (22/112)
Knapper, et al. Blood 2017	–	AML with FLT3-ITD and/or TKD	Newly diagnosed	Standard induction/consolidation f/b Lestaurtinib maintenance	III	CR/CRi	92% (277/300)	(5-yr. PFS 39–40%)	(5-yr. OS 43–50%)	21% (58/277)

5. Mechanisms of Resistance to FLT3 Inhibitors

5.1. Primary Resistance

Resistance to FLT3 inhibitors can be classified as primary resistance (innate resistance) and secondary resistance (acquired resistance). In primary resistance, the effect of FLT3 inhibitors are prevented during the initial administration in an FL-dependent, FGF2-dependent, and stromal CYP3A4-mediated manner as well as by the activation of other signaling pathways (Figure 1). Most FLT3-mutant AML cells also express wild-type (WT) FLT3 concomitantly. Since WT-FLT3 is sensitive to FL and is affected negligibly by FLT3 inhibitors, FL secretion in the bone marrow microenvironment leads to the activation of the FLT3/MAPK pathway and provides survival signals to AML cells during induction and consolidation therapy. Indeed, certain studies have demonstrated that the co-existence of WT-FLT3 attenuated the anti-tumor effects of FLT3 inhibitors on FLT3-mutated AML cells in vitro and in vivo [88,90,91]. In addition to FL, other cytokines, growth factors, and soluble proteins from the bone marrow microenvironment have been studied with respect to their resistance against quizartinib. For example, fibroblast growth factor 2 (FGF2) induces resistance by activating FGFR1 and inducing downstream MAPK signaling. FGF2 expression in bone marrow stromal cells increased in patients with FLT3-ITD-positive AML treated with quizartinib and was maximized prior to clinical relapse and the induction of resistance mutations [92]. CXCL12, a chemokine expressed by osteoblasts in the bone marrow, is a ligand of CXCR4 expressed by hematopoietic stem cells as well as AML cells. Certain reports revealed that the CXCR4 antagonist plerixafor (AMD 3100) selectively reduced the proliferation of FLT3-ITD AML blasts and increased the sensitivity of FLT3-mutated leukemic cells to the apoptogenic effects of FLT3 inhibitors [93,94]; therefore, the activation of the CXCL12/CXCR4 axis may also induce resistance to FLT3 inhibitors in AML cells. The inactivation of TKIs by CYP3A4 is well established. In particular, hepatic CYP3A4 inactivates all TKIs, including FLT3 inhibitors. Additionally, the expression of CYP3A4 in bone marrow stromal cells attenuated the activity of three different FLT3 inhibitors (sorafenib, quizartinib, and gilteritinib) in FLT3-ITD-positive AML [95].

