

## ORIGINAL ARTICLE

# FLT3 K663Q is a novel AML-associated oncogenic kinase: determination of biochemical properties and sensitivity to Sunitinib (SU11248)

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**Somatic mutations of FLT3 resulting in constitutive kinase activation are the most common acquired genomic abnormality found in acute myeloid leukemia (AML). The majority of these mutations are internal tandem duplications (ITD) of the juxtamembrane region (JM). In addition, a minority of cases of AML are associated with mutation of the FLT3 activation loop (AL), typically involving codons D835 and/or I836. We hypothesized that other novel mutations of FLT3 could also contribute to leukemogenesis. We genotyped 109 cases of AML and identified two novel gain-of-function mutations. The first mutation, N841H, is similar to previously described mutations involving amino-acid substitutions of codon 841. The other novel mutation, FLT3 K663Q, is the first AML-associated gain-of-function mutation located outside the JM and AL domains. Of note, this mutation was potently inhibited by Sunitinib (SU11248), a previously described FLT3 kinase inhibitor. Sunitinib reduced the proliferation and induced apoptosis of transformed Ba/F3 cells expressing FLT3 K663Q. The potency of Sunitinib against FLT3 K663Q was similar to its potency against FLT3 ITD mutations. We conclude that FLT3 mutations in AML can involve novel regions of the TK1. Future studies are needed to define the incidence and prognostic significance of FLT3 mutations outside the well-established JM and AL regions.**

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## Introduction

Somatic mutation of FLT3 is the most common acquired genomic abnormality found in acute myeloid leukemia (AML). Notably, internal tandem duplications (ITD) altering the juxtamembrane domain (JM) are found in 20–27% of *de novo* cases and 15% of secondary AML, and patients with these mutations have inferior disease-free and overall survival in most large studies.<sup>1–3</sup> An additional 5–10% of cases of AML are associated with missense or in-frame deletions of critical residues (D835 and/or I836) in the activation loop (AL).<sup>4</sup> These FLT3 mutations cause constitutive activation of FLT3 tyrosine kinase activity and can transform factor-dependent hematopoie-

tic cells as evidenced by conversion to factor-independent growth and formation of tumors in immunodeficient mice.<sup>5–7</sup>

FLT3 is structurally homologous to the KIT and PDGFR receptor tyrosine kinases (RTKs). The protein structure of these RTKs consists of an extracellular (EC) domain with five immunoglobulin-like repeats, a single transmembrane domain, a JM domain and a split kinase domain. The kinase domain is characterized by the amino-terminal (TK1) and carboxy-terminal (TK2) kinase lobes, which are separated by a hydrophilic kinase insert. The TK2 domain contains the kinase AL, a critical hinged region that must assume a particular conformation in order to allow full kinase activation.

KIT can be activated by mutation in a number of human diseases, including gastrointestinal stromal tumors (GIST) (EC, JM, TK1 and AL) (reviewed by Heinrich *et al.*<sup>8</sup>), seminoma (AL),<sup>9</sup> mastocytosis (AL, rarely JM)<sup>10</sup> and AML (JM, AL, EC).<sup>2–4</sup> As mentioned above, activating mutations of FLT3 involving the JM and AL have been previously reported. Based on the homology to KIT, we hypothesized that novel activating mutations of the FLT3 might occur outside the well-established gain-of-function mutations in the JM and AL region that contribute to the pathogenesis of AML.

In this study, we screened genomic DNA from 109 unselected cases of AML patients for activating FLT3 mutations and identified two new mutations. The first mutation, N841H, is similar to previously described mutations involving substitution of isoleucine or tyrosine for asparagine 841, which is located in the FLT3 activation loop. The second novel mutation, K663Q, is a novel gain-of-function mutation involving the TK1 domain. This mutation results in a constitutive activation of FLT3 kinase activity and kinase-dependent activation of downstream pathways involving AKT, mitogen-activated protein kinase (MAPK) and STAT5. Selective inhibition of K663Q kinase activity using a potent FLT3 kinase inhibitor (Sunitinib) abrogated the proliferation of factor-independent Ba/F3 cells expressing FLT3 K663Q and induced apoptosis. Our results indicate that further studies are needed to identify the true frequency and spectrum of FLT3 mutations in AML. Such studies may identify a larger percentage of patients with AML who might benefit from new therapeutic strategies targeting mutant FLT3 kinase.

## Materials and methods

### Cell lines

The wild-type (WT) FLT3 Ba/F3 cell line, a murine interleukin 3 (IL-3)-dependent hematopoietic pro-B-cell line, was obtained from the American Type Culture Collection (ATCC, Manassas,

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VA, USA). WT Ba/F3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 1% penicillin G (10 000 U/ml) and streptomycin (10 000 µg/mg), 2 mM L-glutamine (both Gibco-Invitrogen, Carlsbad, CA, USA) and filtered IL-3-containing supernatant (10%) from WEHI-3 cells (ATCC, Manassas, VA, USA). Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere.

