FLT3 and KIT in acute myeloid leukemia. Translational studies on oncogenic signaling from receptor tyrosine kinases.

Translational studies on oncogenic signaling from receptor tyrosine kinases

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FLT3 and KIT in Acute Myeloid Leukemia

Translational studies on oncogenic signaling from receptor tyrosine kinases

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Abstract

Acute myeloid leukemia (AML) is a life-treatening disease with an uncontrolled expansion of immature blasts in the bone marrow. Current forms of therapy are conventional chemotherapy and in some cases allogeneic stem cell transplantation. New techniques have recently promoted new insights into the complex genetic background of the disease. Around 60% of the genetic aberrations found in leukemia patients involve signaling pathways. The aim of this thesis has been to investigate the altered singnaling properties of two receptor tyrosine kinases, KIT and FLT3, which are frequently mutated in AML.

In our first study, we demonstrated that the ligand-independent activation of the KIT/V560D mutation is totally dependent on the binding to PI3-kinase, and like the KIT/D816V mutation, the contribution of PI3-kinase to ligand-independent activation of KIT/V560D is independent of its lipid kinase activity.

Next, we investigated how BEX1 and the FLT3/ITD mutation interact. By using gene expression data of primary AML patient samples, we found that loss of BEX1 expression reduced the median survival by around 50% (HR 1.697, p = 0.0452) in FLT3/ITD-positive patients. Furthermore we found that expression of BEX1 significantly reduced FLT3/ITD-dependent cell proliferation, enhanced apoptosis and reduced the number of colonies in semi-solid medium of both Ba/F3 and 32D cells. Moreover, BEX1 expression significantly reduced tumor volume and tumor weight in Ba/F3 as well as in 32D cells in xenografted mice.

In the third study we examined the role of HOX proteins in AML. We found that HOXB2 and HOXB3 were up-regulated in FLT3/ITD positive patients and the expression levels of HOXB2 and HOXB3 correlated to prognosis in AML. Lower expression levels correlated to enrichment of oncogenic pathways and induced overexpression of HOXB2 or HOXB3 inhibited FLT3/ITD downstream signaling as well as FLT3/ITD-induced biological events.

In the last study we showed that secondary resistance to sorafenib in FLT3/ITD-positive AML may be caused by up-regulation of the PI3K/mTOR signaling pathway even though the FLT3 receptor stays responsive to inhibition. Furthermore, the highly selective dual PI3K/mTOR inhibitor gedatolisib can override this resistance and block colony formation, decrease cell proliferation, induce apoptosis and block tumor growth in vivo.

To conclude, our studies have gained new insights into the role of mutated receptor tyrosine kinases in AML and suggested some new ways to counteract their oncogenic properties.

Key words: acute myeloid leukemia, KIT, FLT3, tyrosine kinase receptors, FLT3/ITD, HOX, BEX

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“In theory, theory and practice are the same. In practice, they are not.”

-Albert Einstein

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Lindblad O, Kazi JU, Rönnstrand L, Sun J. *Cell Mol Life Sci. 2015 72(22):4399-407.*

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II. BEX1 acts as a tumor suppressor in acute myeloid leukemia.

Lindblad O, Li T, Su X, Sun J, Kabir , Levander F, Zhao H, Lu G, Rönnstrand L, Kazi JU. *Oncotarget. 2015 6(25):21395-405*

III. The role of HOXB2 and HOXB3 in acute myeloid leukemia.

Lindblad O, Chougule RA, Moharram SA, Kabir NN, Sun J, Kazi JU, Rönnstrand L. *Biochem Biophys Res Commun. 2015 467(4):742-7.*

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IV. Aberrant activation of the PI3K/mTOR pathway promotes resistance to sorafenib in AML

Lindblad O, Cordero E, Puissant A, Macaulay L, Ramos A, Kabir NN, Sun J, Vallon-Christersson J, Haraldsson K, Hemann MT, Borg Å, Levander F, Stegmaier K, Pietras K, Rönnstrand L and Kazi JU. *Oncogene, advance online publication, March 21, 2016*

Introduction

Acute myeloid leukemia is a quite uncommon disease with around 350 new cases every year in Sweden. However, it can affect persons from early childhood to high ages and it is always a life-threatening condition requiring acute treatment. At present there is a tremendous development of new targeted drugs in hematology but unfortunately this is not true for AML where the standard treatment has been the same for over 50 years. The only real improvement of survival among these patients has been the introduction of allogeneic stem cell transplantations in the 1980's.

This thesis is based on the concept of translational research where findings in the clinical practice should be easily transferred and investigated in laboratory models. In our studies we have worked both ways. In three studies we started with clinical findings which were transferred and mimicked in the laboratory in order to investigate the underlying principles. In one study we compared two cell lines in the laboratory and found differences that we could confirm also existed between different patient groups.

It is today impossible to have a clear overview of the rapidly accumulating data on cellular signaling. Therefore an improved cooperation between clinicians and laboratory researchers is essential in order to know where to focus. The goal is to find cellular mechanisms that many patients share despite different genetic backgrounds of their diseases and to efficiently inhibit these mechanisms without causing too much side-effects.

List of Abbreviations

AML	Acute Myeloid Leukemia
FLT3	Fms-like Tyrosine Kinase 3
ITD	Internal Tandem Duplication
RTK	Receptor Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
JM	Juxtamembrane
TKD	Tyrosine Kinase Domain
PI3K	Phosphoinositide 3-Kinase
MAPK	Mitogen-activated Protein Kinase
ERK	Extracellular Signal-regulated Kinase
SCF	Stem Cell Factor
FL	FLT3 Ligand
mTOR	Mechanistic (or Mammalian Target of Rapamycin)
STAT5	Signal Transducer and Activator of Transcription 5
CBF	Core-Binding Factor
GIST	Gastrointestinal Stromal Tumor
BEX	Brain-Expressed X-linked

Background

The genomic landscape of AML

As around 50% of all AML cases are cytogenetically normal when using classical cytogenetic methods, the introduction of high-throughput sequencing has revealed the role of smaller-scale genomic abnormalities. Compared to solid tumors AML has a low average number of mutations[1]. The disease is characterized by clonal heterogeneity at the time of diagnosis, including both a founding clone and at least one subclone. During disease progression dynamic clonal evolution occurs and probably contributes to resistance to therapy[2]. The Cancer Genome Atlas Research Network has recently examined the genomes of 200 AML patients using whole-genome or whole-exome sequencing together with RNA and microRNA sequencing and DNA-methylation analysis. Genes that were significantly mutated were classified into eight categories according to their function: Mutations in signaling genes (i.e. FLT3, KIT) enhance proliferation through aberrant signaling pathways whereas mutations in myeloid transcription factors (i.e. RUNX1) or transcription factor fusions by chromosomal rearrangements (i.e. t(8;21)(q22;q22); RUNX1-RUNX1T1), deregulate transcriptional activity and impair hematopoietic differentiation. Mutations of the nucleophosmin (NPM1) gene induce an aberrant cytoplasmic localization of NPM1 and NPM1-interacting proteins whereas mutations of spliceosome-complex genes (i.e.SF3B1) confers deregulation of RNA processing. Mutations of cohesin-complex genes (i.e. STAG2) causes impairment of chromosome segregation and transcriptional regulation. Mutations of genes responsible for the epigenetic control of cells (i.e. ASXL1) impairs chromatin modification whereas DNMT3A and TET2 mutations, as well as IDH1/IDH2 mutations, promote deregulation of DNA methylation. Mutations in tumor-suppressor genes such as TP53 leads to impairment of transcriptional regulation and degradation of TP53[3].

Receptor tyrosine kinases

Around 60% of the identified mutations in AML are found in genes responsible for cell signaling[3]. Two of the most recurrently mutated genes, KIT and FLT3, are part of a family called receptor tyrosine kinases (RTKs). A kinase is defined as an

enzyme that transfers phosphate groups from high-energy donor molecules, such as ATP, to target molecules called substrates. This process is called phosphorylation. Subsequently, kinase enzymes that specifically phosphorylate the amino acid tyrosine are called tyrosine kinases. There are 20 different families of RTK's where the type III family consists of PDGFRα/β, FLT3, KIT and CSF1R. They all share a common structure of five extracellular immunoglobulin-like domains, a single transmembrane domain, a juxtamembrane (JM) domain and a split cytoplasmic kinase domain (TDK). RTK's are cell surface receptors for a wide array of molecules such as growth factors, hormones and cytokines. There are 90 tyrosine kinase genes in the human genome and 58 encode for receptor tyrosine kinase proteins with transmembrane domains. RTK's have been shown not only to regulate normal cellular processes but also to be involved in many types of malignancies[4, 5].

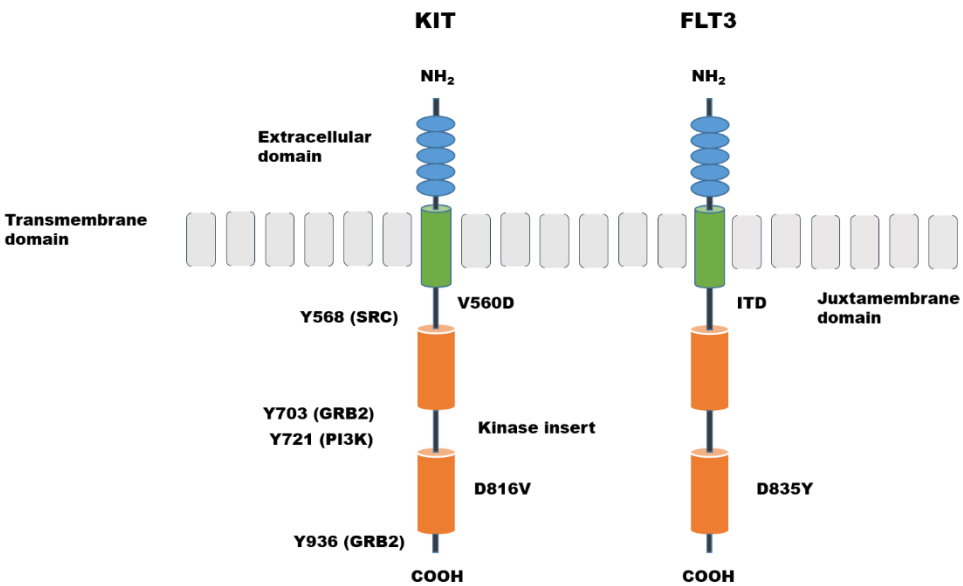

Figure 1. The KIT and FLT3 receptor with localisation of recurrent mutations and binding sites for some of the interacting proteins.

Signaling pathways

Transmembrane cellular receptors, like RTK's, transduce external signals into the different cellular compartments in order to control a wide array of cellular functions. These signaling pathways are biochemical cascades with serial chemical reactions

that finally produce or modify effector molecules. Some of the most studied pathways are briefly summarized below.

The PI3K-AKT-pathway

The family of lipid kinases named phosphatidylinositol 3-kinase was discovered in the 1980s and has been shown to play a major role in controlling cell proliferation and survival[6]. PI3Ks are mostly activated by receptor tyrosine kinases and G protein-coupled receptors, which leads to the generation of phospholipids which transduce signals. PI3Ks can be subdivided into three categories called class I (A and B), II and III where class IA seems to play a predominant role in cancer[7]. They are formed by a catalytic p110 subunit and a regulatory p85 subunit. Whereas p110 generates phosphatidylinositol 3,4,5-trisphosphate (PIP3), p85 interacts with upstream effectors through SH2-domains that bind to pYXXM-motifs in target proteins. There exist three p110 isoforms: p110α and p110β, both being expressed throughout the body, and p110δ whose expression is largely confined to the immune system[8]. Upon activation, Class I PI3Ks generate PIP3 at the plasma membrane, inducing the recruitment of PDK1 and AKT to the membrane[9]. The effects of PI3Ks are opposed by the tumor suppressor PTEN which converts PIP3 back to phosphatidylinositol 4,5-bisphosphate[10]. At the plasma membrane PDK1 phosphorylates AKT on a T308 residue and additional phosphorylation on S473 by mTORC2 fully activates AKT[11]. Activated AKT phosphorylates a large number of downstream effectors including MDM2, FOXO, BAD and Caspase-9, leading to cell growth, survival and proliferation[12].

The mTOR-pathway

mTOR is the target of a molecule named rapamycin or sirolimus® with anti-proliferative properties and was first studied in yeast in the early 1990s[13]. It forms two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2) with different sensitivities to rapamycin as well as upstream inputs and downstream outputs. The best characterized feature influenced by mTORC1 is protein synthesis as it directly phosphorylates the translational regulators eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1)[14]

The MAPK/ERK-pathway

The mitogen-associated protein kinase (MAPK)-pathway was one of the first kinase cascades to be studied. In mammals, several MAPK families have been found including ERK1/2, the JNK family and the p38 MAPK family[15]. ERK1/2 is activated upstream by a family of serine/threonine kinases called MAPK kinases or MEK which is itself activated upon phosphorylation by RAF family kinases (A-RAF, B-RAF or C-RAF)[16]. RAF is itself recruited to and activated in the plasma

membrane by association with RAS which in turn is activated by receptor tyrosine kinases through the GRB2/SOS complex [17].

The KIT receptor

In the early 1900s, mice with a white spot on their bellies were identified to have mutations of their W (dominant White spotting) locus. They also suffered from defects in hematopoiesis and gametogenesis, indicating these predominant functions of the gene along with melanogenesis. The Kit gene was first identified as the viral oncogene v-Kit of the Hardy-Zuckerman 4 feline sarcoma virus (HZ4-FeSV), an acute transforming virus produced by the transduction of feline c-Kit sequences into the Feline leukemia virus[18]. In 1988 the gene product of the W locus was identified as the receptor tyrosine kinase KIT[19].

Another mutation in mice, designated Steel (*Sl*), was observed to have an almost identical phenotype to that of the W locus. *Sl/Sl* homozygotic mice are deficient in both germ cells and coat pigment and die perinatally in macrocytic anemia. The protein encoded by the Steel locus, stem cell factor (SCF), was identified by three different groups in parallel. It was further shown to be the ligand that activates KIT[20-22].

SCF is secreted by fibroblasts and endothelial cells and acts as a multipotent growth factor for early progenitor cells in hematopoiesis with synergies to other factors such as G-CSF, IL-3 and erythropoietin. It further controls the differentiation, proliferation and migration of mast cells, melanocytes, and germ cells[23]. SCF has also been shown to mediate proliferation in acute myeloid leukemia[24].

There are two splice forms of SCF which only differs in exon 6. The longer splice form is rapidly cleaved to generate soluble SCF, whereas the shorter transcript without exon 6 produces a membrane-bound protein[23]. Membrane-bound SCF activates the receptor more persistently, resulting in different biological outcomes[25]. Furthermore, mice lacking membrane-bound SCF are sterile with severe anemia and defects in melanogenesis[26]. In contrast, mice lacking the proteolytic cleavage site display reduced numbers of mast cells and increased sensitivity to γ-radiation[27]. Membrane-bound SCF and soluble SCF display somewhat different signaling properties. One study has shown that p85, the regulatory subunit of PI3-kinase, binds to KIT upon stimulation by both splice forms of SCF but only soluble SCF can induce downstream activation of AKT[28]. It was also shown that PLC-γ is only activated by the membrane-bound isoform of SCF, but not by the soluble isoform

Human KIT consists of 976 amino acids and exists in two forms of 140 and 155 kD due to different N-linked glycosylation[29]. In addition, several isoforms of KIT exist in humans, produced by alternative splicing. The two best characterized splice transcripts differs in the presence or absence of the amino acid sequence GNNK in the extracellular domain[30]. Studies have shown that the absence of GNNK enhances KIT signaling, with qualitatively different signaling outcomes[31].

Other physiological processes that require KIT activity is the proliferation of germ cells[32], the migration of melanocytes from the neural crest to the dermis during embryonal development[33] and the function of interstitial cells of Cajal (ICCs) in the gut[34].

In humans an uncommon genetic disease known as the piebald trait with non-pigmented patches arises in individuals heterozygous for certain loss-of-function mutations in KIT[35]. Activating mutations of KIT are involved in mastocytosis[36], germ cell tumors[37], some subtypes of malignant melanoma[38] and gastrointestinal stroma cell tumors (GISTs)[39]. Other tumors establishes autocrine loops with production of both SCF and KIT (e.g. small cell lung carcinomas and malignant melanoma)[40, 41].

In AML, KIT mutations can be detected in around 30% of patients in a subgroup called core-binding factor leukemia (CBF-AML) which makes it the most frequently mutated RTK in this group. The mutations cluster at exon 17, corresponding to the activation loop of the kinase domain, and exon 8, encoding a part of the extracellular domain of the receptor. More rarely, mutations involving the transmembrane domain and juxtamembrane domains have been reported in CBF-AML[42, 43]. The role of mutated KIT as a sufficient cooperating second event in the development of CBF-AML has been demonstrated in mouse studies and cell lines. Transfection of tyrosine kinase domain mutants (e.g. D816V), as well as juxtamembrane mutants (e.g. 557-558Del) conferred cytokine-independent growth to murine 32D cells[44]. In a recent meta-analysis, KIT mutations in combination with t(8;21) but not inv(16) significantly increased the risk of relapse but did not affect the rate of complete remissions in patients with CBF-AML[45]. Gene expression analyses have detected high expression levels of KIT in CBF-AML regardless of mutational status, making both the overexpressed and mutated KIT a potential target for TKI therapy[46].

KIT inhibition

Several in vitro studies have shown inhibited growth of cells expressing wild-type KIT or various KIT mutants after exposure to different TKIs[47, 48]. Subsequently much effort is put into clinical trials evaluating the combination of TKIs with

conventional chemotherapy in KIT-positive AML, including imatinib, dasatinib and midostaurin[49].