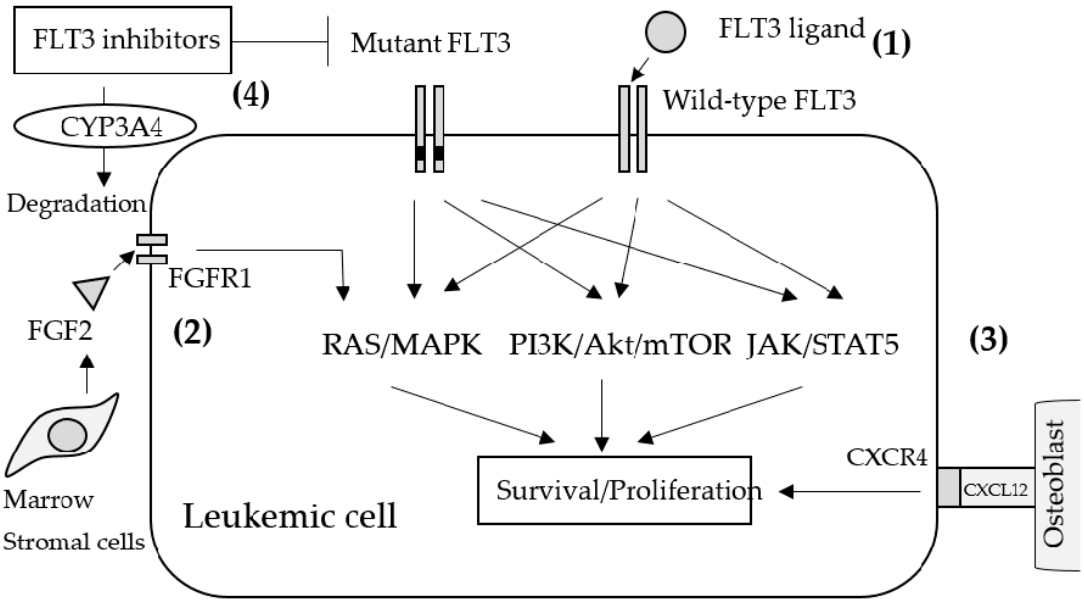

Figure 1. Schematic mechanisms of primary resistance to FLT3 inhibitors. (1) Wild-type FLT3s are a little sensitive to FLT3 inhibitors and allow downstream signaling by binding with FLT3 ligands. (2) FGF2 secreted from bone marrow stromal cells activates FGFR1 on leukemic cells which leads to MAPK activation. (3) Cell adhesion to the microenvironment may also help leukemic proliferation. Antagonizing CXCR4 that binds to CXCL12 on osteoblasts resulted in attenuated leukemia progression. (4) Upregulating CYP3A4 leads to the rapid inactivation of FLT3 inhibitors.

5.2. Secondary Resistance Due to Additional FLT3 Mutations (on-Target Resistance)

Secondary resistance negates the effects of FLT3 inhibitors via the abnormalities acquired by FLT3 inhibition, such as additional mutations in FLT3 ("on-target" resistance) and defective factors apart from FLT3 ("off-target" resistance). Several genetic mutations associated with FLT3 inhibitor resistance have been reported in clinical trials on FLT3 inhibitors. As mentioned earlier, since type II inhibitors originally have no affinity for FLT3-TKD, additional mutations in the TK domain can confer resistance via the elimination of the inhibitory effect on FLT3-ITD. In cases of recurrence after quizartinib treatment in patients with FLT3-ITD-positive AML, secondary mutations at D835 and Y842 residues as well as at the commonly known "gatekeeper residue" F691 in the kinase domain have been reported (Figure 1) [96]. In vitro, Ba/F3 cells expressing FLT3-ITD and one additional TKD mutation, detected in patients with clinical resistance (+D835Y, +D835V, +Y842C, +Y842H, or +F691L), exhibited resistance to the growth inhibitory effect and dephosphorylation activity of quizartinib. These resistance mutations in the A-loop were also observed in patients treated with sorafenib, another type II inhibitor. Furthermore, during the treatment with gilteritinib and crenolanib (a type I inhibitor), the additional appearance of FLT3-TKD mutations in patients with resistance was infrequent, although the appearance of F691L, a gatekeeper mutation, was observed (Figure 2).