### Patient samples

Archival samples of frozen low-density mononuclear cells from consenting patients were obtained from the Oregon Health & Science University Cancer Institute Leukemia Cell bank. Genotyping analyzes were performed as part of an Oregon Health & Science University (OHSU) Institutional Review Board-approved clinical research protocol.

### Site-directed mutagenesis and Ba/F3 generation of Ba/F3 cell lines expressing mutant FLT3

FLT3 cDNA was generously provided by Dr Olivier Rosnet (Molecular Oncology Unit, INSERM, Marseille, France) and cloned into an internal ribosomal entry-enhanced green fluorescent protein (pIRES-EGFP) plasmid (Clontech, Palo Alto, CA, USA) or the pXSN retroviral vector plasmid (BD Biosciences, Palo Alto, CA, USA). Site-directed mutagenesis was used to create the AL (K663Q) mutation (QuickChange Kit, Stratagene, La Jolla, CA, USA), which was confirmed by bidirectional sequencing.<sup>11</sup> Ba/F3 cell lines stably expressing FLT3 K663Q were generated by double selection for G418 resistance and IL-3-independent growth.<sup>6,12,13</sup> The creation of cell lines expressing WT FLT3, FLT3 ITD or FLT3 D835Y mutations was previously described.<sup>12,13</sup>

### Polymerase chain reaction

Genomic DNA was obtained from cryopreserved AML cases using a DNAeasy Tissue Kit (Qiagen Valencia, CA, USA). FLT3 exon 14, 16 and 20 was amplified using the following primer pairs: 5'TCTGCAGAACTGCCTATTCCT3' (FLT3 sense primer, exon 14), 5'TTTCCAAAAGCACCTGATCC3' (FLT3 antisense primer, exon 14), 5'TTTTAAATGCTCCTTCTTTGACA3' (FLT3 sense primer, exon 16), 5'AAGTGGGTTACCTGACAGT3' (FLT3 antisense primer, exon 16), 5'GCACTCCAGGATAATACACATCA3' (FLT3 sense primer, exon 20), 5'AACGACACAACACAAAATAGCCG3' (FLT3 antisense primer, exon 20). PCR amplification of genomic DNA was performed using 500 ng of DNA.<sup>14</sup>

### Denaturing wave high-performance liquid chromatography

Aliquots of 5–20 µl of each PCR reaction were assessed for FLT3 mutations using a Transgenomic WAVE HPLC system (Trangenomic Inc., Omaha, NE, USA).<sup>15</sup> Samples were run at 50°C to distinguish fragments of different lengths in exon 14 and at 56.9°C (exon 14), 61.6°C (exon 16) and 59.1°C (exon 20) to check for point mutations. Amplimers were bidirectionally sequenced on an ABI 310 sequencer using the BigDye terminator kit.<sup>14</sup>

### Antibodies and reagents

An anti-FLT3 rabbit polyclonal antibody, an anti-STAT5b mouse monoclonal antibody (both Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-AKT rabbit polyclonal antibody (New England Biolabs Inc., Beverly, MA, USA) and an anti-MAP

kinase 1/2 (Erk 1/2) rabbit monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA) were used at a 1:1000–1:2000 dilution. An anti-phosphotyrosine (Tyr591) FLT3 antibody, an anti-phosphoserine (Ser473) AKT antibody, an anti-phosphothreonine/tyrosine (Thr202/Tyr204) MAP kinase antibody and an anti-phosphotyrosine STAT5 (Tyr694) antibody were used at dilutions of 1:500–1:2000 (all Cell Signaling Technology, Beverly, MA, USA). Peroxidase-conjugated goat anti-mouse antibody and goat anti-rabbit antibody were used at 1:5000 and 1:10 000 dilutions, respectively (BioRad, Hercules, CA, USA). Protein A/G PLUS-agarose immunoprecipitation reagent was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The small molecule compound Sunitinib was obtained from Sugem (South San Francisco, CA, USA). The compound was dissolved in dimethylsulphoxide (DMSO) to create 10 mM stock solutions, which was frozen at –20°C. The recombinant human FLT3 ligand (FLT3LG) was purchased from R&D Systems (Minneapolis, MN, USA).