Imatinib was introduced in GIST management in 2000 and tremendously affected the long-term prognosis of GIST patients out of which approximately 80% harbor KIT or PDGFRα mutations. It still remains the only approved first line treatment, with sunitinib and regorafenib representing the second and third line treatment[50, 51]. However, patients initially responding well to imatinib commonly develop acquired resistance through different mechanisms[52].

The majority of patients with systemic mastocytosis display activating mutations of KIT, most commonly D816V[53]. As the KIT/D816V mutation confers resistance to imatinib, clinical studies have evaluated the possible usefulness of other TKIs. Masitinib, a KIT and PDGFR-α/β inhibitor, reduced symptoms in 56% of the patients[54]. Similarly, dasatinib reduced symptoms in 6 out of 18 treated patients with indolent systemic mastocytosis in one study[55]. Midostaurin, a multitarget TKI, has also showed promising results in patients with advanced systemic mastocytosis[56].

PI3K inhibition

At the moment there is much activity invested in finding ways to inhibit PI3K in various cancers. In hematology, the first successful example of a drug targeting PI3K that has been taken into clinical practice is idealisib (Zydelig®). It preferentially inhibits PI3Kδ (110kDa) and has recently been approved for the treatment of relapsed/refractory CLL or for high-risk CLL with 17p-deletion.

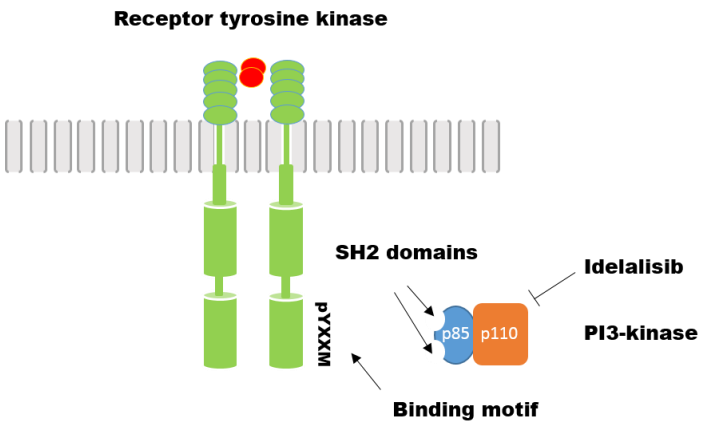

Figure 2. A schematic overview of the binding of PI3-kinase to an activated receptor tyrosine kinase.

In a phase III clinical trial, idelalisib combined with the anti-CD20 antibody rituximab significantly improved progression-free survival and overall survival in relapsed CLL patients compared to placebo plus rituximab. Efficacy appeared consistent in subgroups including those with high-risk prognostic markers (e.g. del17p and unmutated IGHV). Relevant side-effects included common elevations of liver transaminases, acute and chronic diarrhea/colitis, rash and pneumonitis. Neutropenia was another common finding.[57]. In general, the causal relations of idelalisib to these side-effects, as well as potential long term effects of PI3Kδ inhibition in patients are unknown. The disruption of regulatory T cell function for several years could possibly lead to increased risk of development of autoimmune disorders. Colitis has also been reported to occur in a murine model where PI3Kδ kinase activity was disrupted. Moreover, the PI3Kδ isoform is expressed by epithelial cells and has been shown to have a crucial role in lumen formation[58, 59].

Dual PI3K/mTOR inhibition

It has been demonstrated that selective mTOR inhibition paradoxically activate the PI3K pathway, thus providing a rationale for developing dual PI3K/mTOR kinase inhibitors. For that purpose the compound PKI-587 (PF-05212384) was developed and later named gedatolisib (see paper IV)[60]. It has been evaluated in preclinical studies with *in vitro* cell assays and *in vivo* xenograft models and recently the first human phase I study has been reported to show antitumoral effect in solid tumors[61]. There is currently a French phase II study registered for evaluating gedatolisib in t-AML/MDS, relapsed or refractory AML but it is not yet recruiting[49].

The FLT3 receptor

The FLT3 receptor was isolated by two different groups in 1991[62] and is expressed in immature hematopoietic cells, placenta, gonads, and brain[63]. It exists as a less glycosylated immature form of about 130–140 kD and a more glycosylated and plasma membrane expressed form of 155–160 kD[64]. The inactive FLT3 receptor exists in a monomeric, unphosphorylated form. Upon interaction of the receptor with FLT ligand (FL), the receptor unfolds allowing exposure of the dimerization domain, and subsequently receptor-receptor dimerization takes place. The tyrosine kinase enzyme is then activated through phosphorylation of multiple sites in the intracellular domain[65, 66]. The FLT3 ligand called FL was first sequenced in 1994[67].

FL is a homodimer and appears both membrane-bound as well as a soluble form due to alternative splicing or proteolytic cleavage, similar to the KIT ligand[68]. FL is a multipotent cytokine regulating many different cell types in hematopoesis[69] and is expressed in cells of the bone marrow microenvironment, including fibroblasts[70] as well as in hematopoietic cell lines of myeloid, and B- and T-cell lineages[71].

FLT3 in normal and malignant hematopoiesis

FLT3 has a critical role in normal hematopoiesis by regulating a number of cellular processes such as phospholipid metabolism, transcription, proliferation, and apoptosis. FLT3 function requires FL and other growth factors such as SCF and IL-3. Combinations of FL and other growth factors have been found to induce proliferation of primitive hematopoietic progenitor cells as well as more committed early myeloid and lymphoid precursors. FL stimulation appears to mediate differentiation of hematopoietic progenitors into monocytes without significant proliferation[72-74]. Experiments with targeted disruption of FLT3 in mice have resulted in healthy adult mice with apparently normal hematopoiesis. However, further studies showed deficiencies in pro-B and pre-B compartments and that stem cells lacking FLT3 were unable to reconstitute myeloid and T-lymphoid lineages in transplantation assays. Furthermore, combined FLT3 and KIT knock-out mice developed severe life-limiting hematopoietic deficiencies[75]. Mice lacking the FL gene exhibit significant impairment of the immune system with reduction of both myeloid and lymphoid progenitor cells as well as dendritic cells (DCs), and natural killer (NK) cells[76]

Over 90% of B-cell ALL and AML blasts express FLT3 at various levels. FLT3 receptors are also expressed less frequently in myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), T-cell ALL, and chronic lymphocytic leukemia (CLL)[77] There are studies suggesting that high levels of wt FLT3 may induce constitutive activation by itself in leukemic blast and may be associated with a worse prognosis[78, 79].

An internal tandem duplication (ITD) in the juxtamembrane (JM) domain of the FLT3 gene was one of the earliest detected mutations in AML, and is a common finding in approximately 25% of adult patients[80]. The length of duplicated DNA varies between 3 and more than 400 base pairs (bp), and although the exact position differs between cases it is always in frame and subsequently produces a functional protein. In vitro studies have proven that such insertions lead to a constitutively activated receptor [81]. Mice studies have shown that both loss of the wild-type allele and an increased gene dosage potentiate malignant transformation[82].

In wt FLT3 the JM domain is believed to act as a negative regulatory domain by preventing the activation loop from adopting to an active conformation, thereby maintaining the receptor in an auto-inhibited state. Crystallization studies of FLT3/ITD have shown that the ITD mutation disrupts the interaction between the JM domain and the kinase domain and hence the autoinhibitory conformation is lost and the receptor is activated[83].

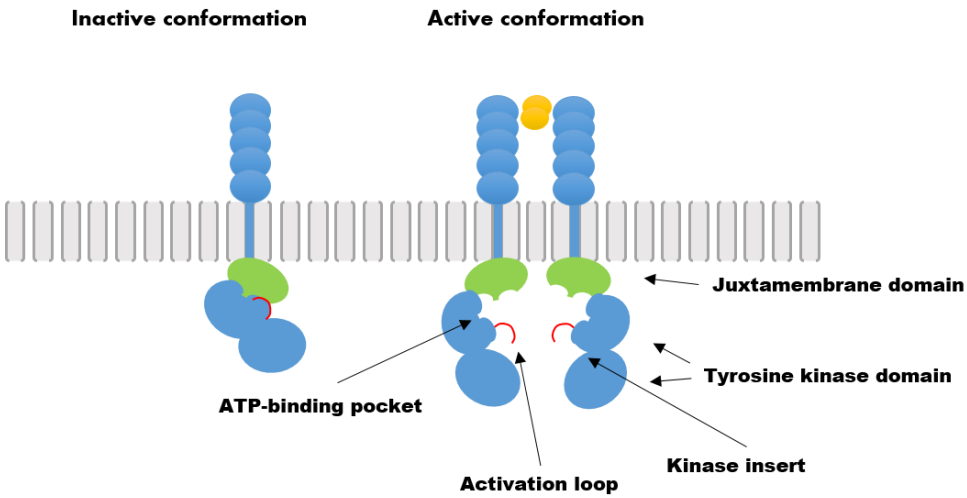

Figure 3. Upon binding to ligand the receptor dimerizes and adopts an active conformation with exposed ATP-binding pocket and kinase insert.

FLT3/ITDs are thought to promote proliferation through activation of multiple signaling pathways including RAS/MAPK, STAT5 and the AKT/PI3 kinase pathways. Demonstration of STAT5 activation in FLT3/ITD has pointed out some of the effects of FLT3/ITDs as unique for the mutated receptor. In contrast, ligand-induced wt FLT3 activation does not activate STAT5 and no STAT5 DNA binding occurs[84]. FLT3/ITD has been shown to be localized predominantly intracellularly to the endoplasmic reticulum with retained STAT5-activating and transforming capacity[85]. PIM-1, CDC25A and BAD are examples of specific downstream targets to STAT5 and key mediators of cell cycle progression and anti-apoptotic signaling[86]. In AML blasts BCL-2 is a transcriptional target of STAT5 and high BCL-2 levels has been shown to correlate with an FLT3/ITD duplication of Y591[87]. Furthermore, constitutively phosphorylated STAT5 has been detected in primary AML blasts with FLT3/ITD mutation and the levels of STAT5 correlates with the inhibition of FLT3/ITD[88].

Other studies have demonstrated that ligand-independent growth induced by FLT3/ITDs rely on aberrantly activated and constitutively phosphorylated AKT[89]. Human wt FLT3 cannot bind directly to the p85α subunit of PI3K but activates the PI3K pathway indirectly by phosphorylation of GAB1 and GAB2[90, 91]. Different aspects of FLT3-induced activation of the MAPK pathway have been demonstrated. For example, the interactions with GRB2 with phosphorylated tyrosines 768, 955 and 969 of FLT3 has been shown to result in ERK phosphorylation, through recruitment of GAB2 to the receptor[91]. Another study has shown that both wt FLT3 and FLT3/ITD activate ERK5 through MEK5 and that inhibition of MEK5/ERK5 induces apoptosis in AML cell lines expressing FLT3/ITD[92]. Furthermore, mutation of the SRC binding sites of FLT3, resulted in reduced ERK phosphorylation[93]. Several studies have shown phosphorylation and activation of SRC by FLT3, and that SRC inhibitors could block the oncogenic effects of FLT3 mutants in AML[94]. In one study the SRC kinase LYN was found selectively and constitutively phosphorylated downstream of FLT3/ITD when compared to wt FLT3, proposing a specific target for inhibition of FLT3/ITD signaling[95]. Furthermore, using phosphospecific antibodies against potential tyrosine phosphorylation sites in FLT3 it has been demonstrated that the FLT3/ITD, FLT3/D835Y and wt FLT3 have different patterns of autophosphorylation and kinetics of phosphorylation[96]

The most frequent kinase domain mutation is a substitution of aspartic acid 835 to tyrosine, D835Y, but it can also be to other amino acids[97]. Animal studies have shown that mice with ITDs primarily develop an oligoclonal myeloproliferative disorder while mice with TKD mutants are more likely to develop an oligoclonal lymphoid disorder[98].

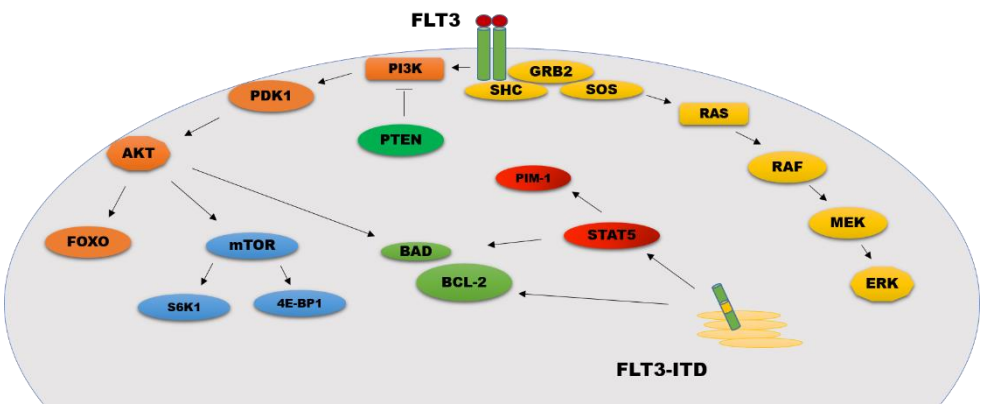

Figure 4. Schematic overview of some of the signaling pathways downstream of wild-type FLT3 and FLT3/ITD.

The clinical significance of FLT3 mutations

FLT3 is an important adverse prognostic marker in its mutated form in AML. The FLT3/ITD occurs at a frequency of 20-30% in AML patients[99, 100] and more frequently in younger (16–60 years) patients[99, 101, 102] Patients with FLT3/ITDs have leukocytosis, higher percentage of blast, higher relapse rate and poor overall survival (OS) compared to patients with wt FLT3[100, 103-105]. Complete remission rates in FLT3/ITD mutated AML are not lower than other AML but patients have poorer responses to salvage therapies[104]. TKD mutations affect around 5% of the AML patients and the prognostic significance is more unclear with studies reporting both favorable and unfavorable outcomes[97, 106, 107]. The correlation between FLT3/ITD allelic burden and clinical outcome has been investigated in several series where patients with high FLT3/ITD allelic burden seems to show a better response to FLT3 inhibitors compared to those with low allelic burden[108, 109]. The poor prognosis associated with FLT3/ITD mutations has drawn attention to the need of new treatment strategies to improve outcome. SCT as a consolidation strategy has been evaluated in several studies and been demonstrated to reduce the risk of relapse and to improve survival[110, 111].

FLT3 inhibition

The development of FLT3 inhibitors has been challenging, partly because of multiple mechanisms of resistance to FLT3 inhibition[112]. Another aspect is the complexity of AML, a disease driven by multiple abnormalities and that clonal evolution creates heterogeneity along with disease progression[2].

The FLT3 receptor has been indicated as a therapeutic target through the finding that FLT3/ITD AML patients under treatment with the FLT3 inhibitor quizartinib develop mutations in the tyrosine kinase domain of the FLT3 gene, which confer resistance to quizartinib. This also points at the status of the ITD as a driver mutation in AML[113]. Initial trials of FLT3 inhibitors used TKIs originally developed to treat solid tumors but these drugs where relatively nonspecific and caused off-target effects resulting in toxicity. This prompted the design of a newer, more specific FLT3 inhibitors with less toxicity[114].

The small molecule inhibitors targeting FLT3 are categorized as type I and type II TK inhibitors[115]. Type I TK inhibitors (e.g. midostaurin), bind in and around the region occupied by the adenine ring of ATP[116], whereas Type II TK inhibitors (e.g. sorafenib and quizartinib) bind to an allosteric site that is directly adjacent to the ATP binding pocket created by the activation-loop[117]. Therefore, type I TK inhibitors are thought to bind to both the inactive and active conformation of the FLT3 receptor and can efficiently block both FLT3/ITD and FLT3/TDK mutations

(i.e. D835), whereas type II TK inhibitors have limited activity against FLT3/TDK mutations[118]

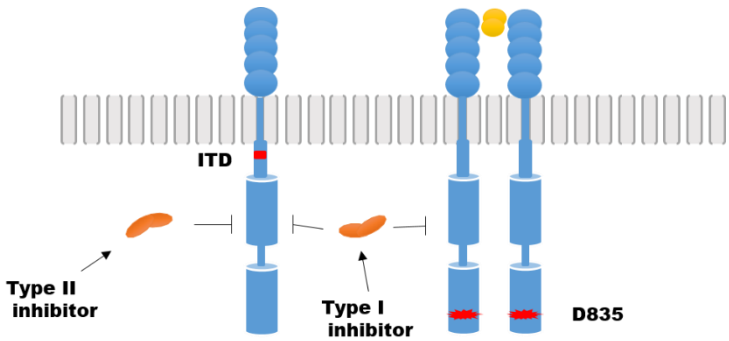

Figure 5. Inhibition of FLT3 by type I and type II tyrosine kinase inhibitors.

One way to measure FLT3 inhibition is to perform a plasma inhibitor activity (PIA) assay in which a cell line with a FLT3/ITD mutation is immersed in plasma from a patient who is taking a TKI. Phosphorylated FLT3 can then be measured by Western blot and followed by comparison with the baseline level. Multiple studies have shown that clinical responses to FLT3 inhibitors have correlated with inhibition to less than 15% of baseline[119, 120].

Single-agent FLT3 inhibition

Sorafenib, a TKI with activity against RAF kinase, VEGFR, KIT, and FLT3 is approved in the United States for unresectable hepatocellular carcinoma and advanced renal cell carcinoma. This drug is active against FLT3/ITD but not against other FLT3 mutants. Despite difficulties for patients to tolerate doses with inhibitory effect, in phase I studies some AML patients did show responses, including a number of patients who achieved complete remission (CR)[121, 122]. Later studies have demonstrated variable response rates in relapsed/refractory FLT3/ITD AML for single-agent sorafenib and that it may have a particular activity in the post-transplant setting[123, 124]. In one study with 29 patients who had relapsed following HSCT, three patients had sustained responses of more than 900 days and later studies have suggested a potential role for sorafenib in both the pre- and post-transplant settings[125, 126].