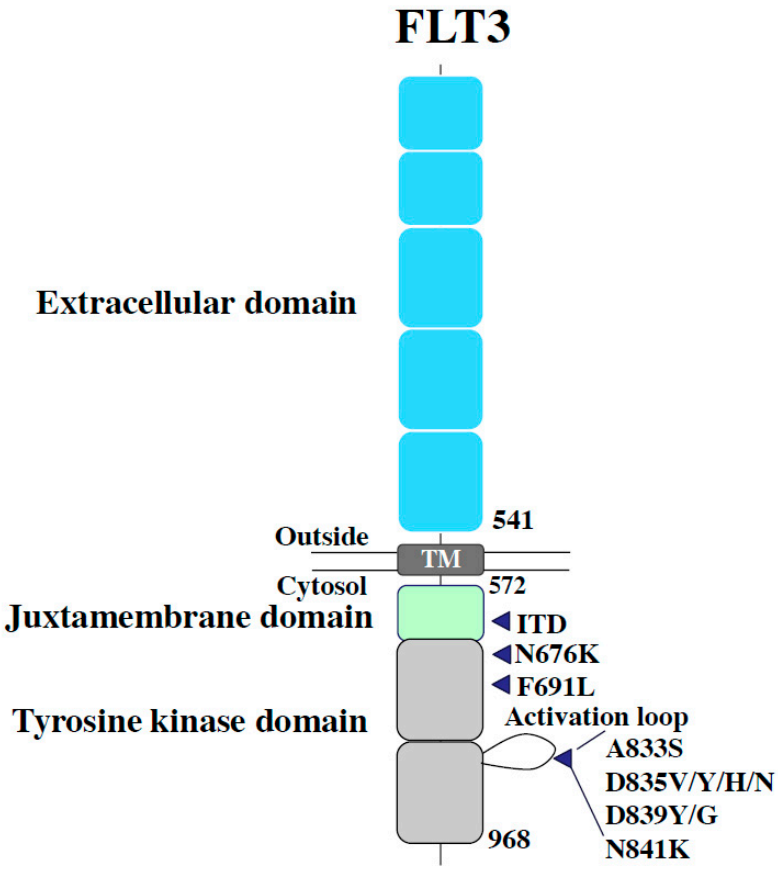

Figure 2. Additional FLT3 tyrosine kinase domain mutations responsible for secondary on-target resistance. These mutations keep the TK domain in active FDG-in form, not allowing the type II inhibitors to bind there. Mutations in a "gate-keeping" residue F691 shows the universal resistance to both type I and II inhibitors.

Although the FLT3-F837K and FLT-C35S mutations occurred after the gilteritinib treatment in one patient each, both were considered silent mutations as these did not induce self-proliferation in Ba/F3 cells[40]. Among the 50 resistant patients treated with crenolanib, five FLT3 (D200N, K429F, Y572C, L601F, and F691L) mutations were observed in six patients; the D200N and L601F mutations

did not result in leukemia [40]. Since the frequency of the acquired mutations in FLT3-ITD in patients with clinical resistance to quizartinib, a type II inhibitor, was 50% or less, other resistance mechanisms are also anticipated. In four out of eight patients treated with quizartinib, one or more resistance mutations were observed in the TK domain [96]. In addition to FLT3-ITD alleles, mutations in the TK domain of the original FLT3 allele were detected in seven individuals. Notably, the patients exhibited different frequencies of mutations between the original FLT3 allele and the FLT-ITD allele. In this study, the AML cells collected from one quizartinib-resistant patient did not acquire resistance mutations in either the original FLT3 allele or the FLT3-ITD allele. No mutations were detected in the genes apart from *FLT3*, although the existence of off-target resistance mechanisms was considered in this patient. These findings suggest the existence of a polyclonal resistance mechanism in patients with AML that relapses after quizartinib treatment.

