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Normal and oncogenic FLT3

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Abstract. FLT3, a member of the class III receptor tyrosine kinases (RTKs), is preferentially expressed on the cell surface of hematopoietic progenitors, and the ligand of FLT3 (FL) is expressed as a membrane-bound or soluble form by bone marrow stroma cells. It has been disclosed that FL-FLT3 interaction plays an important role in the maintenance, proliferation and differentiation of hematopoiesis. FLT3 is also expressed in a high proportion of acute myeloid leukemia (AML) and B-lineage acute lymphoblastic leukemia cells. Activating mutations of FLT3 are the most frequent genetic lesions in AML, and AML patients with FLT3 mutations have a worse prognosis than those with normal FLT3. Exploring the mechanism by which FLT3 mutations cause autoactivation and uncontrolled signaling might lead to a better understanding of how FLT3 becomes oncogenic and provide insights for the development of new drugs.

Key words. FLT3; tyrosine kinase; hematopoiesis; leukemia; tandem duplication; point mutation; target therapy; kinase inhibitor.

Structure and function of FLT3

Murine FLT3 was cloned independently by two groups using testis and a stem cell fraction of fetal liver as an FMS-homologous kinase, and then a human homologue was cloned [1–5]. The FLT3 gene is located on chromosome 13 at q12 and contains 24 exons (formerly 21 exons) [6, 7]. FLT3 has two forms: a 158–160 kDa membrane-bound form which has N-linked glycosylation, and a cytoplasmic 130–143 kDa form that is not glycosylated [8]. FLT3 is predominantly expressed on hematopoietic progenitor cells in the bone marrow, thymus and lymph nodes [3], but is also found in the placenta, brain and gonads [4]. The ligand to FLT3 (FL) is expressed as a membrane-bound and soluble form mainly by bone marrow (BM) stroma cells [8]. FLT3 binding to FL results in dimerization of the receptor, autophosphorylation and the subsequent phosphorylation of cytoplasmic substrates that are involved in signaling pathways regulating the proliferation and differentiation of immature hematopoietic cells [8, 9]. Whereas FL alone shows little proliferation activity in itself, it synergistically enhances the expansion of progenitor cells together with stem-cell factor (the ligand of c-KIT) or other cytokines [9]. Furthermore, FL induces the adhesion of hematopoietic progenitor cells to stroma cells via VLA-4 and VLA-5, which play an important role in hematopoiesis [10, 11]. The functional role of FLT3 expressed in the central nervous system remains unknown.

More convincing evidence of a role for FLT3 has been obtained from FLT3-knockout mice. These animals are born healthy with normal peripheral blood counts, but have reduced numbers of early B-cell precursors in bone marrow. A defect of primitive cells was shown by long-term competitive repopulation assays, indicating a reduced ability to reconstitute B-cell, T-cell and myeloid lineages when transplanted into irradiated mice [12]. Mice deficient in both FLT3 and c-KIT exhibited a more severe phenotype characterized by pancytopenia, suggesting that FLT3 and c-KIT play similar but distinct roles both in multipotent stem cells and in lymphoid differentiation [13]. Similarly, mice lacking FL have significantly reduced cellularity in a number of leukocytes, and myeloid and lymphoid progenitors of the bone marrow and reduced numbers of dendritic cells and natural killer

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(KN) cells in the lymphoid organs [14], but the phenotype improved with the administration of FL. On the other hand, overexpression of FL in transgenic mice leads to develop leukemia with a long latency period [15].

FLT3 expression in malignancies

In leukemia cells, the expression of FLT3 is observed in almost all patients with acute myeloid leukemia (AML) and precursor-B acut lymphoblastic leukemia (ALL), and less frequently in patients with T-ALL [16–18]. FLT3 is not detected in chronic myeloid leukemia (CML) patients samples in the chronic phase, but appears to be highly expressed during disease transformation. Whereas the treatment of clinical AML and ALL samples with FL causes phosphorylation of FLT3, it has little proliferative effect by itself on FLT3-expressing leukemia cells, especially ALL cells, but rather acts additively or synergistically with other cytokines [18, 19]. Recently, quantitative reverse transcription-polymerase chain reaction [RT-PCR] has revealed that some clinical samples of AML expressed remarkably high levels of FLT3 messenger RNA (mRNA), which are associated with the auto-activation of FLT3 and poor prognosis of AML patients [20]. It is unknown how the transcription of FLT3 is regulated in normal and malignant hematopoietic cells.