In two phase II studies, the multitarget TKI midostaurin (or PKC412) showed reduction of peripheral and bone marrow blasts by 50% or greater in patients who had a higher frequency of FLT3 mutations compared to wt FLT3[127, 128].

The FLT3-selective TKI lestaurtinib has been evaluated in a phase II study as monotherapy in older AML patients where three of five patients with FLT3-mutated AML achieved a hematologic response and 5 of 22 wild-type FLT3 patients showed a partial or complete bone marrow response[129].

Quizartinib (or AC220) selectively inhibits class III RTKs and has shown promising results in clinical trials. A phase II study with FLT3/ITD AML patients examined two cohorts: one with patients >60 years with relapsed/refractory AML, and one with patients with refractory disease after two lines of therapy or with relapse after HSCT. In this trial, the OR rate in the first cohort was 72% and in the second group 68%. In the second cohort, 34% of the patients could be bridged to allogeneic transplant with quizartinib monotherapy only[130, 131].

Crenolanib has activity against the FLT3/D835 TKD mutation and has been tested in phase II studies. Of 22 patients with relapsed FLT3/D835 AML, 6 patients reached CR, 11 patients exhibited a clinical benefit and 4 patients were successfully bridged to transplant[132].

Combination therapy

Studies of combination therapy have also been carried out in order to improve the efficacy seen with single-agent FLT3 inhibition. Regarding sorafenib, a phase I/II study of sorafenib combined with standard induction chemotherapy showed promising results but were more recently contradicted by a randomized, controlled trial of 201 AML patients greater than 60 years of age where the addition of sorafenib to induction therapy did not improve EFS or OS[133, 134]. Finally, in the SORAML trial with 267 newly diagnosed AML patients 18- 60 years old, where sorafenib or placebo were given to patients during induction and consolidation therapy and also as maintenance therapy for 12 months after the end of consolidation. Results has shown superior 3-year EFS in the sorafenib arm but also increased toxicity[135]. In another study with patients with relapsed/refractory FLT3/ITD AML, the combination of sorafenib with azacitidine has shown encouraging results[120].

Midostaurin in combination with conventional induction therapy has been investigated in a phase Ib study, with newly diagnosed AML patients. The induction was followed by high-dose cytarabine maintenance and 12 of 13 FLT3-mutated patients achieved CR[136].

The subsequent randomized phase III study of midostaurin with induction chemotherapy and one year of maintenance therapy ("RATIFY") was reported at the ASH (American Society of Hematology) conference 2015 and showed significantly improved EFS and OS in analyses both uncensored and censored for transplants, in patients with both ITD and TDK mutations and in patients with both low or high FLT3 mutational burden [137]. Midostaurin has also been combined with

azacitidine in a phase I/II trial in patients with AML or high-risk myelodysplasia, with an overall response rate of 26%. Patients with FLT3 mutations and no previously exposure to other FLT3 inhibitors achieved longer remissions than other patients[138].

Acquired resistance to FLT3 inhibition

The mechanisms of secondary AML resistance to FLT3 inhibitors remain unclear. Acquired resistance to the type 1 TK inhibitor midostaurin has been reported in a study where a N676K substitution within the FLT3 kinase domain was the only identified cause of resistance in a patient. Reconstitution experiments with 32D cells expressing the N676K mutant demonstrated that FLT3/ITD-N676K conferred an intermediate level of resistance to midostaurin in vitro[139]. In a recent study, eight relapsed ITD-mutated AML patients receiving quizartinib monotherapy were all identified with secondary point mutations in either the TKD1 or TKD2 region of the FLT3 gene including D835 and F691 mutations. These mutations were also cross-resistant to sorafenib[113]. Conversely, sorafenib resistance in two other AML patients with FLT3/ITD mutations was reported not to have any secondary point mutations in the FLT3 gene indicating other underlying mechanisms of reistance[140]. AML cells with TKD2 mutations showed more resistance to sorafenib compared to those with TKD1 mutations possibly due to conformational changes in the TKD2 that might alter sorafenib's binding affinity more than those associated with TKD1. Sorafenib-resistant patients with acquired TKD point mutations have also been reported to have longer length of ITD mutations than those without TKD point mutations, suggesting that a longer ITD mutation confers more instability the FLT3 gene[141] Studies have also showed that patients with longer FLT3/ITD mutations had a significantly shorter event-free survival (7.4 versus 12.6 months)[142].

BEX proteins

The physiological role of BEX proteins

The human BEX family consists of five proteins: BEX1, BEX2, BEX3 or p75NTR-associated cell death executor (NADE), BEX4 and BEX5. All BEX proteins contain a characteristic BEX domain which is also part of another class of proteins called transcription elongation factor A (SII)-like (TCEAL)[143, 144]. The function of this domain is not yet well characterized but recent studies claim BEX domain-containing proteins to be involved in the control of cellular growth[145] Human BEX genes have been mapped to be clustered at q22 on the X chromosome. BEX1 and BEX2 contain a conserved motif, Ser-Leu-Arg, which can be phosphorylated by

protein kinase C (PKC), a family of serine/threonine protein kinases known to be involved in many cancers[146-148] In murine BEX1, another more recent study has identified a serine phosphorylation site for the serine/threonine protein kinase AKT[149].

BEX1 was initially described as a gene down-regulated by retinoic acid treatment of the F9 murine teratocarcinoma cell line[150] and showed high expression in parthenogenetic blastocysts[151]. Further studies have identified high BEX1 expression in the human brain, pancreas, testis, and ovaries while the human heart, placenta, liver, kidney, spleen, thymus, prostate, small intestine, colon, thyroid, spinal cord, and adrenal gland express comparatively low levels of BEX1 mRNA[147]. Another study has demonstrated BEX1 expression in the central nervous system with high levels in the pituitary, cerebellum, and temporal lobe[143]. Human BEX1 localizes to the cytosol while rat BEX1 is found mainly in the nucleus explained by the fact that rat and human BEX1 shows only 67% sequence similarity (see Paper II) [143].

BEX1 has been identified as an important gene for muscle differentiation and has been suggested to play a role in skeletal muscle generation. BEX1 associates with calmodulin (CaM) in a Ca^{2+}-dependent manner[152]. Mice lacking BEX1 expression exhibit altered muscle regeneration but seem otherwise to develop normally[153].

BEX proteins have also been proposed to play a role in neuronal development. For example, BEX1, BEX2 and BEX3 proteins interact with the olfactory marker protein (OMP) and elevated BEX1 expression has been detected in spinal motor neurons (MNs) of peripheral myelin protein 22 (Pmp22) mutants and in mutant mice that develop MN degeneration[154, 155]. Up-regulation of BEX1 expression has also been observed in spinal cord MNs after axonal injury[156]. Moreover, BEX1 knockout mice displayed deficient recovery from sciatic nerve injury suggesting that BEX1 also has a role in neuronal regeneration[157].

The transmembrane receptor p75NTR is a member of the tumor necrosis factor (TNF) receptor superfamily which is involved in cell survival and apoptosis. It is a known receptor for neurotrophins among which nerve growth factor (NGF) is the most studied[158]. NGF treatment has been reported to elevate BEX3 protein levels in oligodendrocytes, suggesting a possible role of BEX3 in NGF-induced apoptosis. Furthermore, BEX3 and p75NTR complexes have been immunoprecipitated from proteasome inhibitor treated PC12 cell lysates after treatment with NGF and activation of caspase-2 and caspase-3 has been observed in BEX3 expressing cells in response to NGF, suggesting that BEX3 mediate apoptosis through activation of caspases[159].

Like BEX3, BEX1 was also found to be associated with p75NTR in PC12 cells where overexpression of BEX1 resulted in cell cycle arrest and neuronal

differentiation but had no effect on cell proliferation, thus suggesting a role of BEX1 in development of the nervous system. Furthermore, overexpression of BEX1 in PC12 cells was associated with reduced NFκB activity in response to NGF while knockdown of BEX1 potentiated NFκB activity in response to NGF. Based on these findings, it has been proposed that both BEX1 and BEX3 interact with p75NTR and regulate NGF-induced apoptosis and differentiation in neural tissues through NF-κB[149].

BEX proteins in malignant disease

In human breast cancer cells MDA-MB-231 overexpression of BEX3 suppressed *in vivo* tumor formation[160]. Playing an opposite role, BEX2 has been shown to act as a tumor promotor. Knockdown of BEX2 blocked the anti-apoptotic response to NGF-treatment in MCF7 breast cancer cells, suggesting that BEX2 is required for p75NTR to transduce the signal from NGF to NF-κB[161, 162].

Epigenetic control of tumor suppressor genes is common in human cancers and is mediated through alterations of chromatin structure, promoter hypermethylation, histone deacetylation, histone methylation, and other histone modifications[163]. DNA methyltransferase and histone deacetylase (HDAC) inhibitors such as Trichostatin A (TSA) and azacitidine have been extensively used to clarify the role of epigenetic modification in cancers[164]. In primary samples of glioma cells, treatment with TSA or azacitidine produced a strong induction of BEX1 and BEX2 expression where the epigenetic suppression was further shown to be mediated through promoter methylation and histone modification[165]. Furthermore, in the U251 glioma cell line BEX2 expression was shown to promote activation of the NF-κB pathway[166] Another study have reported elevated BEX2 expression in wt MLL (mixed-lineage leukemia) AML cell lines after treatment with demethylating agents and inhibitors of histone deacetylases. Moreover, hypermethylation of the BEX2 promotor region was reported to be reversed in wt MLL AML cell lines by demethylating agents and inhibitors of histone deacetylases[167]

BEX1 is reported to be upregulated in neuroendocrine tumors and was identified as one of the most frequently methylated genes in pediatric intracranial ependymoma, possibly silenced in its role as a tumor suppressor[168, 169].

BEX1 has been implied in acquired resistance to imatinib in CML. In a study with the BCR/ABL1 positive K562 cell line long-term treatment with imatinib resulted in loss of BEX1 expression and resistance to imatinib. Azacitidine could not restore BEX1 expression suggesting that downregulation of BEX1 was not related to hypermethylation of the BEX1 promoter region. On the other hand, overexpression of BEX1 could effectively reverse imatinib resistance. The BEX1 expression did

not block AKT or NFκB activation downstream of BCR/ABL1, but it activated the JNK pathway. BEX1 was also demonstrated to activate pro-apoptotic caspase 3/7 through the non-classical pathway in the presence of imatinib. Furthermore, the neuronal receptor protocadherin 10 (PCDH10) could be co-immunoprecipitated with BEX1 and was downregulated in cells where BEX1 expression had been lost. Depletion of PCDH10 resulted in resistance to imatinib in K562 cells, suggesting PCDH10 to be involved in BEX1-induced apoptosis in response to imatinib[170]. A later study identified BCL-2 as an interacting partner to the BEX1 protein using a yeast two-hybrid screen and subsequently confirmed by co-immunoprecipitation assays. Like BCL-2, BEX1 was found to localize to the mitochondria. Additionally, the interaction between BCL-2 and BEX1 promoted imatinib-induced apoptosis by suppressing the formation of anti-apoptotic BCL-2/BCL-2-associated X protein (BAX) heterodimers[171].

The p75NTR signaling pathway seems also to be involved in chemoresistance to daunorubicin in AML as PSIP1 (or LEDGF/p75) has been shown to be upregulated in patient samples. PSIP1 is a transcription coactivator and activates p75NTR. PSIP1 has also been linked to the MLL histone methyltransferase[172, 173].

HOX proteins

The homeobox (HOX) genes are a family of homeodomain-containing transcription factors initially characterized in *Drosophila* as master regulators of trunk and tail development during embryogenesis. The human HOX genes are clustered in four different chromosomes, 7p15 (HOXA), 17q21 (HOXB), 12q13 (HOXC) and 2q31 (HOXD)[174, 175]. HOX proteins are dependent on cofactor interactions where the most important are the pre-B-cell leukemia (PBX) and myeloid ecotropic insertion site (MEIS) families. These cofactors play essential roles in development and hematopoiesis[176]. HOX genes are expressed in HSCs and progenitors in lineage and differentiation stage-restricted patterns. As an example, HOXB3, HOXB4 and HOXA9 are highly expressed in uncommitted hematopoietic cells, whereas HOXB8 and HOXA10 are expressed in myeloid committed cells. The different HOX clusters also show specific patterns of lineage-restricted expression, where HOXA genes are expressed in myeloid cells, HOXB genes in erythroid cells and HOXC genes in lymphoid cells. Despite having similar regulatory regions to the other clusters, the HOXD genes are not expressed in normal hematopoiesis[177]

The role of HOX genes in normal hematopoiesis has been extensively studied using gene expression analysis and knock-in or knock-out studies in HSCs and early hematopoietic progenitors. In general, the overexpression of a HOX gene leads to stem and progenitor cell pool expansion paired with a block on differentiation. To

exemplify, studies on overexpression of HOXA10 in human CD34+ hematopoietic progenitors have showed increased production of blast cells and myelopoiesis concomitant with a complete block of erythroid differentiation and a severe reduction in B-cell development[178]. HOXA9 is the most expressed HOX gene in human CD34+ HSCs and early hematopoietic progenitors and is subsequently down-regulated during differentiation. In mouse models HOXA9 overexpression enhances HSC expansion and myeloid progenitor proliferation and, with a long latency, causes leukemia[179]

Upstream regulators of HOX genes include transcriptional activators such as mixed lineage leukemia (MLL) and a family of caudal-type HOX transcription factors (CDX1, CDX2 and CDX4)[180]. Studies have demonstrated a dramatic reduction in HSCs and hematopoietic progenitors in MLL conditional knockout mice[181].

In breast cancer knockdown of HOXB2 using shRNA resulted in increased tumor growth[182]. In contrast, studies on cervical cancer and other malignancies have suggested that overexpression of HOXB2 is associated with cancer progression[183]. Expression of HOXB3 has also been linked to increased cell proliferation in several tumors[184].

HOX proteins in AML

In numerous studies HOX genes have been shown to advance the development of AML by forming chimeric fusions with other genes. One of the most frequent fusion partners for HOX genes is NUP98, which is a member of the nuclear pore family and serves as a selective transporter for RNA and proteins between the nucleus and cytoplasm. In AML, NUP98-HOXA9 is associated with a t(7;11)(p15;p15) translocation[185]. Among several HOX proteins, HOXB3 has been shown to be a potential leukemogenic partner with NUP98[186]. NUP98-HOX fusion proteins in mice result in AML with a latency of around a year but this can be reduced to two months by co-overexpression of the HOX cofactor MEIS1 and FLT3[187].

MLL fusion proteins are found in about 10% of therapy-related AML and 3% of de novo AML. In normal hematopoiesis MLL induces the transcription of HOX genes whereas MLL fusion proteins activate HOX gene transcription more efficiently than MLL alone especially together with their co-activator MEIS1. A number of studies have tried to demonstrate that expression of HOXA genes is crucial for MLL leukemogenesis[188]. Other studies have contradicted this but found that their expression affects disease phenotype and prevents apoptosis of leukemic blasts[189]. In NPM1-mutated AML the relocation of NPM1 into the cytoplasm causes upregulation of a number of HOX genes including HOXB2 and HOXB3[190].

Present investigation

Aims

The general aim of the present thesis has been to translate clinical findings related to the receptor tyrosine kinases KIT and FLT3 in patients with acute myeloid leukemia into the laboratory in order to improve the understanding of the underlying altered signaling mechanisms that contribute to the disease.

Specific aims

To elucidate the specific downstream oncogenic signaling properties of the KIT/V560D mutant in comparison with the KIT/D816V mutant (Paper I)

To clarify the role of BEX1 as a putative tumor suppressor in patients with FLT/ITD -driven AML (Paper II)

To investigate the role of HOXB2 and HOXB3 up-regulation in a subgroup of AML patients carrying FLT3/ITD (Paper III)

To explore the mechanisms of secondary resistance to FLT3 inhibition by sorafenib (Paper IV)

Results and discussion

I. PI3 kinase is indispensable for oncogenic transformation by the V560D mutant of c-Kit in a kinase-independent manner

In this study, we wanted to investigate the altered signaling properties of the KIT/V560D mutant that has been detected in patients with GIST and CBF-AML. Our group has previously found a difference between wt KIT and KIT/D816V in the use of SRC family kinases and PI3 kinases for receptor activation and downstream cellular signaling[191]. To compare the transforming ability of these two types of mutants, murine Ba/F3 cells were transfected with either wt KIT, KIT/V560D or KIT/D816V. Cell survival and proliferation assays of transfected Ba/F3 cells in the presence or absence of SCF showed that KIT/D816V supported both cell survival and cell proliferation independent of SCF stimulation whereas KIT/V560D showed moderate cell survival and weak cell proliferation regardless of SCF stimulation, suggesting a weaker transforming ability of KIT/V560D compared to KIT/D816V.

By using a phospho-specific antibody, we found that Y568, the binding site of SRC family kinases in KIT, is strongly phosphorylated in cells expressing KIT/V560D independent of SCF stimulation. In contrast, phosphorylation of the GRB2 binding sites Y703 and Y936, and PI3 kinase binding site Y721, were weaker in KIT/V560D than in KIT/D816V. In addition, the total phosphorylation of KIT/V560D was weaker than that of KIT/D816V. The ligand-independent phosphorylation of downstream signaling molecules such as AKT and ERK was also weaker in KIT/V560D-expressing cells compared to KIT/D816V-expressing cells, suggesting that KIT/V560D weakly activates downstream signaling through GRB2 and PI3 kinase leading to weaker cell proliferation and survival. Furthermore, by using phosphotyrosine antibodies, we demonstrated that KIT/V560D ligand-independently phosphorylates GAB2, SHC and SHP2, but weaker than that of KIT/D816V expressing cells.