5.3. Secondary Resistance Due to Non-FLT3 Abnormalities (off-Target Resistance)

Resistant clones formed after treatment with gilteritinib and crenolanib, and a type I inhibitor that exerts an inhibitory effect on FLT3-TKD, have characteristics that are different from those observed after treatment with type II inhibitors. In a comparative genetic analysis before and after relapse in patients treated with gilteritinib, several distinct patterns of clonal selection were observed during the treatment period with gilteritinib [97]. In five out of 41 (12.2%) gilteritinib-resistant patients, FLT3 mutations were not observed in AML cells after the gilteritinib treatment; however, mutations in the RAS/MAPK pathway were present in all of the patients. These results suggest that mutant FLT3-negative clones acquire mutations in the RAS/MAPK pathway and expand as resistant clones. In 36 other patients, the resistant clones contained the original FLT3 mutation, and five of them acquired an F691L TKD mutation in addition to the original FLT3 mutation. In 10 out of the 36 patients with the original FLT3 mutation, additional mutations in the RAS/MAPK pathway, such as *NRAS, KRAS, PTPN11, CBL*, and *BRAF* mutations, were acquired. Of note, the mutations in the RAS/MAPK pathway and FLT-F691L mutations were mutually exclusive. In vitro experiments conducted in MOLM-14, an AML cell line with FLT3-ITD, where either mutant RAS or FLT3-F691L was transduced into the parental cells and gilteritinib was administered at low/high-dose (25 and 250 nmol/L), suggested that the RAS-mutant clones were more likely selected by the high concentration of the inhibitor, besides the FLT3-F691L which was more likely to be selected by a low one. Similar to RAS mutations [97,98], the activation of Axl-1, a member of the TAM family of receptor TKs, may also contribute to FLT3-resistance by constantly activating the RAS/MAPK and PI3K/Akt/mTOR pathway. Axl-1 was observed to be highly phosphorylated in midosutaurin-resistant AML cell lines and its resistance was diminished by the Axl-1 inhibition in vitro [99]. In another experiment, patient-derived AML cells with FLT3-ITD were co-cultured with stromal cells and treated with quizartinib [100]. The surviving cells underwent STAT5 activation, which consequently upregulated AXL, which was further enhanced by the hypoxic environment. Conversely, in patients eliciting poor response to crenolanib, several abnormalities have been observed in the loci encoding epigenetic regulators and granulocyte transcription factors, as well as in the cohesin complex. In particular, *NRAS*, *STAG2*, *CEBPA*, *ASXL1*, and *IDH2* mutations were observed in FLT3-WT clones [40]. These findings suggest that the clones escaped and expanded during crenolanib therapy. However, *TET2*, *IDH1*, and *TP53* mutations occurred simultaneously in FLT3-mutated clones during crenolanib treatment. These results suggest that the off-target resistance mechanism is more frequent when using type I inhibitors, such as gilteritinib or crenolanib, than type II inhibitors. Besides, IDH1 inhibitor ivosidenib [101] and IDH2 inhibitor enasidenib [102,103], both approved by the FDA, are active against IDH1/2-mutant relapsed/refractory AML, though the significance of co-existing FLT3 mutations is not fully understood. In addition, the upregulation of the PI3K/AKT/mTOR pathway in resistant cell lines treated with sorafenib has also been reported [104]. Pim-1 is a proto-oncogene originally detected in hematopoietic cells that functions downstream of STAT5 [105]. Its overexpression induced resistance to lestaurtinib in BaF3/ITD cells and in samples collected from FLT3-ITD-positive patients [106]. Additionally, Pim kinase overexpression has been

observed in the samples collected post sorafenib administration in patients with FLT3-ITD-positive AML compared to the levels observed in the samples collected before administration [107]. Pim-1 was associated with an increased expression of anti-apoptosis proteins, such as Bcl-2, BCL-XL, and MCL-1, in FLT3 inhibitor-resistant cases [25,108–110]. In particular, the observed resistance may be partly induced by Pim-1. Off-target abnormalities along with FLT3 signaling are schematically summarized in Figure 3.