### Immunoprecipitation and immunoblotting

An total of ~5 × 10<sup>7</sup> cells were exposed to varying concentrations of Sunitinib and cultured for 90 min at 37°C in a 5% CO<sub>2</sub> atmosphere. Cell pellets were lysed with 100–150 µl of protein lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate with added inhibitors aprotinin, AEBSF, leupeptin, pepstatin, sodium orthovanadate and sodium pyruvate). A 75–200 µg portion of protein from cell lysates was analyzed by Western immunoblot assay as described previously.<sup>16</sup> Immunoprecipitation of FLT3 was performed as previously described.<sup>11</sup>

### Proliferation assays

Cells were added to 96-well plates at densities of 30 000 cells/well. Sunitinib was added and proliferation was measured after 72 h using an XTT-based assay (Roche Molecular Biochemicals, Indianapolis, IN, USA).<sup>17</sup> Dose–effect plots were created to calculate the IC<sub>50</sub> of proliferation inhibition of Sunitinib for each cell line.

### Apoptosis assays

Cells were incubated with Sunitinib for 72 h and translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane as an early indicator of apoptosis was analyzed using an AnnexinV-FITC Kit (Immunotech, Marseille, France) and a FACScalibur flow cytometer loaded with CellQuest analysis software (BD, Heidelberg, Germany).<sup>17</sup> Dose–effect plots were created to calculate the IC<sub>50</sub> for the treatment effect of Sunitinib for each cell line.

### Data analysis

Dose–effect plots were created to calculate the IC<sub>50</sub> for the treatment effect of Sunitinib using the Calcsyn software available from Biosoft (Cambridge, UK).

### In vivo modeling of tumor formation

BALB/c nude mice (6 weeks old) were injected subcutaneously with 1 × 10<sup>7</sup> parental Ba/F3 cells or Ba/F3 cells expressing FLT3 K663Q or FLT3 ITD. Mice were monitored daily for tumor size and formation and were killed when tumors reached approximately 1 cm<sup>2</sup>.

## Results

Using a combination of denaturing-high-performance liquid chromatography and direct sequencing, we examined genomic DNA of 109 unselected cases of AML from the OHSU Leukemia bank for FLT3 mutations involving the FLT3 JM, TK1 or AL domains. Consistent with previous reports, FLT3 ITD mutations were found in 15.6% of the cases (17/109).<sup>18</sup> In our series, 3.7% of the cases (4/109) had a previously described mutation of the FLT3 AL (two cases with D835Y, one case with del 1836 and one case with D835 H). In addition, we found one case with a novel mutation in the AL (N841 H) in an elderly patient with newly diagnosed AML FAB M5. The interchange of adenine with cytosine at nucleotide 2521 (exon 20) causes a substitution of histidine for asparagine at codon 841 and leads to similar conformational changes of the activation loop compared to the previously described substitutions of isoleucine (N841I) or tyrosine (N841Y) described by Jiang *et al*.<sup>19</sup> Notably, the FLT3 N841 H mutation is homologous to the KIT N822 H mutation found in seminoma or GIST.<sup>9,20</sup>

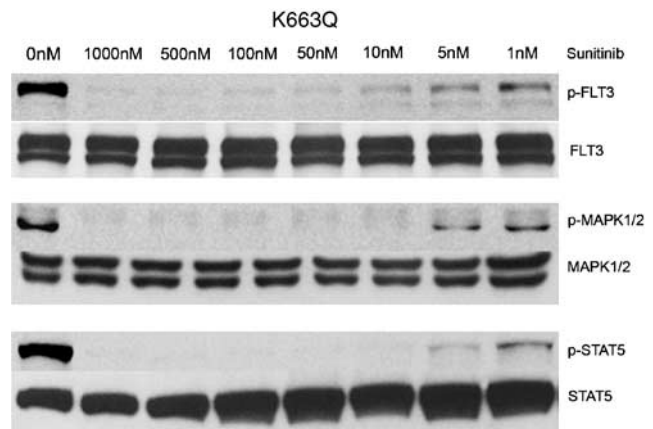
Importantly, we also identified the FLT3 K663Q mutation, which is located in the TK1 domain of FLT3 and represents a novel class of FLT3 mutations (Figure 1a and b). This mutation was found in an elderly patient with newly diagnosed acute monocytic leukemia (FAB M5). More specific clinical and cytogenetic data were not available. Interestingly, the FLT3 K663Q mutation is homologous to the KIT K642E mutation found in GIST.<sup>21</sup>

To verify that the glutamine (Q) substitution for lysine (K) at codon 663 is a gain-of-function mutation (as opposed to previously undescribed single nucleotide polymorphism), we expressed FLT3 cDNA encoding the mutant protein in Ba/F3, a murine IL-3-dependent cell line. Cells expressing FLT3 K663Q were transformed to IL-3-independent growth, consistent with previous reports of transformation by FLT3 ITD or D835 AL mutations.<sup>6</sup> In contrast, Ba/F3 cells expressing WT FLT3 require the continued presence of FLT3 ligand (FLT3LG) for IL-3-independent growth.<sup>22</sup>