Our group has previously found that KIT/D816V circumvents a requirement of SRC family kinases for its activation and signaling[192]. To explore if the KIT/V560D mutant was still dependent on SRC family kinases we used a selective SRC family kinase inhibitor, SU6656, and showed that activation of KIT/V560D was indeed independent of the kinase activity of SRC. Furthermore, using an in vitro kinase assay utilizing the SRC optimal peptide as a substrate, we could show that KIT/D816V displays substrate specificity similar to SRC family kinases while both wt KIT and KIT/V560D do not show any such activity even in the presence of SCF.

Our group has previously shown that KIT/D816V-mediated cell transformation is independent of the lipid kinase activity of PI3K[191]. By adding the M724A mutation

into the KIT/V560D background to block the direct PI3 kinase binding, we found that the ligand-independent activation of KIT/V560D was totally blocked but not the ligand-induced activation. Ligand-dependent phosphorylation of AKT was partially reduced while ERK phosphorylation remained unchanged. These findings suggest that the KIT/V560D mutant can transduce signals without recruiting PI3 kinase but PI3 kinase association is required for constitutive activation of the receptor. By using the PI3 kinase inhibitor LY294002, we aimed to elucidate whether this effect is dependent on the lipid kinase activity of PI3K. LY294002 did not block the activation of KIT/V560D independent of SCF stimulation. Furthermore, AKT phosphorylation was inhibited in the presence of LY294002, indicating that PI3K is activated by KIT/V560D. A cell proliferation assay in the presence of SRC family kinase and PI3 kinase inhibitors further confirmed the lipid kinase independent role of PI3 kinase in KIT/V560D signaling.

In conclusion, we showed that the ligand-independent activation of KIT/V560D is totally dependent on the binding to PI3-kinase, and like KIT/D816V, the contribution of PI3-kinase to ligand-independent activation of KIT/V560D is independent of its lipid kinase activity.

Given the increasing efforts on targeting PI3-kinase with new drugs further studies are needed to explore the different aspects of PI3-kinase in normal and oncogenic signaling. Our study is an example of translational research where a mutation is found in a patient sample, transferred to the laboratory setting and investigated for its specific properties. Since currently available PI3-kinase inhibitors block only its lipid kinase activity and not the binding to other signal transduction molecules, they would have little clinical effect in malignancies driven by mutated KIT such as mastocytosis, GIST and CBF-AML. Moreover, existing PI3-kinase inhibitors in clinical use have shown side-effects such as colitis and pneumonitis[59]. Consequently, to identify this unexplored novel activity of PI3-kinase and develop new inhibitors against the non-lipid kinase activity may be a new direction for drug development.

II. BEX1 acts as a tumor suppressor in acute myeloid leukemia

In this study, we wanted to address the role of BEX1 in FLT3/ITD-mutated AML. The role of BEX proteins in human cancer has only been briefly studied and they seem to act both as tumor suppressors and promoters in different settings[160, 162].

In a gene expression analysis of two FLT3/ITD-positive cell lines, BEX1 was found to be strongly down-regulated in MV4-11 cells compared to MOLM-13 cells and the cell lines showed differential response to drug-induced apoptosis. Furthermore, in a data set of primary AML patient samples BEX1 was also found to be down-regulated in a subgroup. By using gene expression data (GSE6891, N = 525) of

primary AML patient samples, we found that loss of BEX1 expression correlated to a reduction of median survival by around 50% (HR 1.697, p = 0.0452) in patients carrying FLT3/ITD. However, the overall survival of the entire patient group, including both FLT3/ITD positive and negative patients, did not display any correlation to the BEX1 expression levels.

Next we aimed to analyze whether loss of BEX1 expression results in up-regulation of any oncogenic pathways. Using gene set enrichment analysis (GSEA), enrichment of several oncogenic pathways including loss of TP53 function, KRAS and RAF pathways in MV4-11 cells in comparison with MOLM-13 cells was observed. Furthermore, similar enrichment of pathways was observed in FLT3/ITD positive AML patients with lower BEX1 expression.

To assess the biological outcomes of BEX1 expression we generated two cell lines by stably transfecting FLT3/ITD along with BEX1 in the pro-B cell line Ba/F3 and the myeloid cell line 32D. Expression of FLT3/ITD and BEX1 was verified by western blotting. Expression of BEX1 significantly reduced FLT3/ITD-dependent cell proliferation, enhanced apoptosis and reduced the number of colonies in semi-solid medium of both Ba/F3 and 32D cells. Moreover, BEX1 expression significantly reduced tumor volume and tumor weight in Ba/F3 as well as in 32D cells in xenografted mice.

Next we investigated how BEX1 and FLT3/ITD interacts. Sub-cellular localization of BEX1 in FLT3/ITD expressing cells was checked by using confocal microscopy. We observed that BEX1 localized to the cytosol independent of FLT3/ITD activity suggesting that FLT3/ITD is not the direct target of BEX1. Furthermore, BEX1 expression did not alter ubiquitination, tyrosine-phosphorylation or degradation of FLT3/ITD. In order to analyze FLT3/ITD downstream signaling we used phospho-specific antibodies. We observed that BEX1 expression significantly blocked AKT phosphorylation in both Ba/F3 and 32D cell lines, but not ERK1/2 phosphorylation or STAT5 phosphorylation.

In summary, our *in vitro* and *in vivo* data supports the role of BEX1 as a tumor suppressor in AML with a specific role in FLT3/ITD positive AML patients.

Our study suggests that down-regulation of BEX1 has a negative effect on overall survival for patients with FLT3/ITD mutations but not in AML in general. We were not able to establish any direct interaction between the two proteins but high expression of BEX1 was found to block AKT phosphorylation downstream of FLT3/ITD.

A couple of studies have proven BEX proteins to be under epigenetic control. For example, epigenetic suppression of BEX1 and BEX2 in glioma cells was shown to be mediated through promoter methylation and histone modification and could be reversed by treatment with azacitidine[165]. Furthermore, epigenetic silencing by

hypermethylation of the BEX2 promotor region has been reported in wt MLL AML cell lines which could be restored by demethylating agents[167]. Azacitidine has been established as first-line treatment for higher risk groups in MDS[193] and is also used in older AML patient to keep the disease under control when there is no curative option[194]. In a recent study on patients with relapsed/refractory FLT3/ITD AML, the combination of sorafenib with azacitidine has shown promising results[120]. Consequently, a study to test if FLT3/ITD-positive AML patients with suppressed BEX1 expression could benefit from treatment with azacitidine would be valuable.

BEX1 has been found to localize in the mitochondria in one study. Here, by suppressing the formation of anti-apoptotic BCL-2/BCL-2-associated X protein (BAX) heterodimers, imatinib-induced apoptosis was promoted in a K562-derived cell line[171]. BCL-2 has also been evaluated as a drug target in AML. One study showed that ABT-199 (venetoclax), with demonstrated activity against CLL, also had promising effects in AML. Furthermore, it was reported that the FLT3/ITD positive cell line MOLM-13 responded well to ABT-199[195]. Another report showed up-regulation of BCL-2 through STAT5 in AML blasts with the juxtamembrane FLT3 mutant Y591. This report further suggested that high expression of BCL-2 increased the survival of AML blasts by inactivating pro-apoptotic TP53 signaling pathways[87]. Interestingly, we found that low expression of BEX in AML blasts and MV4-11 cells conferred down-regulation of the TP53 pathway. Thus, one possible hypothesis would be that low BEX1 expression confers a shorter overall survival in FLT3/ITD positive AML patients through up-regulation of BCL-2 in the mitochondria. We do not know why the BEX1 expression differs within the group of FLT3/ITD-positive patients but one hypothesis is that different types of ITD mutations confers different signaling properties, exemplified by the study above.

III. The role of HOXB2 and HOXB3 in acute myeloid leukemia

In this study, we aimed at the function of HOXB2 and HOXB3 in AML with FLT3/ITD mutations. The homeobox (HOX) genes are a family of transcription factors involved in body development during embryogenesis. The role of HOXA proteins in AML has been thoroughly investigated whereas the role of HOXB proteins remains more unclear.

First, we analyzed gene expression data from 598 cases of de novo AML and found that several HOXB family genes were up-regulated in FLT3/ITD positive patients. Higher expression of either HOXB2 or HOXB3 correlated with poorer overall survival compared to lower HOXB2 and HOXB3 expression and lower HOXB2 and HOXB3 expression further correlated with better event-free survival. However, as we did not detect any prognostic significance in only FLT3/ITD-dependent AML, this was only seen in the total patient group independent of FLT3 mutations. We

also observed a strong correlation between expression of HOXB2 and HOXB3 (r^2 = 0.8633). In addition, HOXB2 and HOXB3 expression was also up-regulated in patients carrying the NPM1 mutations and expression of both HOXB2 and HOXB3 was significantly down-regulated in the FAB M3 patient group (APL; acute promyelocytic leukemia).

Using gene set enrichment analysis (GSEA) we observed enrichment of several oncogenic pathways including loss of RB and p107 function, loss of SNF function and E2F3 pathways in FLT3/ITD positive AML patients with lower HOXB2 or HOXB3 expression. To further study the role of HOXB2 and HOXB3 in FLT3/ITD signaling we generated two cell lines by stably transfecting the pro-B cell line Ba/F3 with FLT3/ITD together with HOXB2 or HOXB3. Expression of HOXB2 or HOXB3 reduced the size and number of colonies in semi-solid medium, reduced FLT3/ITD-dependent cell proliferation and enhanced apoptosis. To analyze FLT3/ITD downstream signaling we used phospho-specific antibodies and demonstrated that HOXB2 and HOXB3 overexpression blocked FLT3/ITD-dependent AKT, ERK1/2, P38 and STAT5 phosphorylation in Ba/F3 cells.

In summary, HOXB2 and HOXB3 was up-regulated in FLT3/ITD positive patients and the expression levels of HOXB2 and HOXB3 could be correlated to prognosis in AML. Lower expression levels correlated to enrichment of oncogenic pathways and induced overexpression of HOXB2 or HOXB3 inhibited FLT3/ITD downstream signaling as well as FLT3/ITD-induced biological events.

In this study we found that high expression of HOXB2 and HOXB3 was correlated to poor prognosis in a cohort with AML patients. A subgroup with FLT3/ITD mutations had higher expression levels of HOXB2/B3 but their overall survival was not significantly reduced compared to the whole cohort. Both gene set enrichment analysis (GSEA) and biological outcomes of overexpression of HOXB2/B3 in cell lines with FLT3/ITD mutations point out HOXB2/B3 as tumor suppressors. Furthermore, HOXB2/B3 overexpression seems to initiate a transcriptional program that inhibits FLT3/ITD downstream signaling. There are conflicting reports on the role of HOXB2 in other cancers and HOXB3 has been linked to increased cell proliferation[182-184]. Thus, the role of HOXB2/B3 in cancer seems to be context dependent. In AML, both HOXB2 and HOXB3 can be used as an independent prognostic marker where high levels confer a poor prognosis. However, our *in vitro* results suggest that HOXB2/B3 acts as tumor suppressors in FLT3/ITD positive AML. One explanation could be that HOXB2/B3 are up-regulated to balance FLT3/ITD signaling as high levels of FLT3 expression has been reported to induce apoptosis[196].

We and others have shown that also NPM1 mutations are correlated to overexpression of HOXB2/B3[190]. AML patients with concomitant NPM1 and FLT3/ITD mutations have better prognosis than patients with only FLT3/ITD[197].

One hypothesis would be that mutated NPM1 induces overexpression of HOXB2/B3 and subsequently counteract the oncogenic potential of FLT3/ITD.

In the cohort we studied patients with APL were found to have significantly lower expression of HOXB2/B3. Interestingly, one study has reported that in small a subgroup in APL with the fusion protein RARα–PLZF, the possibility for wild-type PLZF (The promyelocytic leukaemia zinc finger gene) to regulate target genes such as HOXB2 is disrupted, resulting in perturbation of cellular growth and differentiation[198].

IV. Aberrant activation of the PI3K/mTOR pathway promotes resistance to sorafenib in AML

Here, we wanted to investigate the mechanisms behind the acquisition of secondary resistance to the tyrosine kinase inhibitor sorafenib in patients with AML. A common problem related to the development of FLT3 inhibitors has been that patients become resistant to the drug during treatment either due to secondary point mutations in the kinase (TKD) domain or by hitherto unknown causes[113, 140].

In order to further elucidate the cellular mechanisms behind secondary resistance to sorafenib, two patient-derived cell lines, MV4-11 ($FLT3^{-/ITD}$) and MOLM-13 ($FLT3^{wt/ITD}$) were put under long-term treatment with sorafenib and after 90 days we observed that both cell lines displayed resistance to sorafenib as well as to quizartinib.

Both sensitive and resistant cells were then treated with sorafenib or DMSO (dimethyl sulfoxide) as a negative control followed by stimulation with FLT3 ligand (FL). Compared to DMSO-treated cells, both cell lines treated with sorafenib displayed poor FLT3 activation. Furthermore, resistant cells treated with DMSO showed a much more robust response to ligand in terms of FLT3 activation and downstream AKT and ERK activation. Resistant cells treated with sorafenib were also able to form colonies similar to DMSO-treated cells.

In order to detect secondary mutations in FLT3, we sequenced the whole coding region of FLT3 using Sanger sequencing. We found a mutation in the extracellular domain of the receptor in all four sensitive and resistant cell lines but no mutations in the inhibitor binding site. Moreover, mass-spectroscopic analysis of affinity-enriched FLT3 indicated no differences in the intracellular domain of FLT3 between sensitive and resistant cells. Using phospho-specific antibodies against known FLT3 residues, we observed an increase in total FLT3 phosphorylation, but no single residue that was selectively hyper-phosphorylated.

As no secondary mutation in the inhibitor binding site of FLT3 was identified, we next sequenced the whole coding region of all genes using next-generation

sequencing in order to determine if the acquired resistance could be due to mutations in genes encoding parallel signaling proteins, leading to hyper-activation of the PI3K/mTOR pathway. Interestingly, we observed novel previously not reported mutations in PIK3C2G in sorafenib-resistant MOLM-13 cells and mTOR in sorafenib-resistant MV4-11 cells.

In order to identify gene expression profiles of resistant cell lines we analyzed mRNA expression of all cell lines and found up-regulation of a group of genes responsible for cell survival and proliferation in the resistant cells while expression of pro-apoptotic genes were down-regulated. Furthermore, using significance analysis of microarrays (SAM) we showed that genes previously known to be associated with cell proliferation or cell cycle progression were significantly up-regulated in the resistant cells. Gene set enrichment analysis (GSEA) of signaling pathways showed an enrichment of the mTOR and AKT pathways in both MOLM-13 and MV4-11 resistant cell lines. An enrichment of the mTOR pathway was also detected in sorafenib-resistant AML patient samples and in AML patient samples expressing FLT3/ITD compared to FLT3/ITD-negative samples. Moreover, by using a phospho-protein antibody array, we found that the phosphorylation of the mTOR substrates S6K and AKT were selectively increased in resistant MOLM-13 cells as well as an increase in STAT3 phosphorylation.

After verifying the dual PI3K/mTOR inhibitor gedatolisib as an inhibitor of proliferation in the sorafenib-resistant cell lines (IC_{50} 23 nM) we tested its specificity in our cell model, using a phospho-specific antibody array. Treatment of cells with a higher concentration of gedatolisib did not alter the phosphorylation of other signaling proteins except for AKT and S6K and this was further confirmed with western blotting using phospho-specific antibodies against AKT, ERK1/2, p38 and S6K.

To assess the biological outcomes of gedatolisib treatment several biological assays were performed. The inhibitor caused dose-dependent impairment of colony formation in both MOLM-13 and MV4-11 sorafenib-resistant cell lines as well as reduced cell proliferation and induction of apoptosis. To test the effectiveness of gedatolisib in an animal model, we made mouse xenografts by injecting MV4-11 and MOLM-13 resistant cells subcutaneously. Mice were treated with 12.5 mg/kg of gedatolisib or vehicle for 25 days. As a result, in mice treated with gedatolisib tumors developed with a significant delay compared to vehicle-treated animals and gedatolisib treatment reduced tumor weight in xenografts with both resistant cell lines.

To address further the efficacy of gedatolisib, we generated a patient-derived xenograft model using primary blasts from a sorafenib-resistant AML patient. Mice treated with gedatolisib showed a lower number of circulating CD45 positive cells

compared to vehicle-treated mice and treatment with gedatolisib significantly increased survival of mice engrafted with the sorafenib-resistant AML patient cells.

To conclude, we show in this study that secondary resistance to sorafenib in FLT3/ITD-positive AML can be caused by up-regulation of the PI3K/mTOR signaling pathway even though the FLT3 receptor stays responsive to inhibition. Furthermore, the highly selective dual PI3K/mTOR inhibitor gedatolisib can override this resistance and block colony formation, decrease cell proliferation, induce apoptosis and block tumor growth in vivo.

Other groups have reported that aberrant activation of parallel signaling pathways, such as STAT5 and MAPK, can lead to FLT3 inhibitor resistance even though FLT3 inhibition is maintained[199]. In this study we show that resistance to sorafenib in two FLT3/ITD-dependent cell lines could be mediated through aberrant activation of the PI3K/mTOR pathway paired with concomitant loss of dependency on FLT3 signaling. Furthermore, we establish the dual PI3K/mTOR inhibitor gedatolisib as a potent drug to overcome this resistance.