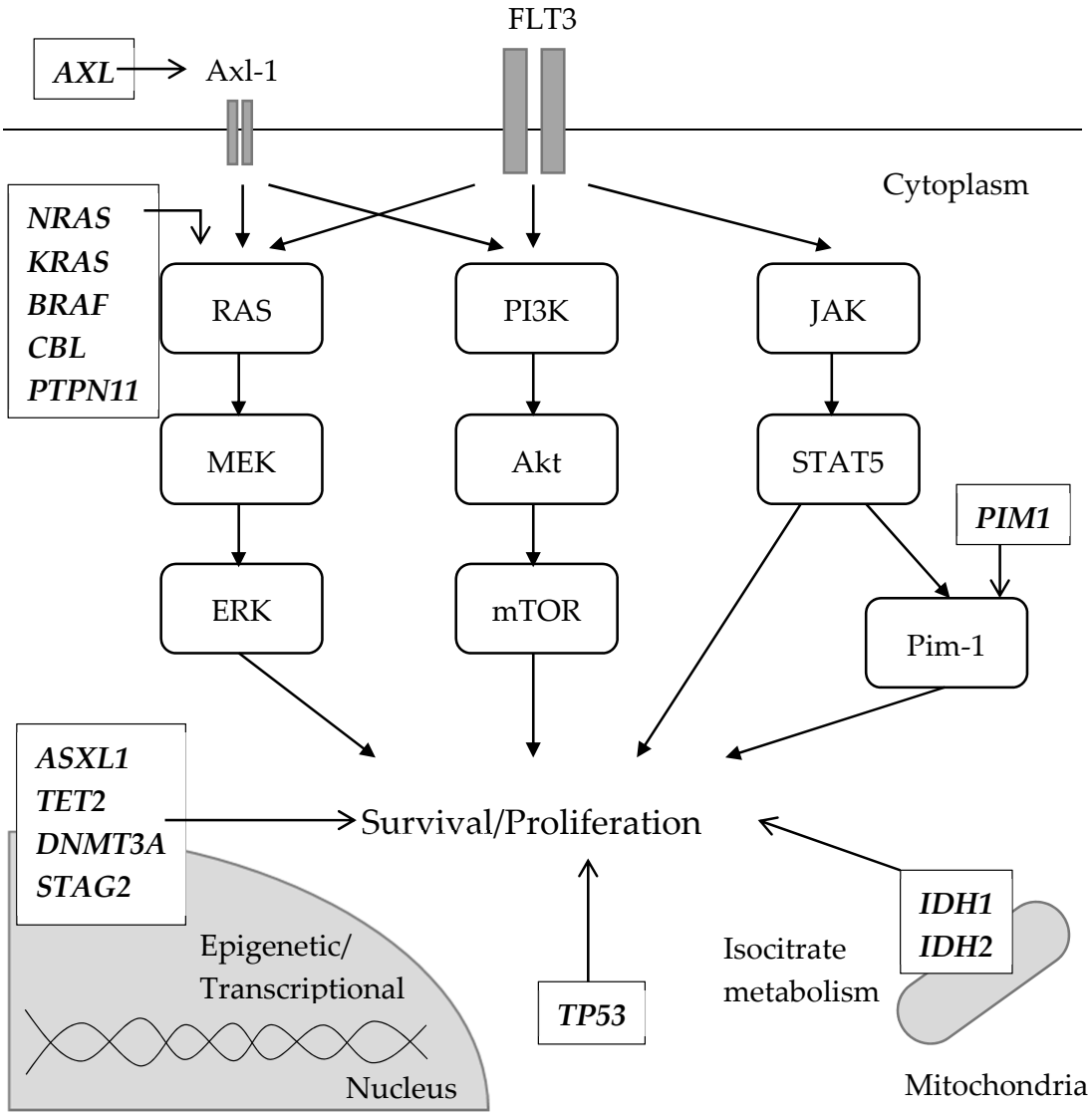

Figure 3. Schematic description of genetic abnormalities (mutations or upregulation) associated with secondary off-target resistance to FLT3 inhibitors. Mutations involved in the RAS/MAPK pathway were reported. *NRAS* mutation is the most common among them. Axl-1, coded by the *AXL* gene, is a receptor tyrosine kinase that leads to the activation of RAS/MARK and PI3K/Akt/mTOR pathway. The upregulation of the *AXL* gene was observed in midostaurin-resistant AML cell lines. Pim-1 is part of the downstream signaling of STAT5, contributing cell survival and proliferation as well as cell migration. A lestaurtinib-resistant AML cell line showed the overexpression of Pim-1. Other gene mutations commonly seen in AML regardless of FLT3 status were also detected. Although a direct relationship with FLT3 signaling was not suggested, these mutations have an essential role in maintaining leukemic clones by modulating epigenetic/transcriptional regulations (e.g., *ASXL1*, *TET2*, *DNMT3A* and *STAG2*), altering the metabolism of the citrate acid cycle (e.g., *IDH1* and *IDH2*) and preventing apoptosis (e.g., *TP53*).