FLT3 with an internal tandem duplication (FLT3/ITD)

FLT3 mutations were discovered during an investigation into the expression of FLT3 mRNA in clinical samples from AML patients [21]. Elongated fragments of the JM domain were detected in the PCR products of mRNA as well as genomic DNA in 5 of 30 AML patients. All samples contained tandemly duplicated sequences within the JM domain encoded by exons 14 and 15, sometimes with an insertion of additional nucleotides. Although the duplicated region varied in both size and location in each individual, the resulting transcripts were always in-frame and would therefore be expected to produce a functional kinase domain with an elongated JM region. Thereafter, numerous studies investigated the distribution, incidence and clinical significance of the internal tandem duplication of FLT3 (FLT3/ITD) [22–28].

The incidence of FLT3/ITD is reported to be between 13.2 and 32% in adult patients with AML [22–28]. FLT3/ITD occasionally emerges at relapse or during disease progression of MDS [29, 30]. It is rarely observed in patients with ALL [31], and FLT3/ITD-positive ALL had sometimes biphenotypic characteristics [32]. It has not been found in patients with other hematological malignancies [31].

The most characteristic feature of FLT3/ITD is its modality of mutation. The length of an ITD varies significantly from 3 to 400 bp ([28] and unpublished observation), and the insertion varies from 3 to 36 bp [22, 26–28]. However, ITD usually involves at least one of Y589, Y591, Y597 or Y599. Since the DNA corresponding to D593 to K602 of FLT3 potentially forms a palindromic intermediate, a hairpin formation of a lagging strand and replication slippage might generate ITD during the DNA replication process [33]. Accordingly, the mechanism of ITD is similar to that of microsatellite instability rather than MLL tandem duplication (MLL/TD), in which nearly a 50 Kb-fragment is duplicated, although genotoxic agents have been suggested to be involved in both FLT/ITD and MLL/TD [34].

The biological significance of FLT3/ITD was first studied using Cos7 cells transfected with FLT3/ITD [33]. FLT3/ITD is ligand-independently dimerized and phosphorylated irrespective of the location and length of the ITD, and dominantly activated wild-type FLT3 expressed in the same cell. It has been shown to confer growth factor independence on factor dependent cell lines such as Ba/F3 and 32D cells, and to induce the constitutive activation of downstream signaling molecules such as signal transducer and activation of STAT5, MAP kinase, Akt, SHC, Cbl, Vav and SHP2 [35–38]. FLT3/ITD is likely to transmit not only constitutive but also additive signals such as STAT5a, implying an oncogenic function of FLT3/ITD such as anti-apoptosis and differentiation block [39, 40]. Furthermore, FLT3/ITD but not wild-type FLT3 is stabilized with HSP90 chaperone complex, which may explain the sensitivity of FLT3/ITD-transformed cells to HSP90 inhibitors [41]. Further study is necessary to elucidate the molecular difference between wild-type and mutant FLT3.

The molecular mechanism of how an ITD is associated with auto-activation of FLT3 remains unclear. Extensive analysis has revealed that not only ITD but also an insertion, substitution of particular amino acid residues and even shortening causes constitutive phosphorylation of FLT3 [37]. Accordingly, the JM domain is likely to negatively regulate the kinase activity, which is disrupted by the ITD mutation [37, 42]. Recently, the structural basis for auto-inhibition of FLT3 by the JM domain was elucidated [43]. The normal JM domain contains three distinct topological components: the binding motif (JM-B), the switch motif (JM-S) and the zipper segment (JM-Z). The JM-B is buried almost in the catalytic center of the kinase (fig. 1). The JM-S contained anti-parallel beta-sheets, one of which also interacts with a small beta-sheet structure within the catalytic loop (S806 to H809, indicated by arrow in fig. 1). The JM-Z is the C-terminal JM domain and physically associated with the N-lobe as it loops around the outside of alphaC. Accordingly, the JM domain totally stabilizes the inactive kinase con-