Gedatolisib has been tested in clinical trials for solid tumors with promising results[61] but there are no reports of its use in AML. However, other dual PI3K/mTOR inhibitors have been shown to be effective in MLL-rearranged AML cell lines[200]. A French phase-II study for gedatolisib in the treatment of t-AML/MDS, relapsed or refractory AML is planned[49]. Gedatolisib has also been reported to potently inhibit leukemia proliferation with synergistic activity in combination with JAK or SRC/ABL inhibition in JAK-mutant or ABL/PDGFR-mutant Ph-like ALL in a mouse model[201].

As the development of new FLT3 inhibitors has met problems with secondary resistance our study highlights that a more efficient way would be to attack the leukemic clone with a combination of drugs.

With next-generation sequencing we observed novel mutations in mTOR and PIK3C2G in sorafenib-resistant cells, possibly involved in the aberrant activation of the PI3K/mTOR pathway. Consequently, to identify a direct link between the mutations observed in the resistant cells and hyper-activation of the PI3K/mTOR pathway would be a future direction.

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This thesis is dedicated to two persons:

My father, **Carl-Johan Lindblad**, abrubtly taken away from life in the midst of family life and career with an unfinished thesis on his desk.

My grandfather, **Tage Bengtsson**, great inventor and the real scientist of the family.

Populärvetenskaplig sammanfattning

Varje år drabbas ca 50000 personer i Sverige av cancer. Ungefär 10% av fallen har sitt ursprung i blodet och lymfkörtlarna. Av dessa får ca 350 personer en aggressiv form med ursprung i benmärgens stamceller som blir snabbt livshotande utan behandling. Denna sjukdom, akut myeloisk leukemi (AML), är till stor del den kliniska grunden till frågeställningarna i denna avhandling.

Vårt laboratorium har specialiserat sig på studiet av en typ av cellytereceptorer som kallas tyrosinkinasreceptorer. Dessa tar emot signaler från omgivningen som förmedlas in i cellen för att styra dess öde till att dela sig, mogna ut till en specialiserad celltyp eller genomgå programmerad celldöd. I benmärgen spelar dessa receptorer en viktig roll hos en frisk person. Hos patienter med AML har dock dessa receptorer fått mutationer som gör att deras signaler in i cellen orsakar ohämmad celldelning och att cellerna inte mognar ut. Följden blir en kraftig ansamling av icke-funktionella celler i benmärgen och blodet som gör att blodets vanliga funktioner inte kan upprätthållas.

I den första studien gjorde vi en modell av en känd mutation i tyrosinkinasreceptorn KIT genom att introducera muterat DNA i odlade blodceller från mus. Genom att studera och på olika sätt påverka signalerna från receptorn inne i cellen kunde vi visa att mutationens förmåga att orsaka ohämmad celldelning var beroende av ett protein kallat PI3-kinas. Vi kunde också visa att de läkemedel som idag finns för att blockera funktionen av detta protein inte skulle fungera på denna mutation.

I den andra studien tittade vi på en annan tyrosinkinasreceptor kallad FLT3. Ungefär 30% av patienterna med AML har en mutation i receptorn (FLT3/ITD) som bidrar till att driva sjukdomen. Genom att studera provdata från en stor grupp patienter fann vi att ett protein kallat BEX1 verkade påverka överlevnaden positivt för de som hade mutationen FLT3/ITD. Vidare när vi introducerade DNA för FLT3/ITD och BEX1 i celler från mus så delade sig cellerna mindre och graden av programmerad celldöd ökade. Vi kunde således visa att BEX1 verkar fungera som en så kallad tumörsuppressor hos AML-patienter med FLT3/ITD och bromsar sjukdomen.

Även i en den tredje studien tittade vi på sjukdomsmodifierande faktorer relaterat till FLT3/ITD. Studier på prover från AML-patienter tydde här på att en ökning av proteinerna HOXB2 och HOXB3 försämrade prognosen för gruppen med FLT3/ITD. Trots detta visade våra laboratorieförsök att en ökad mängd HOXB2/B3

i musceller motverkade celldelning och stimulerade till ökad programmerad celldöd. Man kan här spekulera i om HOXB2 och HOXB3 liksom BEX1 har en roll som tumörsuppressor hos patienter med FLT3/ITD och möjligen ökar proteinnivåerna hos patienter med aggressiv sjukdom för att försöka motverka en skenande celldelning.

Det pågår sedan ett decennium tillbaka en stor aktivitet inom forskarvärlden och läkemedelsindustrin för att hitta läkemedel som kan hämma mutationer i FLT3-receptorn. Dessa så kallade tyrosinkinashämmare utvärderas nu i kliniska studier på patienter men ett återkommande problem är att patienterna utvecklar resistens och medlen blir då verkningslösa. I den sista studien behandlades cellinjer från AML-patienter i laboratoriet under 90 dagar med tyrosinkinashämmaren sorafenib. Cellerna utvecklade då resistens och vi kunde studera vilka mekanismer som gjorde att cellerna kunde försvara sig mot läkemedlet. Vi fann att sorafenib fortfarande kunde blockera den muterade FLT3-receptorn men att cellerna istället använde sig av en alternativ signaleringsväg kallad PI3K/mTOR. Denna signaleringsväg lyckades vi blockera med ett nytt läkemedel under utveckling kallat gedatolisib och kunde på så vis häva resistensen.

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Paper I

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Cellular and Molecular Life Sciences

RESEARCH ARTICLE

PI3 kinase is indispensable for oncogenic transformation by the V560D mutant of c-Kit in a kinase-independent manner

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Abstract Oncogenic mutants of c-Kit are often found in mastocytosis, gastrointestinal stromal tumors and acute myeloid leukemia. The activation mechanism of the most commonly occurring mutation, D816V in exon 17 of c-Kit, has been well-studied while other mutations remain fairly uncharacterized in this respect. In this study, we show that the constitutive activity of the exon 11 mutant V560D is weaker than the D816V mutant. Phosphorylation of downstream signaling proteins induced by the ligand for c-Kit, stem cell factor, was stronger in c-Kit/V560D expressing cells than in cells expressing c-kit/D816V. Although cells expressing c-Kit/V560D showed increased ligand-independent proliferation and survival compared to wild-type c-Kit-expressing cells, these biological effects were weaker than in c-Kit/D816V-expressing cells. In contrast to cells expressing wild-type c-Kit, cells expressing c-Kit/V560D were independent of Src family kinases for downstream signaling. However, the independence of Src family kinases was not due to a Src-like kinase activity that c-Kit/D816V displayed. Point mutations that selectively block the association of PI3 kinase with c-Kit/V560D inhibited ligand-independent activation of the receptor, while inhibition of the kinase activity of PI3 kinase with pharmacological inhibitors did not affect the kinase activity of the receptor. This suggests a lipid kinase-independent key role of PI3 kinase in c-Kit/V560D-mediated oncogenic signal transduction. Thus, PI3 kinase is an attractive therapeutic target in malignancies induced by c-Kit mutations independent of its lipid kinase activity.

Keywords Receptor tyrosine kinase · Ba/F3 · Cancer · LY294002 · GDC0941 · Signaling

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Introduction

The type III receptor tyrosine kinase c-Kit belongs to the same subfamily as the platelet-derived growth factor (PDGF) receptors, Fms-like tyrosine kinase 3 (FLT3), and the macrophage colony stimulating factor (M-CSF) receptor. C-Kit plays important roles in hematopoiesis, melanogenesis, and gametogenesis under normal physiological conditions [1]. Binding of its ligand, stem cell factor (SCF) induces dimerization of c-Kit followed by activation of its intrinsic tyrosine kinase activity. This leads to phosphorylation of key tyrosine residues that constitute binding sites for Src homology 2 domain containing signaling proteins. These events result in the phosphorylation and activation of downstream signaling molecules that mediate the biological effects, such as cell proliferation, survival and migration.

Mutations of c-Kit have been identified in around 90 % of mastocytosis and 80 % of gastrointestinal stromal tumor (GIST) cases. It also exists, less commonly, in sub-group of acute myeloid leukemia (AML), in melanoma and in germ cell tumors such as seminomas and ovarian dysgerminomas [1]. Ligand-independent activation of oncogenic mutants of receptor tyrosine kinases has long been recognized as important mediators of oncogenesis. However, we and others have shown that some oncogenic mutants of receptor tyrosine kinases, in addition to being constitutively active, also gain altered substrate specificity [2, 3].

The Src family kinases are a group of non-receptor tyrosine kinases that are important signal mediators in receptor signaling. Activation of wild-type c-Kit is partially dependent on the activity of Src family kinases, and inhibition of Src family kinases leads to decreased c-Kit activation [4]. The D816V mutation is the most commonly identified and well characterized mutation of c-Kit in malignancies. We have previously found that c-Kit/D816V gains a Src family kinase-like activity and thus circumvents a requirement of Src family kinases for its activation and cell transformation [2]. This suggests that oncogenic c-Kit mutations, in addition to being ligand-independent, have different mechanisms of activation and signal transduction compared to wild-type c-Kit.

PI3 kinases are intracellular lipid kinases that are involved in receptor signal transduction under both normal and pathological situations. Unlike Src family kinases, PI3 kinases play an important role in c-Kit/D816V-mediated cell transformation which has been shown to be independent of its lipid kinase activity [5].

Since there is a difference between the way wild-type c-Kit and c-Kit-D816V utilize Src family kinases and PI3 kinases for receptor activation and downstream cellular signaling, it is of interest to investigate the role of these two kinase families in the signal transduction and cell transformation mediated by other oncogenic c-Kit mutants. By introducing mutations in c-Kit and by using selective inhibitors, we observed that the commonly identified exon 11 mutation V560D of c-Kit is not dependent on Src family kinases for its signaling. In contrast, the ligand-independent activation of c-Kit/V560D is totally dependent on an intact binding site for PI3 kinase in c-Kit but is independent of its lipid kinase activity. These results confirm that oncogenic c-Kit mutants have different mechanisms of both activation and signaling than wild-type c-Kit.

Materials and methods

Kits and reagents

The PI3-kinase inhibitor LY294002 and Src Family Kinase inhibitor SU6656 were from Sigma-Aldrich (St. Louis, MO), another PI3 kinase inhibitor GDC0941 was from Apexbio (Boston, MA). Lipofectamine 2000 was from Life Technologies (Carlsbad, CA). Chemiluminescent HRP substrate was from Millipore (Billerica, MA). QuikChange mutagenesis kit was from Agilent Technologies (Santa Clara, CA). Annexin V-PE apoptosis detection kit was from BD Biosciences (San Jose, CA). [γ-^{32}P]ATP and protein G-Sepharose beads were from GE Healthcare (Little Chalfont, UK). All the reagents and kits were used according to the manufacturer's instructions.

Cytokines and antibodies

Recombinant human SCF was purchased from Prospec Tany (Rehovot, Israel). The rabbit antibody KitC1, recognizing the C-terminal tail of human c-Kit, was purified as described [6]. Antibodies recognizing pTyr-568, pTyr-703, pTyr-Y721, and pTyr-936 in human c-Kit were previously described [2]. Antibodies against Akt, pErk and SHP2 were from Santa Cruz Biotechnology (Texas, TX). pAkt antibody was from Epitomics (Burlingame, CA). β-actin antibody were from Sigma-Aldrich (St. Louis, MO). SHC antibody was from BD Biosciences (San Jose, CA). Phosphotyrosine antibody 4G10 and Gab2 antibody was from Millipore (Billerica, MA). PE labeled c-Kit antibody (104D2) was from Biolegend (San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Life Technologies (Carlsbad, CA).

Cell culture

EcoPack virus packaging cell line (Clontech, Mountainview, CA) was grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Ba/F3 cells (DSMZ, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, and 10 ng/ml recombinant murine IL-3 as recommended previously [7]. To establish Ba/F3 cell lines expressing c-Kit, EcoPack cells were transfected with either wild-type or mutant of c-Kit constructs in pMSCVpuro vector. Supernatants were collected to infect Ba/F3 cells followed by 2-weeks selection in 1.2 μg/ml puromycin. Expression levels of c-Kit were confirmed by flow cytometry and immunoblotting. C-Kit expressing Ba/F3 cells were grown in the same medium as untransfected Ba/F3 cells.

Cell survival and proliferation assay

Cell survival and proliferation assay was performed as described [5].

Cell stimulation, immunoprecipitation and Western blotting

Cell stimulation, immunoprecipitation and Western blotting were performed as described [5].

In vitro kinase activity assay

Ba/F3 cells were starved for 4 h in medium without serum and IL-3 and subsequently stimulated with SCF (100 ng/

ml) for 5 min. Thereafter, cells were washed once in ice-cold PBS, lysed, immunoprecipitated with the KitC1 antibody, and processed for in vitro kinase assay as described [8] with the exception that Src optimal peptide [9] was used as substrate.

Results

The V560D mutant is less oncogenic than the D816V mutant of c-Kit

The exon 11 mutation V560D of c-Kit is frequently identified in GISTs [10] while the exon 17 mutation D816V of c-Kit is frequently identified in mastocytosis and less frequently in certain types of AML [1]. To compare the transforming ability of these two types of mutants, we transfected Ba/F3 cells, which lack endogenous c-Kit expression, with either wild-type c-Kit or c-Kit mutants. Cell surface expression of c-Kit was shown to differ between the three different mutant cell lines. While cells expressing wild-type c-Kit displayed the highest level of cell surface expression, c-Kit/V560D had intermediate surface expression, and cells expressing c-Kit/D816V displayed the lowest cell surface expression (Fig. 1a). C-Kit is expressed as a mature, fully glycosylated 145KD and an immature, partially glycosylated 125 kD protein [11], the ratio between the two c-Kit protein bands are also different between wild-type c-Kit and the two c-Kit mutants. The 145 kD band is dominant in cells expressing wild-type c-Kit while the two bands are more equal in intensity in the mutants (Fig. 1b). To investigate the oncogenic potential of these mutants, we examined cell survival and cell proliferation of transfected Ba/F3 cells in the presence or absence of SCF. Cell survival (Fig. 1c) and cell proliferation (Fig. 1d) of cells expressing wild-type c-Kit were dependent on the presence of SCF. In contrast, c-Kit/D816V supported both cell survival and cell proliferation independent of SCF stimulation. In comparison, Ba/F3 cells expressing c-Kit/V560D showed moderate cell survival and weak cell proliferation regardless of SCF stimulation, suggesting a weaker transforming ability of c-Kit/V560D compared with c-Kit/D816V.

The activation kinetics of c-Kit/V560D is different from that of c-Kit/D816V

To investigate the mechanism behind the weak transforming ability of c-Kit/V560D, c-Kit activation was compared between wild-type c-Kit, c-Kit/V560D and c-Kit/D816V expressing cells. Both c-Kit/V560D and c-Kit/D816V can induce ligand-independent activation of the receptor (Fig. 2a). It is well established that Src family kinases play a crucial role in the activation of wild-type c-Kit. The binding site of Src family kinases in c-Kit is Tyr568 [12]. By using a phospho-specific antibody, we show that Tyr568 is strongly phosphorylated in cells expressing c-Kit/V560D independent of SCF stimulation. In contrast, phosphorylation of the Grb2 binding sites Tyr703 and Tyr936 [13], and PI3 kinase binding site Tyr721 [14] was weaker in c-Kit/V560D than in c-Kit/D816V. In addition, the total phosphorylation of c-Kit/V560D was weaker than that of c-Kit/D816V. Similar to the receptor activation, the ligand-independent phosphorylation of downstream signaling molecules such as Akt and Erk was also weaker in c-Kit/V560D-expressing cells compared to c-Kit/D816V-expressing cells (Fig. 2b), although ligand stimulation transiently enhanced phosphorylation further in c-Kit/V560D expressing cells. These results suggest that c-Kit/V560D can weakly activate downstream signaling through Grb2 and PI3 kinase leading to weaker activation of Akt and Erk signaling as well as weaker cell proliferation and survival. The adapter proteins Gab2 and SHC are important intermediates in the activation of both Akt and Erk [15, 16], and the protein tyrosine phosphatase SHP2 is involved in Erk activation [17]. By using phosphotyrosine antibodies, c-Kit/V560D was shown to be able to phosphorylate Gab2, SHC and SHP2 even in the absence of ligand stimulation and similar to Akt and Erk phosphorylation, phosphorylation of these three proteins was weaker than that of c-Kit/D816V expressing cells (Fig. 2c).

The kinase activity of c-Kit/V560D is not dependent on Src family kinases

Src family kinases are important for the activation of wild-type c-Kit [4]. We have previously found that the D816V mutation of c-Kit circumvents a requirement of Src family kinases for its activation of c-Kit and downstream signal transduction [2]. Thus, we sought to investigate whether the V560D mutant of c-Kit is still dependent on Src family kinases. By using a selective Src family kinase inhibitor, SU6656, we showed that both ligand-independent and ligand-dependent activation of c-Kit/V560D was independent on the kinase activity of Src family kinases (Fig. 3a). Likewise, phosphorylation of downstream signaling molecules such as Akt and Erk were not inhibited by the Src family kinase inhibitor SU6656 (Fig. 3b).