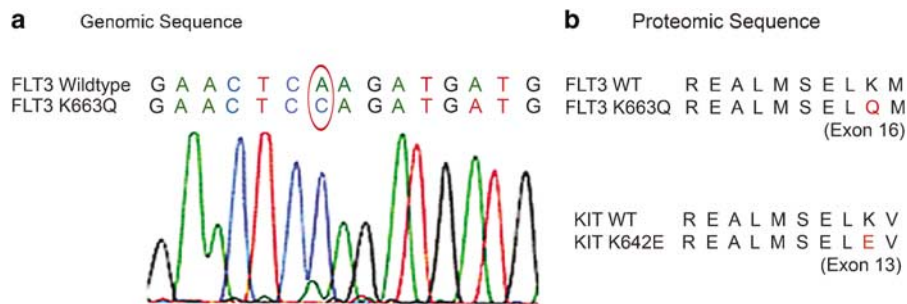
To further investigate the biological properties of these mutations, we examined the activation status of FLT3 by measuring FLT3 autophosphorylation. As shown in Figure 2, FLT3 K663Q was constitutively tyrosylphosphorylated in the absence of FLT3LG. In contrast, WT FLT3 expressed in Ba/F3 was only minimally phosphorylated in the absence of exogenous FLT3LG (data previously shown<sup>22</sup>). We tested the effect of a potent small molecule FLT3 inhibitor, Sunitinib, on the kinase activity of FLT3 K663Q. There was a clear dose-dependent effect of Sunitinib on the autophosphorylation of FLT3 K663Q with an IC<sub>50</sub> in the low nanomolar range (1–10 nM).

FLT3 ITD mutations are reported to constitutively activate the PI3K/AKT, RAS/MAPK and JAK/STAT5 pathways in cell lines and primary AML specimens. We assessed the activation status of these pathways in isogenic Ba/F3 cells stably expressing various FLT3 JM, TK1 or AL mutations using immunoblotting and reagents specific for activated (phosphorylated) forms of FLT3, AKT, MAP kinase (MAPK1/2) and STAT5. In our experiments, the STAT5, MAPK1/2 and AKT pathways were activated, regardless of the type of mutant FLT3 isoform expressed in Ba/F3 cells (Figures 2 and 3). However, there were some subtle differences in pathway activation between these cell lines, which may be due to quantitative differences in signaling between different FLT3 mutant isoforms (e.g. STAT5 activation is greater in Ba/F3 ITD and D835Y than in K663Q cells). More detailed biochemical studies are required to determine the molecular mechanisms underlying these differences in intracellular signaling.

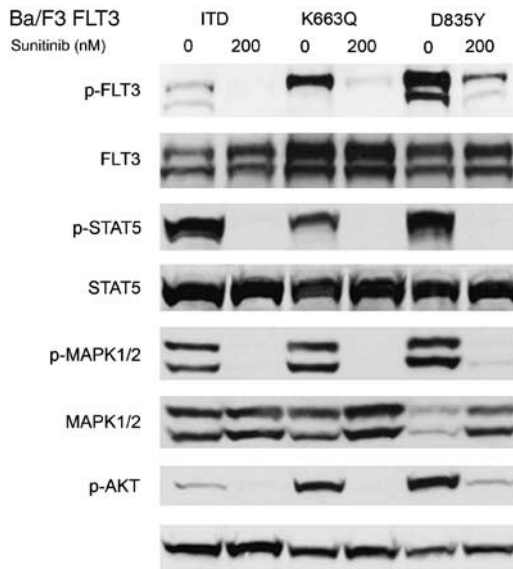
Notably, the kinase activities of FLT3 ITD, D835Y and K663Q were all sensitive to inhibition by Sunitinib with IC<sub>50</sub>s <100 nM (Figure 3).<sup>11,12</sup> Sensitivity of constitutive inhibition of the activity of downstream effectors (STAT5, MAPK1/2 (ERK1/1) and AKT) followed the sensitivity of inhibition of FLT3 autophosphorylation for each isoform (Figure 3). In addition, we demonstrate the dose-dependent effect of Sunitinib on the consecutive down-



**Figure 2** Sunitinib potently inhibits the constitutive kinase activity of FLT3 K663Q. Protein lysates were sequentially immunoblotted for phosphorylated and total forms of FLT3, and in addition, for forms of MAPK1/2 and STAT5 that are major effectors of FLT3 signaling. Sunitinib potently inhibited the autophosphorylation of FLT3 K663Q with an IC<sub>50</sub> of approximately 1–10 nM. FLT3 K663Q-dependent activation of MAPK1/2 and STAT5 signaling pathways was inhibited in the same dose range.



**Figure 1** Nucleotide and protein sequences of the FLT3 K663Q mutation. Genomic sequencing demonstrates heterozygous interchange of adenine with cytosine in exon 16 at nucleotide 1987 (a), producing a substitution of glutamine for lysine at codon 663 (b). The position of this mutation is homologous to the K642E KIT mutation associated with some cases of GI stromal tumors.