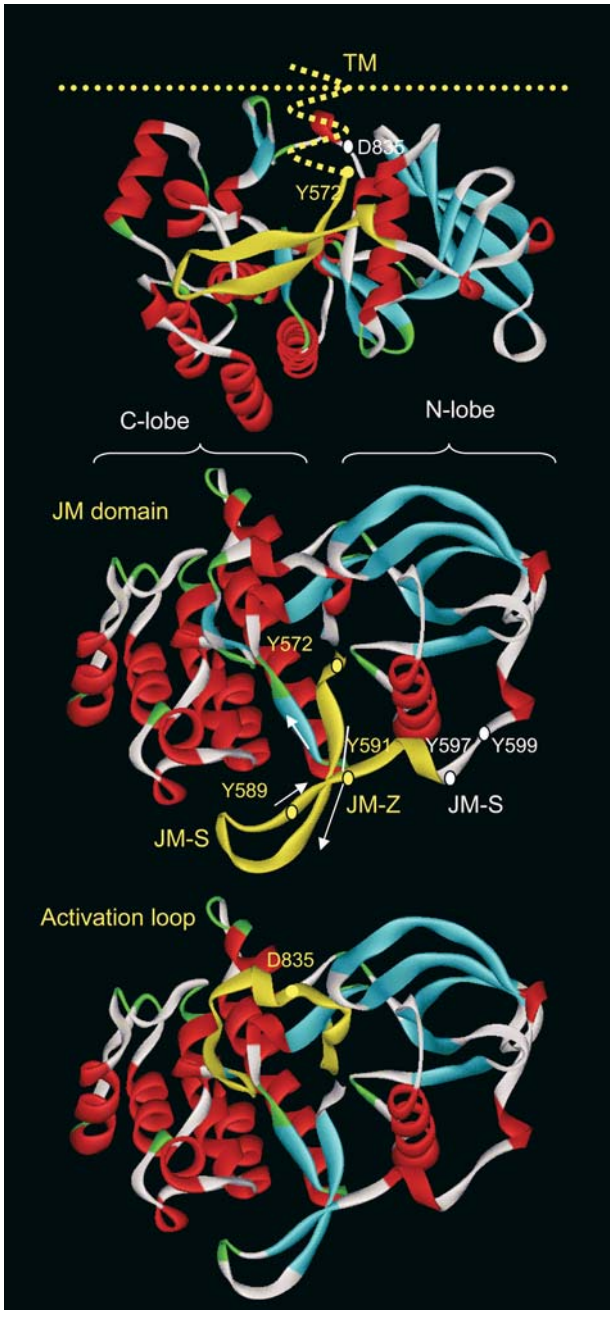

Figure 1. Ribbon diagrams of inactive FLT3 for the side view (upper panel) and upper view (middle and lower panel) [43]. The JM domain and the activation loop are presented by yellow in the upper and middle panels and the lower panel, respectively. Both domains are folded between the N-lobe and C-lobe, and located in the neighborhood. The beta-sheets described in the text are shown by white arrows. TM, trans-membrane domain.

formation. Ligand binding overcomes the inhibitory effect of the JM domain by inducing a conformational change. Mutations of the JM domain may therefore weaken or block the protective association between the JM domain and kinase domain, exposing the latter to constitutive activation. It may further recruit molecules that could stabilize this conformation or alter downstream signaling. Recently, point mutations within the JM domain were reported, although the incidence was low and their biological significance remains unknown [44].

The in vivo oncogenic potential of FLT3/ITD was first demonstrated by injection of 32D cells carrying a FLT3/ITD into syngeneic mice, which led to the development of leukemia [45]. However, transplantation of BM cells transfected with FLT3/ITD into mice led to a myeloproliferative disorder but not leukemia [46]. This indicates the requirement for additional co-operating mutations for a fully transformed phenotype in this model. The transplantation of FLT3/ITD-transfected BM cells into PML/RARalpha transgenic mice clearly shortened the latency period and increased the penetrance for developing an APL-like disease [47]. This finding is interesting when taken together with the clinical evidence that APL with t(15;17) is most frequently associated with FLT3 mutations [22, 48]. A two-hits model for AML has been proposed, the mutation of transcription factors (e.g. PML/RARalpha) producing a differentiation block, and the mutation of signaling molecules (e.g. FLT3) providing a proliferative or survival signal [49].