C-Kit/V560D has not gained Src-like substrate specificity

We have previously found that c-Kit/D816V has gained substrate specificity similar to Src family kinases and that it thereby loses its dependence on Src family kinases for

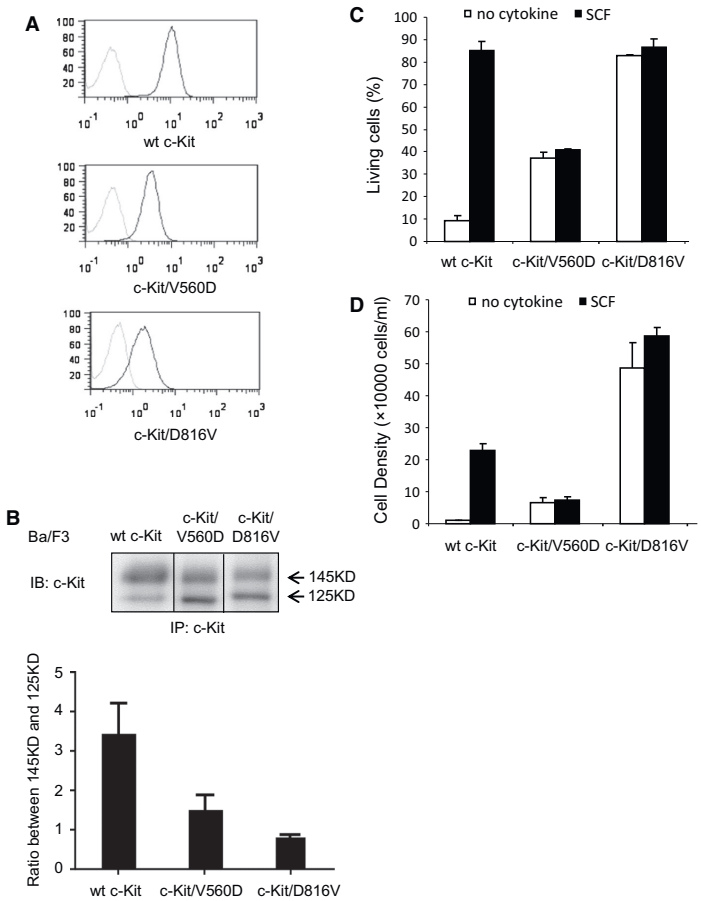
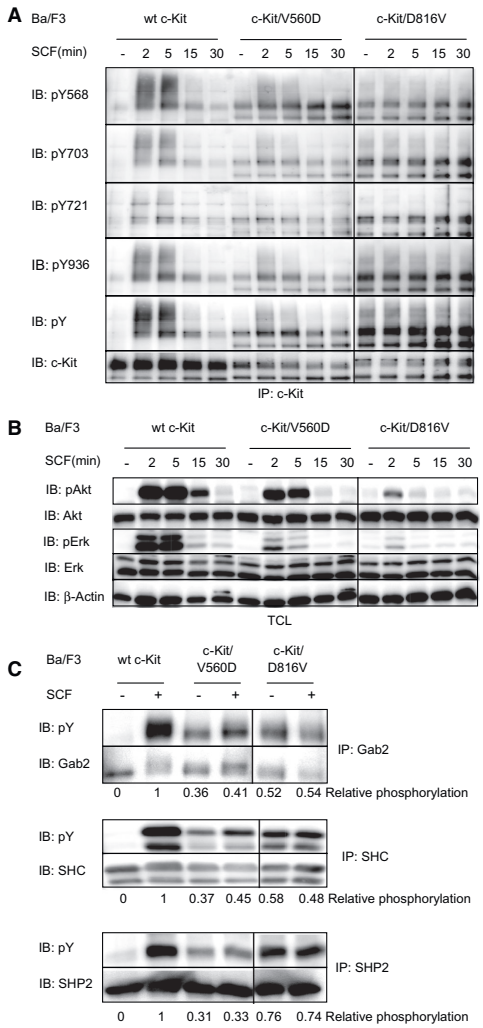


Fig. 1 Cell surface expression of c-Kit in Ba/F3 cells, cell survival and cell proliferation of Ba/F3 cells expressing wild-type c-Kit and c-Kit mutants. **a** Cell surface expression of c-Kit in Ba/F3 cells stably transfected with either wild-type c-Kit, c-Kit/V560D or c-Kit/816V was measured by flow cytometry (*gray curve* isotype control, *black curve* anti-c-Kit). **b** Ba/F3 cells expressing c-Kit were lysed and subjected to immunoprecipitation using a c-Kit antibody, and thereafter subjected to Western blotting using c-Kit antibody. The signal intensity of the 145 kD band and 125 kD band were quantified, the ratio between the 145 kD band and 125 kD band was calculated. **c**, **d** Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D or c-Kit/816V were analyzed for their proliferative response to SCF. Cells were washed free from IL-3, and subsequently seeded at a density of 70,000 cells/mL in 6-well plates. Thereafter cells were SCF-stimulated for 48 h (*black column*) or left untreated (*empty column*) as control. Living cells were counted under the microscope to examine cell proliferation (**c**) or stained with the apoptosis (**d**) detection kit (BD biosciences) and subsequently subjected to flow cytometry

its activation and downstream signaling. Similar to c-Kit/D816V, the V560D mutant of c-Kit has also lost its dependence on Src family kinases for its activation and downstream signaling (Fig. 3). We, therefore, wanted to investigate whether also c-Kit/V560D, similar to c-Kit/D816V, has gained altered substrate specificity. Using an in vitro kinase assay utilizing the Src optimal peptide as a substrate, we could show that c-Kit/D816V displays substrate specificity similar to Src family kinases while both wild-type c-Kit and c-Kit/V560D do not show any such activity even in the presence of ligand (Fig. 4). These results indicate that the mechanism of activation and signaling is different between c-Kit/V560D and c-Kit/D816V.

Ligand-independent activation of c-Kit/V560D is dependent on PI3 kinase in a lipid kinase-independent manner

PI3 kinase plays an important role in the ligand-independent signaling by c-Kit/D816V, and c-Kit/D816V-mediated cell transformation is independent of its lipid kinase activity [5]. PI3 kinase binds to pY721 in c-Kit [18] and the binding requires the motif pYXXM in c-Kit [19]. By introducing the M724A mutation in c-Kit/V560D to block the direct PI3 kinase binding without affecting c-Kit tyrosine phosphorylation, we show that the ligand-independent activation of c-Kit/V560D is totally blocked although ligand can still activate the receptor (Fig. 5a).



◀**Fig. 2** Activation of wild-type c-Kit and c-Kit mutants, and downstream signaling pathways. **a** Western blot analysis using phospho-specific antibodies against known c-Kit phosphorylation sites. Ba/F3 cells expressing c-Kit were starved of serum and IL-3 for 4 h followed by SCF (100 ng/ml)-stimulation for 0, 2, 5, 15 or 30 min. Lysates were subjected to immunoprecipitation using a c-Kit antibody, and thereafter subjected to Western blotting using the following antibodies: pY568, pY703, pY721, pY936 and anti-phosphotyrosine antibodies. C-Kit antibody was used as control. **b** Phosphorylation of Akt and Erk in whole cell lysates (from the experiment 2A) was investigated by Western blot analysis using the following antibodies: anti-phosphoAkt (Ser473), anti-Akt, anti-phosphoErk (Thr202/Tyr204), anti-Erk and anti-β-actin. **c** Ba/F3 cells expressing c-Kit were starved of serum and IL-3 for 4 h followed by stimulation with SCF (100 ng/ml) for 2 min or left untreated. Lysates were subjected to immunoprecipitation using antibody against Gab2, SHC and SHP2, respectively. Tyrosine phosphorylation of Gab2, SHC and SHP2 was evaluated by Western blot analysis using anti-phosphotyrosine antibodies. Phosphorylation of Gab2, SHC and SHP2 was quantified and normalized against total protein

Ligand-dependent phosphorylation of PI3 kinase downstream signaling component Akt was partially reduced other than complete diminution, while PI3 kinase independent component Erk phosphorylation remained unchanged (Fig. 5b). These findings suggest that the c-Kit/V560D mutant can transduce signals without recruiting PI3 kinase but PI3 kinase association is required for constitutive activation of the receptor. In order to elucidate whether this effect is dependent on the lipid kinase activity of PI3 kinase, the effect of a PI3 kinase inhibitor on the activation of c-Kit/V560D was examined. The PI3 kinase inhibitor LY294002 did not block the activation of c-Kit/V560D independent of SCF stimulation (Fig. 5c). Furthermore, Akt phosphorylation was inhibited in the presence of LY294002 (Fig. 5d) indicating that PI3 kinase is activated by c-Kit/V560D. A cell proliferation assay in the presence of Src family kinase and PI3 kinase inhibitors further confirmed lipid kinase independent role of PI3 kinase in c-Kit/V560D signaling (Fig. 6).

Discussion

Mutations in c-Kit have been identified in exon 9, 11, 13 and 17 in different malignancies. In GISTs, most of c-Kit mutations were mapped to exon 11, and to a lesser extent also to exon 9 and 13 [20]. In mastocytosis, the D816V mutation in exon 17 of c-Kit is the most commonly identified mutation, found in about 80–90 % of patients [1]. The different mutants of c-Kit have different sensitivity to the current clinically approved c-Kit inhibitors. Mutations in exon 11 of c-Kit usually respond well to imatinib while c-Kit/D816V is insensitive to imatinib. After treatment with imatinib, relapsed GISTs many times gain secondary mutations in exon 13 and 17 of c-Kit that are resistant to imatinib [20, 21]. The difference in response of c-Kit mutations to inhibitors is most likely partially due to differences in activation mechanism. It is known that imatinib binds to the inactive kinase conformation of c-Kit [22], while c-Kit/D816V is constitutively in its active conformation [23] and thus does not bind imatinib. Moreover, different c-Kit mutants display considerable difference in intrinsic kinase activity. For example, c-Kit/D816V has a higher intrinsic kinase activity than c-Kit/V560D [21]. Therefore it is important to elucidate the detailed activation mechanism of these mutants to design more efficient drugs.

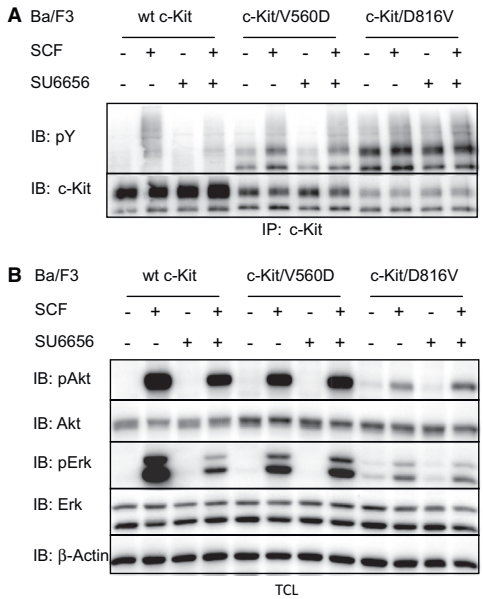


Fig. 3 Src family kinases are not required for the activation of c-Kit/V560D and downstream signal transduction. **a** Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D and c-Kit/816V were starved of serum and IL-3 for 4 h, thereafter cells were pre-incubated with the Src inhibitor SU6656 (2 μm) for 30 min before SCF stimulation (100 ng/ml) for 2 min followed by immunoprecipitation using a c-Kit antibody. After SDS-PAGE and electrotransfer, membranes were probed with phosphotyrosine antibody and reprobed with c-Kit antibody. **b** Phosphorylation of Akt and Erk in whole cell lysates was investigated by Western blot analysis as in Fig. 2

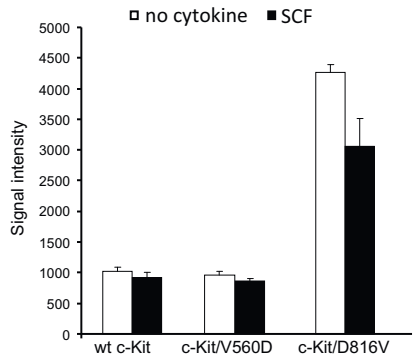


Fig. 4 C-Kit/V560D does not gain Src family kinase activity. Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D or c-Kit/816V were starved of serum and IL-3 for 4 h, stimulated with SCF (100 ng/ml) for 2 min. Cells were then lysed and immunoprecipitated using a c-Kit antibody. The immunoprecipitated c-Kit proteins were used in a kinase assay together with Src optimal peptide as substrate. the phosphorylation of the substrate by wild-type c-Kit or c-Kit/D816V was detected by Fuji FLA 3000. Signal intensity from multiple blots was quantified by the Multigauge software. *Error bars* indicate standard deviation

Since Src family kinases are very important in wild-type c-Kit signaling, it is tempting to consider them as therapeutic targets in tumors driven by c-Kit mutant. However, we have previously found that the c-Kit/D816V mutant circumvents a requirement for Src family kinases by displaying altered substrate specificity. For that reason, it is likely that Src family kinase inhibitor will probably not be useful for the treatment of malignancies expressing c-Kit/D816V. It is of importance to investigate whether the independency on Src family kinases of receptor activation and signaling is a common mechanism among other activating c-Kit mutants. By using inhibitors and generating oncogenic mutants of c-Kit, we demonstrate that the commonly occurring c-Kit mutation V560D in GISTs also is able to circumvent a requirement of Src family kinases for its signal transduction. These results further suggest that the use of Src family kinase inhibitors in the treatment of cancers carrying c-Kit mutations will not be a viable option.

PI3-kinases are lipid kinases that play important roles in cell survival, proliferation and metabolism. Overactivation of PI3 kinase contributes to transformation in several malignancies. As with Src family kinases, PI3-kinases are also attractive treatment targets for cancer. Various small molecule inhibitors have been developed and taken into clinical trials [24].

PI3 kinases contribute to wild-type c-Kit-mediated cell survival and proliferation. Ligand-dependent activation of wild-type c-Kit is not dependent on PI3-kinases [25]. In contrast, the most commonly identified c-Kit mutant, D816V, has been shown to be dependent on PI3 kinase for its transforming ability [18], thus making PI3-kinase an attractive target in the treatment of malignancies carrying the c-Kit/D816V mutant. In addition to its lipid kinase activity, we have recently demonstrated that the important role of PI3-kinase in c-Kit/D816V mediated cell transformation is independent on its lipid kinase activity [5]. Since currently available PI3-kinase inhibitors block the lipid kinase activity and no other functions of PI3-kinase, such as binding to other signal transduction molecules, they are probably of low clinical efficacy in this type of malignancies.

Mutations in the c-Kit gene lead to activation of its kinase activity by different mechanisms and display differential sensitivity to c-Kit inhibitors. It is interesting to know whether the dependence on PI3 kinases is a common mechanism among all c-Kit mutants. By introducing

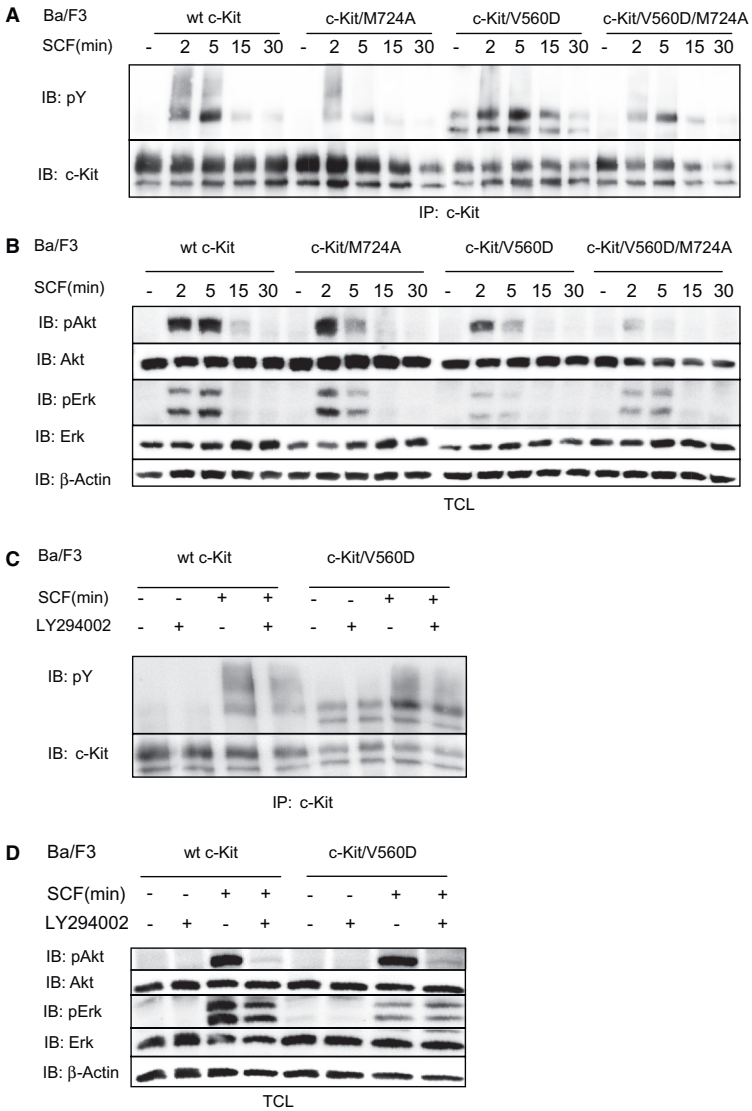


Fig. 5 Ligand-independent activation of c-Kit/V560D is dependent on PI3 kinase in a lipid kinase independent manner. **a** Ba/F3 cells expressing wild-type c-Kit, c-Kit/M724A, c-Kit/V560D or c-Kit/V560D/M724A were starved of serum and IL-3 for 4 h followed by SCF(100 ng/ml)-stimulation for 0, 2, 5, 15 or 30 min. Lysates were subjected to immunoprecipitation using a c-Kit antibody, and thereafter subjected to Western blotting using anti-phosphotyrosine antibodies and c-Kit antibodies. **b** Phosphorylation of Akt and Erk in whole cell lysates was investigated by Western blot analysis using the following antibodies: anti-phosphoAkt (Ser473), anti-Akt, anti-phosphoErk (Thr202/Tyr204), anti-Erk and anti-β-actin. **c** Ba/F3 cells expressing wild-type c-Kit or c-Kit/V560D were starved of serum and IL-3 for 4 h, thereafter cells were pre-incubated with the PI3 kinase inhibitor PY294002 (2 μM) for 30 min before SCF stimulation (100 ng/ml) for 2 min followed by immunoprecipitation using a c-Kit antibody. After SDS-PAGE and electrotransfer, membranes were probed with phosphor–tyrosine antibody and reprobed with c-Kit antibody. **d** Phosphorylation of Akt and Erk in whole cell lysates were investigated using Western blotting

mutations in c-Kit that block the association of PI3-kinase with c-Kit, we showed that the ligand-independent activation of c-Kit/V560D is totally dependent on PI3-kinase. The loss of PI3-kinase binding completely abolishes ligand independent activation of c-Kit/V560D. As is the case with c-Kit/D816V, the contribution of PI3-kinases to ligand-independent activation of c-Kit/V560D is independent of its lipid kinase activity. These results suggest a new activity or function of PI3-kinase in addition to its lipid kinase activity.