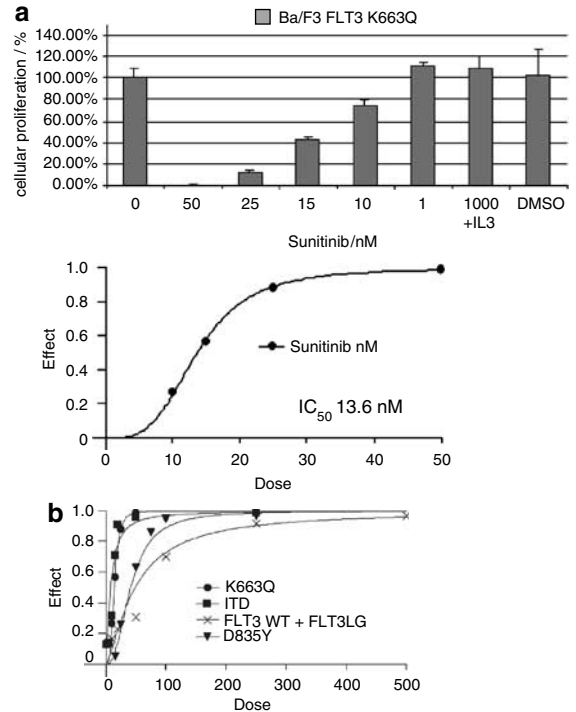
**Figure 3** Comparison of the activation of downstream signaling pathways in Ba/F3 cells expressing FLT3 ITD, K663Q or D835Y. A comparison of the signal-transduction pathway activation of Ba/F3 cells expressing different JM, TK1 or TK2 domain mutations is shown in a representative experiment. A 200  $\mu$ g portion of protein lysate was analyzed for each cell line. Regardless of the amount of phosphorylated FLT3 protein, the downstream pathways affecting phosphorylation of STAT5, MAPK1/2 and AKT were activated in all cell lines. Treatment with Sunitinib (90 min) led to a marked decrease in the expression of activated forms of FLT3, STAT5, MAPK and AKT in these cell lines. Consistent with previous reports, the D835Y mutant isoform was slightly less sensitive to Sunitinib than the other three FLT3 mutants.<sup>11,41</sup> This result is consistent with the cellular proliferation data shown in Figure 4b.

stream signaling in a dilution series for the MAPK1/2 and STAT5 phosphorylation, which was similar to that observed for inhibition of FLT3 autophosphorylation in the mutant FLT3 K663Q isoform (Figure 2).

As further proof of the transforming nature of these mutations, we examined the effects of Sunitinib on cellular proliferation. Using XTT-based proliferation assays, Sunitinib was found to have an estimated  $IC_{50}$  of 10–15 nM for Ba/F3 cells expressing K663Q (Figure 4a). Notably, in the presence of IL-3, a dose of 1000 nM Sunitinib did not significantly inhibit the proliferation of Ba/F3 K663Q cells. The inhibitory effect of any given dose of Sunitinib on the proliferation of Ba/F3 K663Q cells was very similar to that seen with Sunitinib-treated Ba/F3 FLT3 ITD or Ba/F3 D835Y. Consistent with previous reports, Sunitinib was slightly less potent in inhibiting the proliferation of cells expressing WT FLT3 (FLT3LG-dependent growth) or FLT3 D835Y<sup>11,12</sup> (Figure 4b).

To assess the oncogenic potential of FLT3 K663Q *in vivo*, we injected BALB/c nude mice with Ba/F3 cells expressing FLT3 K663Q or ITD as well as Ba/F3 parental cells as a control. Daily monitoring of tumor size and formation over a 30-day period indicated that parental Ba/F3 cells were incapable of inducing tumors *in vivo*, whereas Ba/F3 cells expressing FLT3 K663Q or ITD had oncogenic capacity to induce tumors. Notably, all five mice injected with either FLT3 ITD or K663Q developed tumors. A significantly shorter time to tumor formation was noted for Ba/F3 cells expressing FLT3 ITD (14 days) compared with Ba/F3 cells expressing FLT3 K663Q (22 days).