6. Strategies to Overcome Resistance to FLT3 Inhibitors

6.1. Development of Novel Agents

Previous reports suggest that on-target resistance tends to occur in patients after type II inhibitor treatment, while off-target resistance is likely to occur after type I inhibitor treatment. Since these reports are currently limited to patients recruited during clinical trials, for a better understanding of the mechanism underlying the resistance to each FLT3 inhibitor, it is necessary to determine the characteristics of patients with resistance in real-word settings. In addition, to counter the gatekeeper mutation (F691L) in *FLT3*, which confers resistance to all existing FLT3 inhibitors, it is necessary to develop a novel FLT3 inhibitor. As described above, while type I inhibitors can also inhibit FLT3-TKD, they exhibit low selectivity, whereas although type II inhibitors cannot inhibit FLT3-TKD, they exhibit high selectivity. FLT3-TKD inhibitory activity and FLT3 selectivity share a trade-off relationship. To resolve these issues, a novel FLT inhibitor known as FF-10101 was designed, which would form covalent bonds with the C695 residues of FLT3. The creation of covalent bonds by FF-10101 enables the selective and irreversible inhibition of FLT3 in either the active or the inactive form [111]. Furthermore, the unique binding method of FF-10101 exerts wide inhibitory action against various *FLT3* mutations, including F691L. Currently, phase 1/2 trials are underway to evaluate its safety, tolerability, pharmacokinetics, and efficacy against recurrent refractory AML (NCT03194685). In addition, several agents that may overcome or prevent resistance are currently under investigation. A pan-PIM/FLT3 inhibitor SEL24 [112], a type II FLT3 inhibitor MZH29 [113], a MERTK/FLT3 inhibitor MRX-2843 [114], a BCR-ABL inhibitor ponatinib [115], and a multiple tyrosine kinase inhibitor cabozantinib [116] have exhibited anti-tumor activity in cases with FLT3-TKD, including those with the F691 pointmutation.

6.2. Combination with Different Class Agents

Existing FLT3 inhibitors are now being tested in combination with HMAs, standard chemotherapy, bortezomib (proteasome inhibitor), atezolizumab (anti-PD-L1 antibody), venetoclax (BCL-2 inhibitor), milademetan (MDM2 inhibitor) and homoharringtonine (STAT inhibitor). Ongoing trails of combination strategy are summarized in Table 3 Preclinically, FLT3 ligand-mediated resistance was attenuated by the dual inhibition of AKT/FLT3 in vivo [117]. The combination of the MEK and FLT3 inhibitors as well as the dual inhibition of MEK/FLT3 proved to be effective against resistance-conferring FLT3 mutations in in vivo and in vitro mutations [97,118]. The sensitization of FLT3 inhibitors can serve as an alternate strategy. Proteasome inhibitors, arsenic trioxide (ATO), and a CDK4/6 inhibitor palbociclib downregulated FLT3 molecules in FLT3-ITD AML cells by promoting cytotoxic autophagy, inhibiting the expression of FLT3 RNAs, and dysregulating the transcription of *FLT3* and *PIM1*, respectively [119–121]. The inactivation of *ATM* or its downstream effector G6PD also induced synthetic lethality along with *FLT3* inhibition by enhancing mitochondrial oxidative stress, which eventually resulted in tumor apoptosis [122].

Table 3. Clinical trials of FLT3 inhibitors.