Currently available PI3 kinase inhibitors are based on the inhibition of its lipid kinase activity. However, as we showed in this study, PI3-kinase inhibitors cannot inhibit the activation of c-Kit mutants and downstream signaling

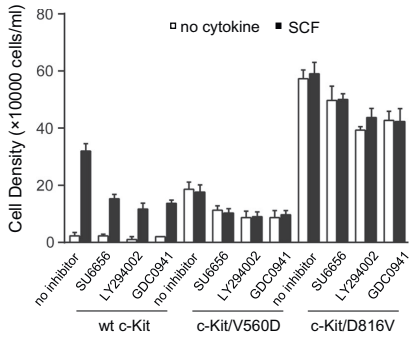


Fig. 6 Ba/F3 cells expressing wild-type c-Kit, c-Kit/V560D or c-Kit/D816V were analyzed for their proliferative response in the presence of Src family kinase inhibitor or PI3 kinase inhibitor. Cells were washed free from IL-3, and subsequently seeded at a density of 70,000 cells/mL in 6-well plates. Thereafter cells were SCF-stimulated for 48 h (*black column*) or left untreated (*empty column*) as control in the presence or absence of Src family kinase inhibitor SU6656 (2 μM) or PI3 kinase inhibitor LY294002 (10 μM) or GDC0941 (0.5 μM). Living cells were counted under the microscope to examine cell proliferation

pathways and they will probably not be efficient in the treatment of malignancies carrying c-Kit mutations. To identify the novel activity of PI3 kinase and develop new inhibitors against the non-lipid kinase activity will be a new direction. The novel inhibitors of PI3 kinases might be useful in the treatment of malignancies driven by oncogenic mutants of c-Kit.

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Conflict of interest The authors declare that they have no conflicts of interest.

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Paper II

BEX1 acts as a tumor suppressor in acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous disease of the myeloid lineage. About 35% of AML patients carry an oncogenic FLT3 mutant making FLT3 an attractive target for treatment of AML. Major problems in the development of FLT3 inhibitors include lack of specificity, poor response and development of a resistant phenotype upon treatment. Further understanding of FLT3 signaling and discovery of novel regulators will therefore help to determine additional pharmacological targets in FLT3-driven AML. In this report, we identified BEX1 as a novel regulator of oncogenic FLT3-ITD-driven AML. We showed that BEX1 expression was down-regulated in a group of AML patients carrying FLT3-ITD. Loss of BEX1 expression resulted in poor overall survival (hazard ratio, HR =2.242, p = 0.0011). Overexpression of BEX1 in mouse pro-B and myeloid cells resulted in decreased FLT3-ITD-dependent cell proliferation, colony and tumor formation, and in increased apoptosis *in vitro and in vivo*. BEX1 localized to the cytosolic compartment of cells and significantly decreased FLT3-ITD-induced AKT phosphorylation without affecting ERK1/2 or STAT5 phosphorylation. Our data suggest that the loss of BEX1 expression in FLT3-ITD driven AML potentiates oncogenic signaling and leads to decreased overall survival of the patients.

INTRODUCTION

FMS-like tyrosine kinase-3 (FLT3) is a receptor tyrosine kinase belonging to the type III receptor tyrosine kinases family. FLT3 expression has been detected in almost all acute myeloid leukemia (AML) patients, and the activating mutations in FLT3 occur in as high as 35% of AML patients [1] and less frequently in acute lymphoblastic leukemia (ALL) patients [2]. The most common FLT3 mutation is an internal tandem duplication (ITD), and other oncogenic mutations include point mutations in the kinase domain. Clinically, FLT3-ITD mutations are seen frequently in AML with normal karyotype, t(6:9), t(15:17) and trisomy 8 [3, 4] where it significantly increases the risk of relapse without affecting complete remission rates [5]. Consequently, FLT3-ITD expression limits disease-free and overall survival [6]. FLT3-ITD is an in frame duplication of 3 to 400 base pairs occurring in the region of the gene encoding the juxtamembrane domain of the receptor, and the length of the ITD mutation correlates with overall survival [7]. Thus, FLT3 is an attractive target to inhibit in AML patients with constitutive active FLT3 mutants. Wild-type FLT3 and its oncogenic mutants activate several downstream signaling cascades including PI3K-AKT and MAPK pathways resulting in cell survival [8–14]. Additionally FLT3-ITD activates STAT5 signaling [15].

BEX1 belongs to the Brain-Expressed X-linked (BEX) gene family. The initial description of the BEX genes was made in 1999 and included three mouse genes on the X chromosome with high expression levels in the brain. Up to six paralog BEX genes have since been identified in rodents and humans. BEX1 and its closest homolog BEX2 share a similar protein sequence (87% identity), while BEX3/NADE, which was characterized as an interactor of the neurotrophin receptor p75NTR death domain, is only 30% identical to either BEX1 or BEX2 and represents a more distant member of the family [16–21]. Human BEX1 is located at Xq22.1 while BEX2 is located at Xq22.2. Both BEX1 and BEX2 contain a characteristic BEX domain. Human BEX1 is expressed in the central nervous system with high levels in pituitary, cerebellum, and temporal lobe, but also widely expressed outside of the central nervous system with high expression in the liver [19, 22]. A later study showed that BEX1 interacts with p75NTR regulating the cell cycle and neuronal differentiation in response to nerve growth factor (NGF) [23]. BEX1 and BEX2 have also been shown to act as a tumor suppressor in malignant glioma [24]. Furthermore, the higher BEX1 expression was detected in AML cell lines with MLL-mutations compared to MLL-WT cell lines [25]. Treatment with the hypomethylating agent 5-Aza and with the HDAC inhibitor TSA induced expression of BEX1 in MLL-WT cells indicating that BEX1 is epigenetically regulated [25–27]. In the BCR-ABL positive K562 cell line, silencing of BEX1 in association with protocadherin 10 (PSDH10) induced resistance to imatinib [28]. In conclusion, current studies suggest that BEX1 is expressed in a variety of cells where it acts as a tumor suppressor.

In this report, we show that BEX1 is down-regulated in a group of FLT3-ITD driven AML patients. Loss of BEX1 expression resulted in activation of oncogenic signaling and reduced patient overall survival. BEX1 localized to the cytosolic compartments and overexpression of BEX1 resulted in decreased cell proliferation and colony formation, delayed tumor formation and increased apoptosis by inhibiting AKT signaling induced by FLT3-ITD.

RESULTS

BEX1 expression is downregulated in the MV4-11 compared to the MOLM-13 cell line

In our previous study, we observed that MV4-11 and MOLM-13 cell lines displayed differential response to drug-induced apoptosis, where MOLM-13 cells were more sensitive than MV4-11 [29]. To understand the basic differences between those cell lines we analyzed gene expression using microarray. We observed that both cell lines displayed differences in gene expression patterns (Fig. 1A) suggesting that although both cell lines are known to be dependent on FLT3-ITD, additional genetic and epigenetic mutations in different genes led to expression of unique genes in each cell lines. Genes up-regulated or down-regulated in the respective cell lines were determined by SAM tools. We observed that several genes displayed significant up-regulation or down-regulation. *BEX1, LOC550643, SLC22A16, CCND2, PRG2, CBS and NPW* genes were down-regulated in MV4-11 cells, whereas *MPO, IL8, APOC1, CECR1 and CCL4L1* genes were up-regulated (Fig. 1B). Interestingly *BEX1* expression was found to be 28-fold down-regulated in MV4-11 cells (Fig. 1C) compared to MOLM-13 cells. However, next-generation sequencing of MOLM-13 and MV4-11 cell lines did not identify any loss-of-function mutations in BEX1 gene in the MV4-11 cell line (data not shown). Furthermore, we observed differential BEX1 expression in a data set of primary AML patient samples (Fig. 1D). Therefore, we suggest that BEX1 expression is down-regulated in a group of AML patients.

Loss of BEX1 expression correlates with poor survival of FLT3-ITD positive AML patients

Because we observed that *BEX1* was down-regulated in MV4-11 cells and a group of AML patients, we hypothesized that *BEX1* may play a role in AML. We analyzed the prognostic significance of *BEX1* in AML using gene expression data (GSE6891, $N = 525$) of primary AML patient samples. We observed that the loss of BEX1 expression significantly correlated with poor overall survival in patients carrying FLT3-ITD and reduced median survival of around 50% (HR 1.697, $p = 0.0452$) (Fig. 2A). Furthermore, comparison between FLT3-ITD negative patients and BEX1 higher and lower expression (Fig. 2B), and patients with lower BEX1 expression and FLT3-ITD negative versus higher BEX1 and FLT3-ITD positive (Fig. 2C) did not display any difference in patient survival. The patient group with lower BEX1 and FLT3-ITD mutation versus higher BEX1 expression without FLT3-ITD mutation displayed a significant difference in patient survival (HR 2.242, $p = 0.0011$) (Fig. 2D). With other deregulated genes, we did not observe any significant correlation (Fig. S1A-S1D). The BEX1 expression did not display any correlation to the overall survival of the entire patient group regardless of FLT3-ITD mutation (Fig. S1E). Therefore, we suggest that the loss of BEX1 expression in AML patients carrying an FLT3-ITD mutation leads to an elevated risk compared to other groups of patients.

Loss of BEX1 expression correlates with up-regulation of survival pathways

Since the loss of BEX1 expression correlated with poor survival in FLT3-ITD positive patients, we wanted to analyze whether loss of BEX1 expression results in up-regulation of any oncogenic pathways. To that end,

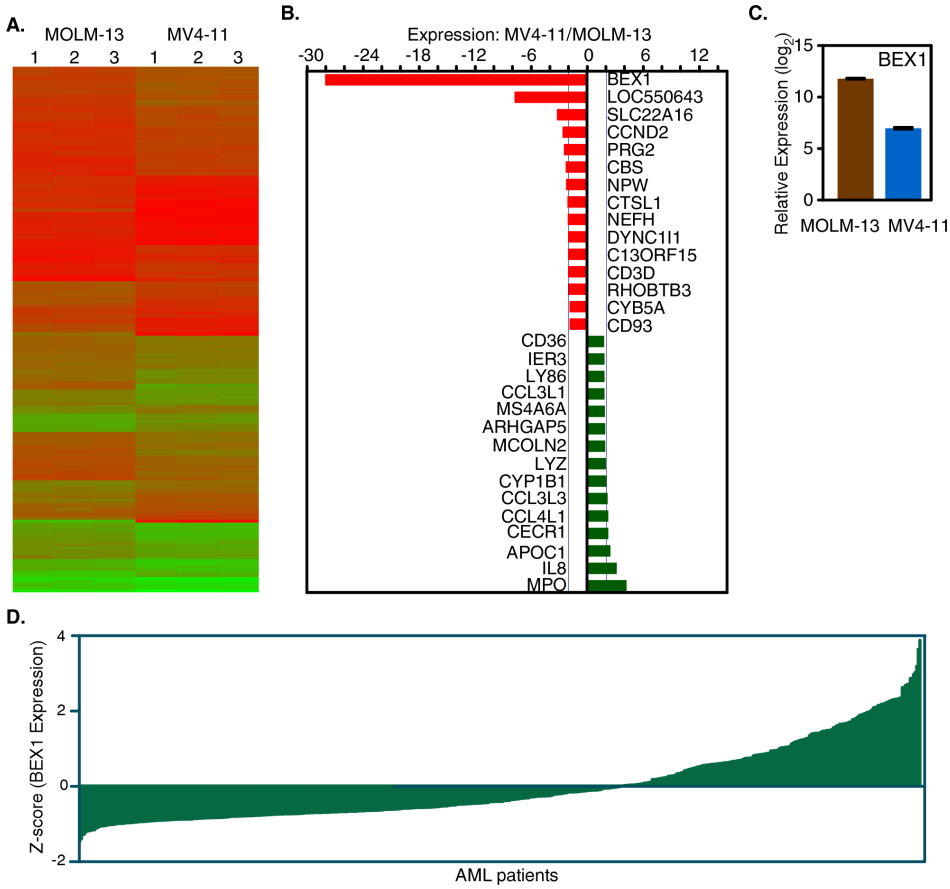

Figure 1: Deregulated gene expression in MV4-11 and MOLM-13 cell lines. A. Deregulated gene expression patterns in MV4-11 and MOLM-13 cell lines. **B.** Up-regulated and down-regulated genes in MV4-11 versus MOLM-13 cell lines. **C.** Relative BEX1 expression in MOLM-13 and MV4-11 cell lines. **D.** The BEX1 expression is deregulated in AML patients. Data set GSE14468 was used.

we analyzed enrichment of oncogenic pathways using gene set enrichment analysis (GSEA). We observed enrichment of several oncogenic pathways including loss of p53 function, KRAS and RAF pathways in MV4-11 cells in comparison with MOLM-13 cells (Fig. 3A). Moreover, similar enrichment of pathways was observed in FLT3-ITD positive AML patients with lower BEX1 expression (Fig. 3B). These results indicate a possible link between the loss of BEX1 expression and enhancement of oncogenic signaling in AML, which has already been shown in other malignancies [24].

BEX1 expression leads to impaired cell proliferation, inhibits colony formation and induces apoptosis

Results from survival assays and GSEA suggest that BEX1 plays a role in FLT3-ITD positive AML patients. To assess the role of BEX1 in FLT3-ITD signaling we generated two cell lines by stably transfecting FLT3-ITD along with BEX1 or empty control vector in the pro-B cell line Ba/F3 and the myeloid cell line 32D. Expression of FLT3-ITD and BEX1 was verified by western blotting (Fig. 4A).

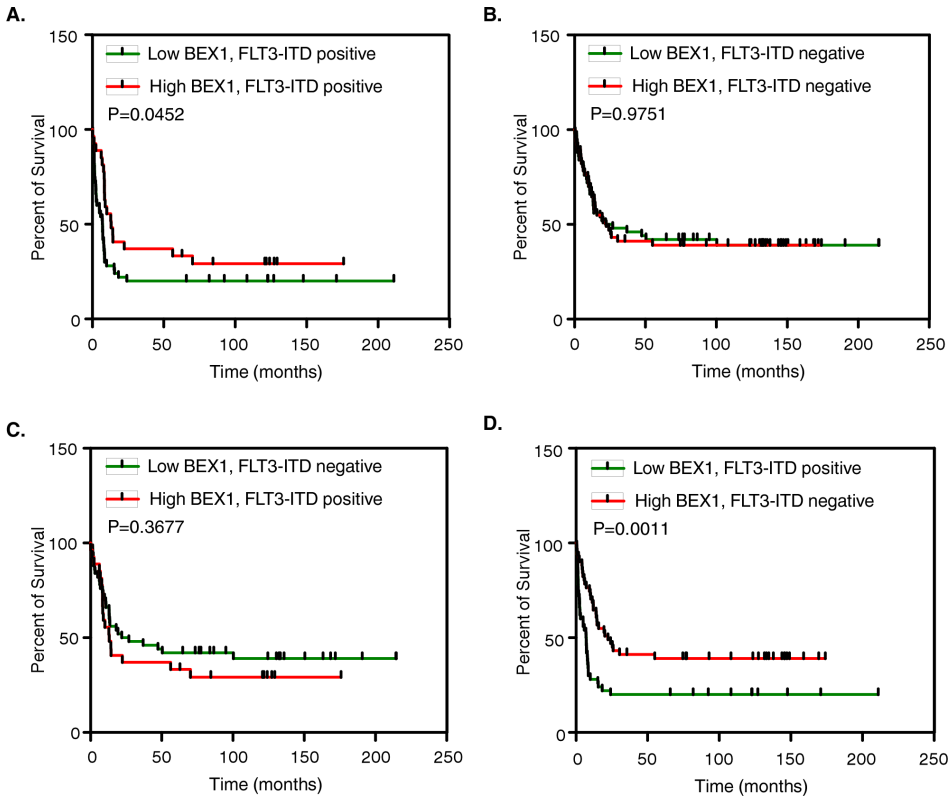

Figure 2: Overall survival of AML patients with higher and lower BEX1 expression. Data set GSE14468 was used in this analysis. Z-score was used to divide higher (n = 50) and lower (n = 50) BEX1 expressing patients. **A-D.** Overall survival of AML patients with FLT3-ITD positive and BEX1 higher or lower expression (A), FLT3-ITD negative and BEX1 higher or lower expression (B), FLT3-ITD positive plus BEX1 higher versus FLT3-ITD negative plus BEX1 lower expression (C) and, FLT3-ITD negative plus BEX1 higher versus FLT3-ITD positive plus BEX1 lower expression (D).

Expression of BEX1 significantly reduced FLT3-ITD-dependent cell proliferation of both Ba/F3 and 32D cells (Fig. 4B). Furthermore, cells expressing BEX1 displayed reduced number of colonies in semi-solid medium (Fig. 4C) and significantly enhanced apoptosis (Fig. 4D) in both cell lines, suggesting that BEX1 expression is essential for controlling FLT3-ITD-induced biological events.