In order to assess whether Sunitinib is a potent inducer of apoptosis via inhibition of FLT3 signaling, we used a flow

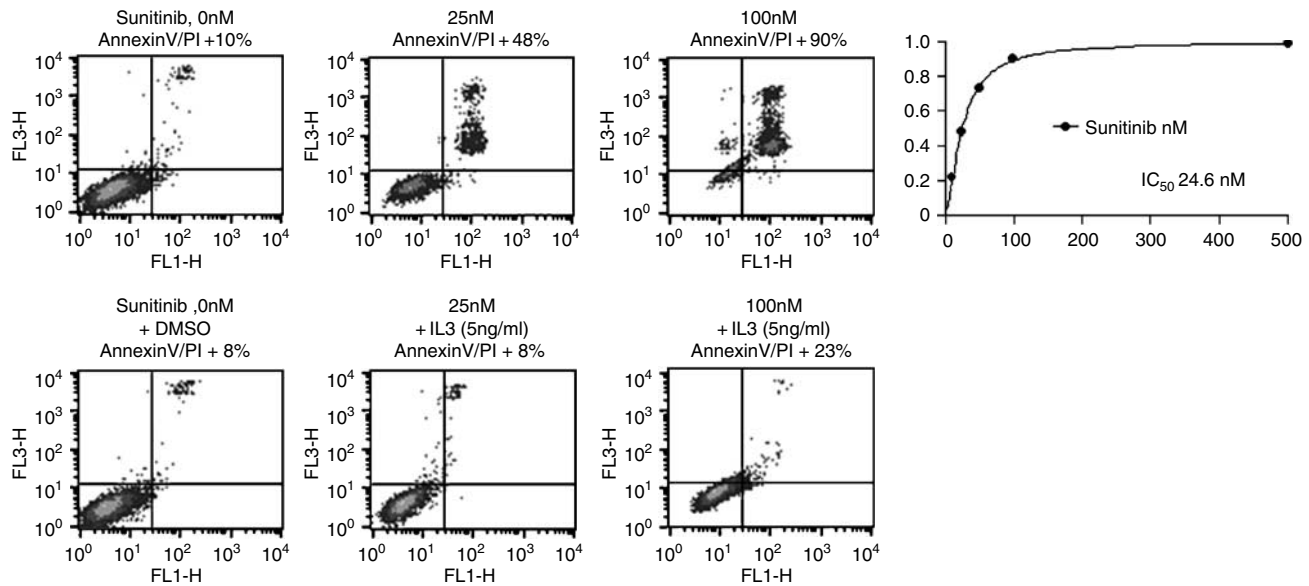


**Figure 4** Sunitinib inhibits cellular proliferation of Ba/F3 FLT3 K663Q cells in a dose-dependent manner. (a) Ba/F3 FLT3 K663Q cells were treated with Sunitinib  $\pm$  murine IL-3 (5 ng/ml, 12 h in advance of Sunitinib treatment) or vehicle only for 72 h and cellular proliferation was measured using an XTT-based assay. The results of a representative experiment are shown. Error bars indicate one standard deviation. Sunitinib successfully inhibits proliferation of Ba/F3 FLT3 K663Q with an  $IC_{50}$  of 13.6 nM, as shown in the dose-effect plot. The addition of IL-3 rescues these cells from the antiproliferative effects of Sunitinib, even using an Sunitinib concentration that is 100-fold greater than the  $IC_{50}$  for inhibition of FLT3 kinase (1000 nM). (b) Ba/F3 cells expressing WT or mutant isoforms of FLT3 were treated for 72 h with escalating doses of Sunitinib. Comparative dose-effect plot analysis of representative XTT-based proliferation assays reveals that the K663Q mutation has similar sensitivity to Sunitinib as cells with the ITD mutation. Sunitinib was slightly less potent in inhibiting the proliferation of cells expressing WT FLT3 (FLT3LG-dependent growth) or FLT3 D835Y.

cytometry-based assay (Annexin-V FITC/propidium iodide). Consistent with our biochemical data, Sunitinib induced apoptosis in Ba/F3 K663Q cells in a dose-dependent fashion with Sunitinib concentrations  $>50$  nM leading to an overwhelming induction of programmed cell death (Figure 5). The  $IC_{50}$  for induction of apoptosis was  $\sim 15$  nM for the FLT3 K663Q isoform. The addition of IL-3 rescued these cells from the proapoptotic effects of Sunitinib.

## Discussion

Somatic mutations in the JM and AL of FLT3 are the most common acquired genomic abnormalities found in AML. In most studies, patients whose AML cells express an FLT3 ITD have a worse outcome compared with cases of AML lacking this genomic abnormality.<sup>3,23–25</sup> The importance of FLT3 mutations in the leukemogenesis is further underlined by the hypothesis that so-called class I mutations that confer a proliferative and/or survival advantage (e.g. FLT3) cooperate with a class II mutation that impairs hematopoietic differentiation (e.g. AML1/ETO or PML/RARalpha).<sup>26</sup> Notably, it is the combination of class I and