Trial Number	Objectives	Disease Status	Agents * Controls Not Shown	Phase/Design
SORAFENIB (BAY 43-9006)				
NCT01371981	AML with FLT3-ITD (high allelic ratio)	Newly diagnosed	Sorafenib + Bertezomib	III
NCT03170895	AML with FLT3-ITD	Newly diagnosed or relapsed	Sorafenib + Homoharringtonine (STAT inhibitor)	II
MIDOSTAURIN (PKC412)				
NCT03686345	Core binding factor AML	Newly diagnosed	Midostaurin + Standard induction	II
NCT03280030	AML with FLT3-ITD and/or TKD	Newly diagnosed	Midostaurin + Standard induction/consolidation f/b Midostaurin maitenance	II
NCT03512197	AML with FLT3-ITD and/or TKD	Newly diagnosed	Midostaurin + Standard induction/consolidation f/b Midostaurin maitenance	III
NCT03379727	AML with FLT3-ITD and/or TKD	Newly diagnosed	Midostaurin + Standard induction/consolidation f/b Midostaurin maitenance	III
GILTERITINIB (ASP2215)				
NCT02236013	AML	Newly diagnosed	Gilteritinib + Standard induction/consolidation	I
NCT02752035	AML with FLT3-ITD and/or TKD	Newly diagnosed and ineligible to standard therapy	Gilteritinib + Azacitidine	III
NCT03730012	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Gilteritinib + Atezolizumab (anti-PD-L1 antibody)	I/II
NCT02310321	AML	Newly diagnosed	Gilteritinib + Standard induction/consolidation	I/II
NCT03182244	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Gilteritinib + Salvage chemotherapy	III
QUIZARTINIB (AC220)				
NCT02668653	AML with FLT3-ITD	Newly diagnosed	Quizartinib + Standard induction/consolidation	III
NCT03723681	AML	Newly diagnosed	Quizartinib + Standard induction/consolidation	I
NCT02834390	AML	Newly diagnosed	Quizartinib + Standard induction/consolidation	IB
NCT03552029	AML with FLT3-ITD	Relapsed or refractory or ineligible to standard therapy	Quizartinib + Milademetan (MDM2 inhibitor)	I
NCT03135054	AML with FLT3-ITD	Newly diagnosed or relapsed	Quizartinib + Homoharringtonine (STAT inihibitor)	II
NCT03661307	AML with FLT3-ITD	Newly diagnosed or relapsed	Quizartinib + Decitabine + Venetoclax (BCL-2 inhibitor)	I/II
NCT03735875	AML with FLT3-ITD	Relapsed or refractory	Quizartinib + Venetoclax (BCL-2 inhibitor)	IB/II
CRENOLANIB (CP868596)				
NCT03258931	AML with FLT3-ITD and/or TKD	Newly diagnosed	Crenolanib + Standard induction/consolidation	III
NCT02283177	AML with FLT3-ITD and/or TKD	Newly diagnosed	Crenolanib + Standard induction	II
NCT03250338	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Crenolanib + Salvage chemotherapy	III
NCT02400281	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Crenolanib + Salvage chemo. or Azacitidine	I/II
NCT02626338	AML	Relapsed or refractory	Crenolanib + Salvage chemotherapy	I/II
NCT01522469	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Crenolanib + Standard induction/consolidation	IB
OTHERS				
NCT00783653	AML with FLT3-ITD and/or TKD	Newly diagnosed	Sunitinib + Standard induction	I/II
NCT00469859	AML with FLT3-ITD and/or TKD	Relapsed or refractory	Lestaurtinib + Salvage chemotherapy	I/II