Mutations in the activation loop of the kinase domain (kinase domain mutation: KDM)

Mutations at D835 in the activation loop of the kinase domain were serendipitously found in AML patients, since c-KIT has point mutations at the same position in mast cell leukemia [50]. The substitutions are from D835 to Y or H, less frequently to V or E [50, 51]. Additionally, I836M and an insertion of glycine and serine between S840 and N841 are reported in AML patients [52]. The incidence is 6.4%–7.7%, and KDM and ITD mutations are mutually exclusive. KDMs are also found in ALL (2.8%) and MDS (3.4%) [50]. The mutations were found to cause the constitutive tyrosine phosphorylation of FLT3 and confer IL-3-independent growth on 32D cells, indicating a similar property to ITD [50]. In the inactive form, the activation loop has been thought to block the access of ATP and the substrate to the kinase domain [43]. Ligand-induced activation leads to phosphorylation (Y842) within the loop, causing an active configuration to be adopted and allowing kinase activity. The KDMs are thought to mimic the latter by interfering with the inhibitory effect of the loop, resulting in constitutive kinase activation. According to the three-dimensional model, D835 is found to be located near the key 'hook' (Y572) within the JM-B [43] (fig. 1). The KDMs are therefore similar to ITD mutations in disrupting the auto-inhibitory mechanisms of FLT3. However, the dimerization is not

needed for the constitutive activation in the case of KDM [unpublished data], whereas the JM domain with ITD is involved in homo- and hetero-dimerization by itself [37].

Clinical prevalence of FLT3 mutations

The overall frequency of FLT3/ITD reported in adult patients is between 13.2 and 34%, as described previously. Leukocytosis is usually associated with FLT3/ITD. Although ITDs are found across the spectrum of FAB types, certain associations have been observed. Several studies have reported high frequencies in AML M3 and M5, and low frequencies in M6 and M7 [24–27, 48]. Although studies of pediatric patients are fewer, the incidence is generally lower than that of adults [25].

A careful search for ITDs in clinical samples has revealed that the ITD band detected by gel electrophoresis or gene scanning is not always single [25, 27, 28, 48, 53]. The mutants arose independently because they differed in the duplicated sequence, implying an underlying genomic instability. There is also evidence for partial or almost complete loss of the wild-type allele in 6–10% of patients with FLT3/ITD [25, 27, 28, 53]. Since neither loss of wild-type FLT3 gene nor amplification of mutant FLT3 gene was reported by fluorescent in situ hybridization (FISH) analysis, this may have arisen as a consequence of homologous recombination [27].

It is biologically interesting whether FLT3 mutations are associated with specific cytogenetic or genetic alterations. As described above, a high frequency of FLT3 mutations (30–39%) has been observed in patients with t(15;17) [48], in particular with the M3 variant together with the short isoform of PML/RARakpha mRNA [54, 55]. Another possible association of FLT3 mutations is with abnormalities of the MLL gene. Tandem duplication of the MLL gene (MLL/ITD) and FLT3 mutations are recurrently found in AML cells [34], whereas FLT3/ITDs are uncommon in AML patients with 11q23 abnormalities. A high level of expression of FLT3 mRNA has been reported in ALL patients with MLL abnormalities [20, 56], and a high level of TKD mutations (20%) has been found in 40 infant ALLs with an MLL rearrangement [57]. In contrast, infrequent associations have been observed in core-binding factor (CBF) leukemia such as t(8;21) and inv(16) [22–28]. Both of these translocations are thought to lead to a block in differentiation by causing the aberrant recruitment of the nuclear co-repressor complex associated with the AML1 complex. Other subgroups with a low frequency of FLT3 mutations include complex karyotype, and translocations at 11q23, t(3;3) and t(9;22); all of them belong to the poor prognostic group [26–28, 50, 55]. The co-incidence rate of N-Ras mutation or BCR/ABL with FLT3 mutations in the same individual is very low [23, 30, 58]. These results suggest that there is no biological advantage in having both mutations, as each alone provides a selective growth advantage.