**Figure 5** Sunitinib induces apoptosis of Ba/F3 FLT3 K663Q cells in a dose-dependent manner. Ba/F3 FLT3 K663Q cells were treated with Sunitinib  $\pm$  IL-3 (5 ng/ml, 12 h in advance of Sunitinib treatment) or vehicle only (DMSO) for 48 h before assessing apoptosis using a flow cytometry-based assay. Representative density dot blots illustrating the apoptotic cell subpopulation in the upper right quadrant are shown. Sunitinib strongly induces apoptosis of Ba/F3 FLT3 K663Q cells with an  $IC_{50}$  of 24.6 nM in the computed dose–effect analysis, whereas addition of IL-3 potently rescues cells from programmed cell death.

class II mutations that results in the pathogenesis of AML. This hypothesis is supported by murine experimental models in which retroviral transduction of primary murine bone marrow with FLT3-ITD cDNAs results in a lethal myeloproliferative syndrome.<sup>27</sup> In contrast, retroviral transduction of bone marrow derived from PML-RARalpha transgenic mice with FLT3-ITD results in a marked increase in the incidence of APL-like leukemia in mice when compared to those transduced with PML-RARalpha transgene alone.<sup>28</sup> These findings highlight the potential importance of FLT3 mutations in the development of AML and suggest that targeted inhibition of the kinase activity of dysregulated FLT3 kinase activity may be a promising treatment approach for this disease.

FLT3 is a type III receptor tyrosine kinase with close homology of the cytoplasmic domain to the related RTKs, KIT and PDGFRA. Activating mutations of KIT are found in the vast majority of GISTs and systemic mastocytosis, as well as a minority of cases of AML and seminoma. Activating mutations of PDGFRA are found in approximately 5–10% of GISTs (approximately 40% of GISTs lacking KIT mutations). Notably, these mutations of KIT or PDGFRA can involve the extracellular domain (KIT), JM (KIT and PDGFRA), TK1 (KIT and PDGFRA) and AL (KIT and PDGFRA).<sup>20,21,29</sup> The homology of described KIT, PDGFRA and FLT3 mutations is shown in Figure 6.<sup>2,18,30,31</sup>

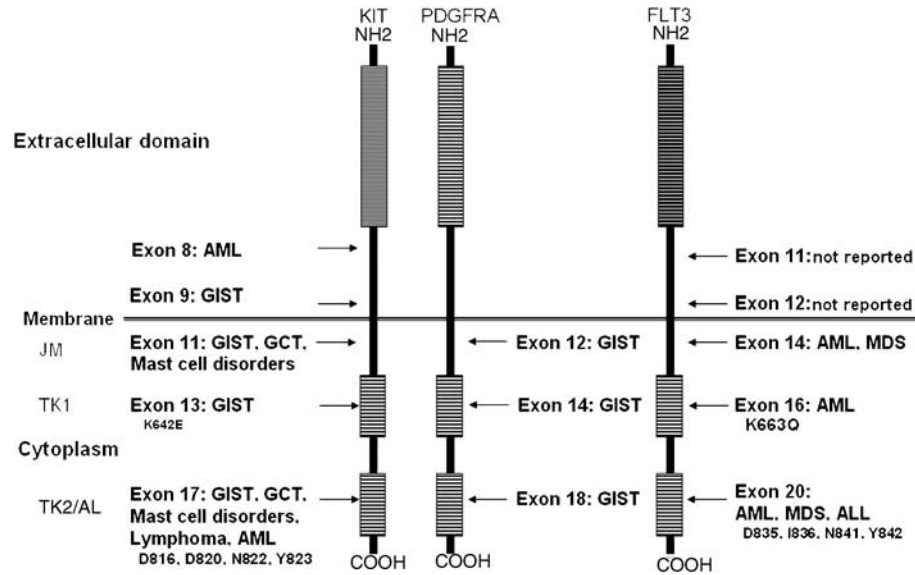
The vast majority of published studies on the clinical and/or biological aspects of FLT3 mutations have focused largely, if not exclusively, on FLT3 ITD mutations of the JM domain that are found in 15–30% of patients with AML. In addition, point mutations of FLT3 codon 835 or 836 are found in approximately 5–10% of cases of AML. Many of the studies examining AML cases for AL mutations have used methodology that would only detect mutations involving codon 835 or 836. To date, there has been no large systematic search for FLT3 mutations in AML beyond the well-described JM mutations and the D835/I836 mutations in the AL. Notably, we found a similar frequency of novel FLT3 mutations (K663Q and N841 H, one case each out of 109 cases) and previously reported D835/D836 mutations

(4/109 cases). Our results suggest that the true frequency of FLT3 mutations in AML may be underestimated, as there have been no large studies of FLT3 mutations in AML that have used a mutation detection strategy that would allow identification of TK1 or novel AL mutations.