6.3. Genetic Mutation Analysis

As described, the presence or absence of mutations in *FLT3* has become an important determinant of the treatment methods in AML. Currently, a companion diagnostic tool LeukoStratCDx (Invivoscribe, Inc., San Diego, CA, USA) is widely used for the clinical use of FLT3 inhibitors in Japan, the United States, and Europe, among others. However, LeukoStratCDx is only able to detect D835 and I836 mutations and cannot detect any other FLT-TKD mutations, including F691L. Therefore, the instrument might incorrectly analyze the condition in patients with FLT3-TKD that is potentially treatable by FLT3 inhibitors. Although intensive chemotherapy has ensured substantial clinical benefit in AML patients, several patients eventually require targeted therapy, particularly young patients. In addition, *CEBPA* and *NPM1*, and recently *TP53*, *ASXL1* and *RUNX1*, have been determined to be important markers prognosis [27,31,123], transplant eligibility, and treatment strategy. Even after FLT3-ITD/TKD becomes undetectable in remission, the expression of persistently mutated genes such as *DNMT3A*, *TET2*, *SRSF2*, and *ASXL1* continues to be associated with high relapse rates and poor prognosis [124]. Although the negative prognostic impact of FLT3-ITD might be, at least partially, attenuated by upfront haploidentical stem cell transplantation (haplo-SCT) [125], FLT3 inhibitors remain one of the useful choices for treating the majority of FLT3-mutated AML patients, especially elderly and/or unfit people. To overcome resistance to FLT3 inhibitors, mutation analyses in patients with resistance to FLT3 inhibitors are required to identify the genetic abnormalities that contribute to drug-resistance and determine additional therapeutic targets. Genome-wide analysis using the CRISPR-Cas9 single-guide RNA (sgRNA) library, a vector-mediated technique for the knockdown of particular genes, revealed that the loss of *SPRY3* and *GSK3* confers resistance to quizartinib by inducing the reactivation of the FGF/RAS/ERK pathway and Wnt signaling [126]. Likewise, in addition to FLT3 mutations, it is necessary to comprehensively evaluate various genetic abnormalities; comprehensive mutation testing by next-generation sequencing (NGS) is expected to enable this. Accordingly, we analyzed the cancer-related genetic abnormalities (i.e., in an NGS panel) in patients with AML who were ineligible for intensive chemotherapy or developed recurrent/refractory cancer after initial therapy (Foundation One Heme; we planned HM-SCREEN-JAPAN, an observational study that analyzes and evaluates the relationship between prognosis by F1H). The primary goal of this project is the development of F1H and the promotion of targeted therapy for AML [127].

7. Conclusions and Future Perspectives

This paper described the principal mechanisms of resistance to FLT3 inhibition and the current investigations to overcome it. Secondary on-target mutations (i.e., FLT3-TKD) can be managed by choosing type I inhibitors such as gilteritinib that are consistently active against FLT3-TKD as well as FLT3-ITD, except for a "gatekeeper" F691L mutation. Covalently-coupling FF-10101 and other novel FLT3 inhibitors are now under investigation and have shown promising data on FLT3 F691L. Strategies for secondary off-target abnormalities and a part of primary resistance cannot be simple, regarding the diverse relating genomic abnormalities and complex patterns of clonal evolution. Nevertheless, some genetic abnormalities are/will be clinically targetable, expecting a synergistic anti-tumor effect with FLT3 inhibition. For example, several agents targeting BCL-2, MDM2 and STAT as well as conventional chemotherapy are being evaluated in combination with FLT3 inhibitors. Similarly, abnormal *RAS* and *PIM1* pathways as well as metabolic modifications (e.g., G6PD inactivation) are subject to preclinical investigations. Recent studies have suggested the non-negligible importance of clone-evolutional patterns in terms of acquiring resistance, which possibly affects clinical strategy in managing FLT3-mutated AML. Simply, when you find two distinct targetable mutations and the corresponding agents are available (e.g., FLT3-ITD and *IDH2* mutation), you can choose either one agent if both mutations are limited in a single leukemic clone, but if each mutations are found in different clones, it is worth considering combination or sequential therapy, if allowed. Routine and successive gene sequencing will help detecting additional targetable mutations and determining

individual patterns of clone evolution, which would improve our clinical approaches along with the development of novel agents and combination strategies.

Several new agents such as FLT3 inhibitors can create overlapping treatment options, especially in the elderly, unfit AML patients as well as in relapse/refractory AML patients. A lot of clinical trials evaluating the efficacy of promising investigational drugs in AML are ongoing and more drugs will go to the market than ever before. Based on the resistant mechanisms during treatment, how to use these new agents properly is one of the issues with the treatment of AML. Physicians should select an optimal treatment depending on factors such as age, performance status, comorbidities, and genome profiling analysis upon new diagnoses and during treatment.

Author Contributions: M.E. and S.C. wrote the first draft and all authors revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This paper was supported by the National Cancer Research & Development expenses grant.

Conflicts of Interest: Y.M. received research funding from Ono and honoraria from Bristol-Myers Squibb, Novartis, and Pfizer. The other authors declare no conflict of interest.

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