Tyrosine kinase inhibitors

Since one-third of AML patients have FLT3 mutations and they had a worse prognosis than patients with wild-type FLT3, FLT3 signals would be an important target for treatment [59]. Success in the treatment of CML with the ABL-specific kinase inhibitor imatinib mesylate encouraged investigators to develop FLT3-targeted therapy [60]. To date, many tyrosine kinase inhibitors (TKIs) are shown to have potency in inhibiting the kinase activity of FLT3 and some have entered clinical trials [61], although they are cross-inhibitory to other kinases in varying degrees.

MLN518 (CT53518), a derivative of quinazolines, possesses inhibitory activity against FLT3/ITD-expressing cells both in vitro and in vivo, but not against FLT3/KDM-expressing cells [62]. This compound also inhibits wild-type FLT3, PDGFRbeta and c-KIT. In phase I study, no complete or partial response was observed in 40 patients with relapsed or refractory AML; however there was a 40 – 50% reduction in bone marrow blasts in 3 patients [63]. No specific adverse effects were reported, though pharmacokinetic properties showed the peak plasma concentration of this agent could not be approached to a biologically effective level. Since the phase I study included only 2 patients with FLT3/ITD, a phase II study is currently in progress with additional patients with AML harboring FLT3/ITD.

PKC412 is a benzoylstaurosporine and initially developed as a protein kinase C (PKC) inhibitor. This compound inhibits the kinase activity both of ITD and KDM as well as wild-type FLT3 [64]. A phase II study including 61 patients consisted of 57 AML, 3 MDS and 1 CMMoL, and 11 and 4 cases revealed FLT3/ITD and FLT3/KDM, respectively [65]. Half of the enrolled patients showed over 50% reduction in the number of bone marrow blasts, and one AML patient with FLT3/ITD achieved complete remission, though the duration was short. Although plasma concentration of PKC412 in patients is above micro mol/L its bioavailability is limited, since it binds tightly to acid-alpha-glycoprotein (AGP) in human plasma. Two patients died by pulmonary edema, which was considered a drug-induced toxicity. Therefore, it was concluded that single-agent therapy with PKC412 showed insufficient activity in AML patients even with FLT3 mutations. Now a combination of PKC412 and conventional chemotherapeutic agents is projected.

CEP-701, another derivative of indolocarbazole, shows preferential inhibition of wild-type and mutated FLT3 kinases, as well as TrkA, KDR and PKC [66]. In a phase IIa study of 12 AML patients with FLT3 mutations, reduc-

tion of peripheral blast cells less than 5% or the disappearance of bone marrow blasts was observed in 4 patients, while complete remission was not achieved in any patients [67]. Since this compound has also indolocarbazole structure, it binds to AGP with high affinity and reducing bioavailability. Although pharmacokinetics and pharmacodynamics showed a biological effect, inhibition of FLT3 phosphorylation, in some patients with partial response, clinical efficacy seemed to be limited. Additional trials such as combination with Ara-C are being planned.

SU11248, a derivative of indolinone, inhibits a different spectrum of kinases, which include c-KIT, PDGFR and KDR, more sensitively than FLT3 [68]. A phase I study including 15 patients with advanced AML showed tentative reduction of peripheral blast cells in 7 patients [69]. Since 2 patients died from cardiotoxicity, and the plasma concentration was reached a biologically effective level of SU112480, combination therapy with chemotherapy is being considered.

The early clinical studies targeting FLT3 for AML might thus seem unimpressive. But because these studies incorporated AML patients refractory to conventional chemotherapy, it is not unexpected for the response rates of FLT3 inhibitors to be lower than those of imatinib in CML patients. However, these early reports have disclosed several problems (e.g. plasma concentration and adverse events), which should be carefully investigated in preclinical and clinical studies.

Conclusion

The presence of FLT3 mutations in such a high proportion of patients with AML demonstrates the need to carefully search for the activation mutation of tyrosine kinases in malignancies. Since FLT3 mutations are significantly associated with a poor prognosis in younger AML patients, routine screening of FLT3 mutations is needed to stratify AML according to prognostic factors. The development of FLT3-specific small-molecule inhibitors for use in combination with conventional chemotherapy will lead to improved outcome.

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