The K663Q mutation is located in the TK1 domain of FLT3 and is homologous to the K642E mutation associated with GISTs. The activating nature was confirmed by the following lines of evidence: (1) transformation of BA/F3 cells to factor independence; (2) constitutive autophosphorylation of FLT3 in BA/F3 cells expressing the FLT3 K663Q isoform; (3) constitutive FLT3-dependent activation of the RAS/MAPK, PI3K/AKT and JAK/STAT5 pathways; (4) dose-dependent inhibition of FLT3 autophosphorylation by Sunitinib; (5) dose-dependent inhibition of downstream mediators such as MAPK1/2 and STAT5 by Sunitinib; (6) dose-dependent inhibition of proliferation and induction of apoptosis by Sunitinib in Ba/F3 cells expressing the FLT3 K663Q isoform; and (7) the ability of Ba/F3 K663Q cells to form tumors *in vivo*.

Jiang *et al.* reported the finding of FLT3 N841I and N841Y mutations in AML.<sup>19</sup> The transforming and biochemical properties of these alternative mutations involving codon 841 are likely similar to those of the N841 H mutation that we found in our series. Recently, other reports were published describing novel mutations in the JM<sup>32</sup> and TK2,<sup>33</sup> as well as in the TK1 domain (T680C).<sup>34</sup> Functional studies of the latter mutation are still ongoing, but in conjunction with our findings, it further supports the notion that mutations of the FLT3 receptor in the TK1 domain may be associated with leukemogenesis in a subset of cases of AML.

These data suggest that FLT3 mutations are more common and occur in a broader spectrum than previously described. Based on homology to KIT, we hypothesize that mutation of FLT3 codon 839 might also result in constitutive activation of FLT3. Indeed, screening of our AML cell bank revealed a case of AML with a D839G mutation (data not shown). In addition, the existence of activating mutations involving the KIT



**Figure 6** Comparison of the protein structure of KIT, PDGFRA and FLT3 and location of reported oncogenic mutations. The arrows indicate the regions of KIT, PDGFRA or FLT3 where mutations have been reported in association with different human neoplasms, including the novel mutations described herein. Based on homology considerations, we speculate that extracellular domain mutations involving FLT3 exons 11 and 12 could also result in constitutive activation of FLT3 kinase. Abbreviations: JM, juxtamembrane; TK1, kinase N-lobe; TK2/AL, activation loop of the kinase C-lobe; MDS, myelodysplastic syndrome; GIST, GI stromal tumor; GCT, germ cell tumor; ALL, acute lymphoblastic leukemia.

extracellular domain suggests the possibility that mutations of this region of FLT3 might also result in constitutive activation of FLT3. Further studies will be required to determine the true spectrum, frequency and prognostic significance of FLT3 mutations in AML.

Several potent small molecule FLT3 tyrosine kinase inhibitors (TKI) including Sunitinib, MLN518, PKC412 and CEP-701 show promising *in vitro* and *in vivo* anti-leukemic activity,<sup>11,35,36</sup> and clinical phase I and II trials are ongoing. In these studies, FLT3 TKIs inhibited phosphorylation of FLT3 with reduction of leukemic blast count and induction of clinical responses in patients with advanced disease.<sup>35,37,38</sup> To date, although some patients have had significant reductions in peripheral blood and to a lesser extent bone marrow blasts, there have been few if any durable complete remissions. More recent data suggest that combining FLT3 TKIs and conventional chemotherapy results in synergistically kills of leukemia cell lines or primary AML specimens expressing mutant forms of FLT3 kinase. Based on these studies, future clinical studies of FLT3 TKIs used in combination with conventional chemotherapy are being planned.<sup>12,39</sup> To date, clinical studies of FLT3 TKIs have largely targeted patients with FLT3 ITD-positive AML. However, our studies clearly indicate that the kinase activity of the FLT3 K663Q mutation is at least as sensitive as FLT3 ITD mutants to inhibition by Sunitinib. Therefore, patients with these mutations might benefit from treatment with FLT3 inhibitors such as Sunitinib.

Heidel *et al.* recently described a TK1 domain mutation at codon 676 as a cause of resistance in an FLT3 ITD-positive patient treated with the TKI PKC412.<sup>40</sup> Thus, FLT3 TK1 mutations can occur as primary gain-of-function mutations or as secondary mutations in the setting of acquired resistance to particular FLT3 TKIs. This is analogous to the situation in GIST, where the K642E TK1 mutation can be found as a primary oncogenic kinase in some patients, whereas the V654A TK1 mutation is a common secondary mutation found in imatinib-resistant GISTs.

## Conclusion

To the extent that FLT3 TKIs become part of the management of patients with AML associated with FLT3 mutations, it will be important to develop diagnostic testing strategies that would allow identification of all patients who may benefit from such FLT3 targeted therapies. In addition, further studies will be required to determine the true frequency of mutations lying outside of the JM and AL – in the *de novo* setting as well as in pretreated patients.

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