BEX1 expression leads to delayed tumor formation in a mouse xenograft model

Because BEX1 expression reduced cell proliferation, inhibited colony formation and induced apoptosis, we aimed to check whether BEX1 expression delays FLT3-ITD-induced tumor formation in mice. Nude mice were injected subcutaneously with Ba/F3-FLT3-ITD and 32D-FLT3-ITD cells along with BEX1 or with empty control vector. We observed that BEX1 expression significantly reduced tumor volume (Fig. 5A) and tumor weight (Fig. 5B) in Ba/F3 as well as in 32D cells (Fig. 5C and 5D) in xenografted mice.

BEX1 localizes to the cytoplasm but does not affect FLT3 stability

To understand how BEX1 acts on FLT3-ITD-induced leukemogenesis, we first checked sub-cellular localization of BEX1 in FLT3-ITD expressing cells. We observed that BEX1 localized to the cytosolic compartment of cells and that localization was independent of FLT3-ITD activity (Fig. 6A) suggesting that FLT3-ITD is not the direct target of BEX1. Furthermore, BEX1 expression did not

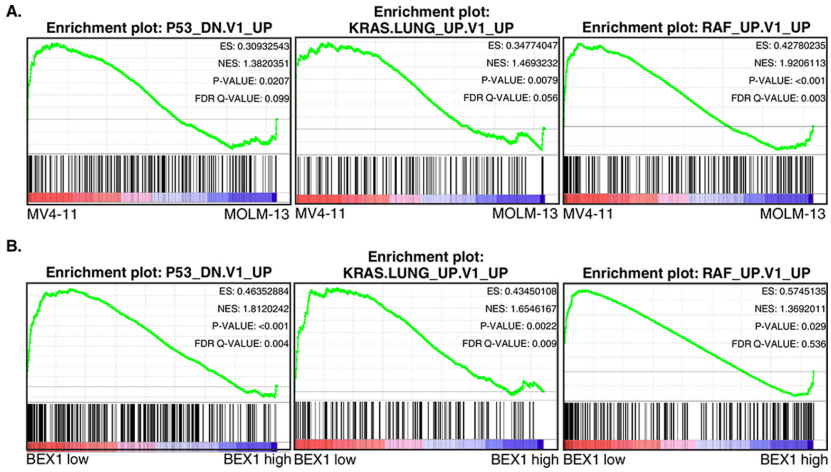

Figure 3: GSEA showed enrichment of oncogenic pathways in lower BEX1 expressing cells and patients. Data set GSE14468 was used in this analysis. Z-score was used to divide higher ($n = 50$) and lower ($n = 50$) BEX1 expressing patients. **A.** MV4-11 cells display enrichment of several oncogenic pathways in comparison to MOLM-13 cells. **B.** AML patients with lower BEX1 expression showed enrichment of several oncogenic pathways compared to patients with higher BEX1 expression.

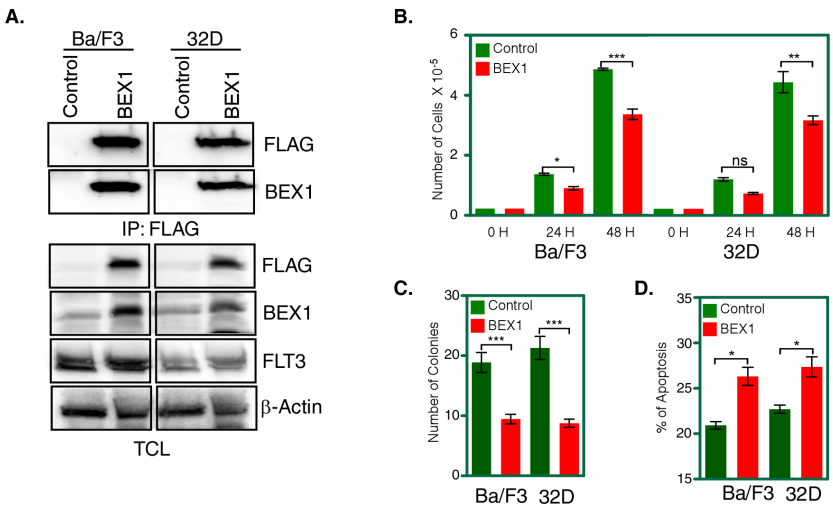

Figure 4: BEX1 expression inhibited cell proliferation, colony formation and enhanced apoptosis. Cells were washed three times with RPMI-1640 to remove IL3. **A.** Expression of BEX1 and FLT3-ITD in stably transfected Ba/F3 and 32D cells was measured by western blotting analysis. **B.** FLT3-ITD dependent cell proliferation in presence and absence of BEX1 expression was measured after 24 and 48 hours using stably transfected Ba/F3 and 32D cells. **C.** Stably transfected Ba/F3 and 32D cells were used to determine colony formation potential in the semi-solid medium. **D.** Apoptosis induced by BEX1 expression was measured using Annexin-V and 7-AAD kit.

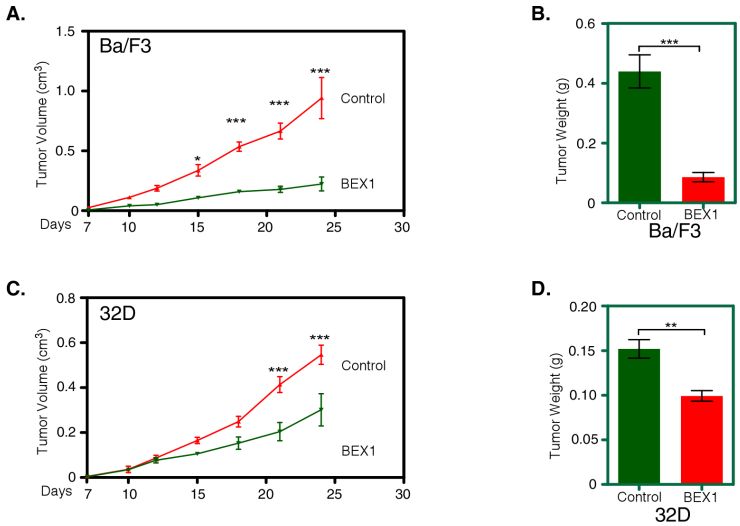

Figure 5: BEX1 delayed tumor growth in mouse xenograft. Cells were washed three times with RPMI-1640 to remove IL3. **A-D.** Cells expressing BEX1 or control were xenografted into mice and tumor growth was monitored for 24 days. Tumor volume (A) and weight (B) in Ba/F3 cells as well as in 32D cells (tumor volume (C) and weight (D)) were analyzed.

alter ubiquitination or tyrosine-phosphorylation (data not shown) nor did it influence the degradation of FLT3-ITD (Fig. 6B). In addition, we were unable to detect any interaction in between FLT3-ITD and BEX1 (data not shown). Thus, it is more likely that FLT3-ITD is not itself a target of BEX1 but that signaling proteins downstream of FLT3-ITD might be a target.

BEX1 expression selectively inhibits FLT3-ITD-induced AKT phosphorylation

Because BEX1 did not alter FLT3-ITD stability or tyrosine-phosphorylation, we analyzed FLT3 downstream signaling using phospho-specific antibodies. We observed that BEX1 expression significantly blocked AKT phosphorylation (Fig. 7A) in both Ba/F3 and 32D cell lines, but did not block ERK1/2 phosphorylation (Fig. 7B) or STAT5 phosphorylation (Fig. 7C). Thus, we suggest that BEX-1 inhibits FLT3-ITD signaling by blocking FLT3-ITD-induced AKT phosphorylation.

DISCUSSION

In this study, we aimed to address the role of BEX1 in FLT3-ITD expressing AML. The BEX1 expression was down-regulated in MV4-11 cells and also a subset of AML patients, and loss of BEX1 expression correlated with poor overall survival. BEX1 localized to the cytosol and controlled FLT3-ITD signaling by negative regulation of AKT phosphorylation.

The role of BEX1 in human cancer has not been thoroughly studied. BEX1 has been shown to be overexpressed in ER-positive breast cancer, but its role has not been defined [30]. In this study, we observed that BEX1 expression was down-regulated in MV4-11 cells compared to MOLM-13 cells as well as in a group of AML patients. AML patients positive for FLT3-ITD mutation along with reduced BEX1 expression displayed poor overall survival suggesting that BEX1 acts as a tumor suppressor in AML. A role of BEX1 as a tumor suppressor has previously been suggested in malignant glioma, where BEX1 expression was silenced by extensive promoter hyper-methylation [24]. Next generation sequencing of MV4-11 and MOLM-13 cells did not identify any mutations in the BEX1 gene, suggesting that BEX1 expression was probably also down-regulated due to the epigenetic modifications in MV4-11 cells as well as in a group of AML patients similar to in malignant glioma. It will be of interest to analyze the promoter region of those cell lines as well as of AML patient samples.

A demethylating agent, azacytidine, is being used in the clinic for treating patients with myelodysplastic syndrome (MDS) [31]. It is also used for AML patients to keep the disease under control when conventional

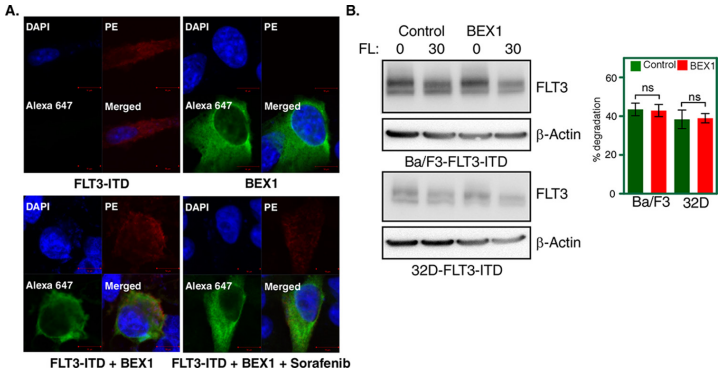

Figure 6: BEX1 localized to the cytosol and did not affect FLT3-ITD stability. A. Localization of BEX1 was visualized by confocal microscope. FLT3-ITD was stained with PE-conjugated antibody and BEX1-FLAG was stained with Alexa Flour 647-conjugated anti-FLAG antibody. **B.** Cells were washed three times with RPMI-1640 to remove IL3. FLT3 degradation was measured in transfected Ba/F3 and 32D cells after 30 minutes of ligand stimulation.

cytostatics failed or when the physical status or the age of the patient does not permit intensive therapy. Azacytidine enhances the efficacy of chemotherapy [32] probably through inducing expression of BEX1, as this drug has been shown to induce expression of BEX2 in MLL-positive AML cell line [26, 27]. Expression of BEX1 increased the sensitivity to chemotherapy-induced apoptosis in malignant glioma [24] and, furthermore, down-regulation of BEX1 in the BCR-ABL positive K562 cell line led to resistance to imatinib treatment [28, 33]. Therefore, AML patient with loss of BEX1 expression might benefit from azacytidine treatment combined with conventional cytostatics in order to lower the risk of relapse. In fact, a recently presented phase-2 study showed promising results in treating refractory FLT3-ITD positive AML with a combination of sorafenib and azacytidine [34].

The observation that BEX1 expression promotes apoptosis and inhibits cell proliferation, colony formation and tumor formation induced by FLT3-ITD suggests that BEX1 expression is favorable for AML patients who are positive for the FLT3-ITD mutation. A recent study suggested that BEX1 expression is suppressed in pediatric intracranial ependymoma due to epigenetic modifications and that overexpression of BEX1 significantly suppressed cell proliferation and colony formation in cell lines [35], in line with our observation that BEX1 acts as a tumor suppressor. The mechanism by which BEX1 displays its tumor suppressor activity might be cellular context dependent. It has been shown that BEX1 suppresses NF-κB signaling in oral squamous cell carcinoma [36]. In our study, we observed that BEX1 selectively suppresses AKT phosphorylation without affecting ERK1/2 and STAT5 phosphorylation suggesting that BEX1 controls FLT3-ITD signaling by blocking AKT activation. Although BEX1 inhibited FLT3-ITD-induced AKT phosphorylation, it neither interacted with FLT3-ITD nor regulated FLT3-ITD activation or stability. These observations indicate that FLT3-ITD is not a direct target of BEX1-mediated regulation. Otherwise it would also inhibit ERK1/2 and STAT5 phosphorylation, but a selective regulator of FLT3-ITD-induced AKT signaling.

Taken together, our study suggests that BEX1 has a tumor suppressor role in AML and that loss of BEX1 expression results in poor overall survival in FLT3-ITD positive AML patients. Since BEX1 is capable of limiting cell proliferation, colony formation, tumor formation and inducing apoptosis, drugs that enhance BEX1 expression would be beneficial for the treatment of patients with loss of BEX1 expression in FLT-ITD driven AML.

MATERIALS AND METHODS

Cell culture

The human AML cell lines, MV4-11, and MOLM-13, were maintained in RPMI-1640 media (Hyclone, Thermo Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA) and 1% penicillin and streptomycin. The murine hematopoietic cell line Ba/F3 and the myeloid cell line 32D were cultured in the same medium with addition of 10 ng/ml murine interleukin 3 (IL3) as recommended before [37]. COS-1 cells were cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum

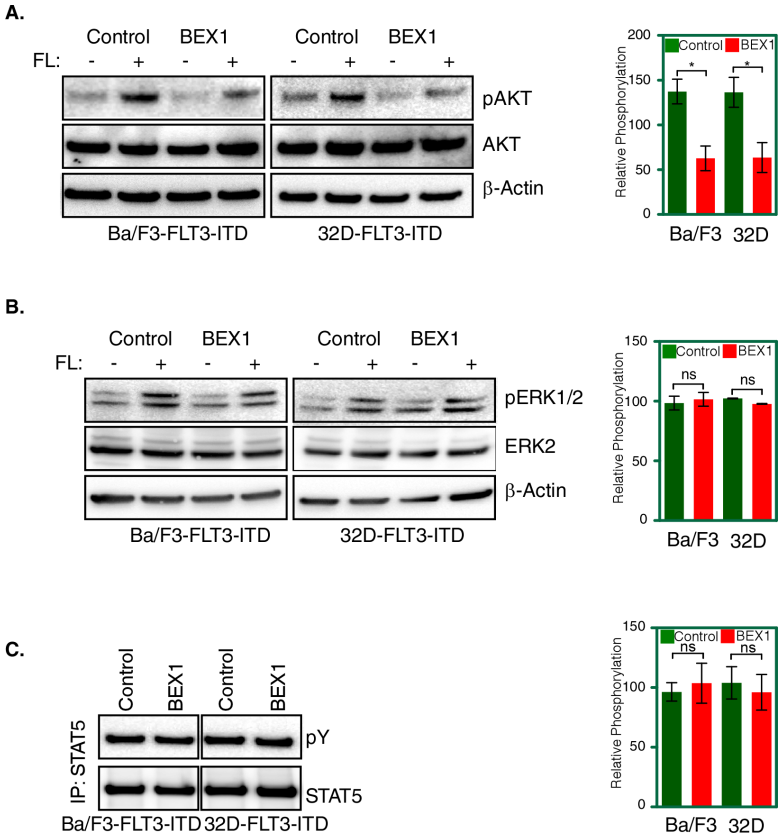

Figure 7: BEX1 expression decreased AKT phosphorylation. Cells were washed three times with RPMI-1640 to remove IL3. **A-B.** Transfected Ba/F3 and 32D cells were stimulated with a ligand for 5 minutes before lysis. Total cell lysates were used for SDS-PAGE and western blotting analysis with AKT (A) and ERK (B) antibodies. Ligand stimulated samples were used for quantification. **C.** Cell lysates for stimulated and unstimulated cells were immunoprecipitated with an anti-STAT5 antibody followed by western blotting analysis.

(Life Technologies, Carlsbad, CA) and 1% penicillin and streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmids, antibodies and inhibitors

Plasmid expressing human BEX1, pCMV- BEX1-WT-Myc-DDK (FLAG) was purchased from Origene, Rockville, MD. For retroviral transduction, the pMSCV-BEX1-WT-Myc-FLAG plasmid was generated by ligating full-length BEX1 into the pMSCVneo vector. Anti-FLT3 antibody was described previously [38]. Anti-phosphotyrosine antibody 4G10 was from Millipore (Life Technologies, Carlsbad, CA). Anti-phospho AKT was from Epitomics (Abcam, Cambridge, UK) and anti-phospho ERK antibody was form Santa-Cruz, Dallas, Texas. Anti-β-actin antibody was from Sigma-Aldrich, St. Louis, MO. Flag-Alexa 647 was from Cell Signaling Technology, Inc. Danvers, MA. DAPI was from Molecular Probes. FLT3-PE was from BD Biosciences Franklin Lakes, New Jersey.

Stable transfection of Ba/F3 and 32D cells

To establish Ba/F3 and 32D cells stably expressing FLT3-ITD, EcoPack packaging cells were transfected with

pMSCV-puro-FLT3-ITD construct, and virus-containing supernatants were collected 72 h after transfection. Retroviral infection of Ba/F3 and 32D cells was followed by a 2-week selection in 1.2 μg/ml puromycin. Expression of FLT3-ITD was confirmed by flow cytometry and western blotting. FLT3-ITD-transfected Ba/F3 and 32D cells were then further transfected with the pMSCV-neo-BEX1-Myc-FLAG construct or empty vector. Cells were selected with 0.8 mg/ml G-418 for 2 weeks, and BEX1 expression was verified by Western blotting.

Immunoprecipitation and western blotting

After required treatments such as ligand-stimulation, cells were washed once with cold PBS. Cells were then lysed using Triton X-100 based lysis buffer. Cell lysates were mixed with DDT and SDS containing loading buffer in a 1:1 ratio and boiled before separation by SDS-PAGE. For immunoprecipitation cell lysates were mixed with specific primary antibodies for 1 hour on ice followed by purification on protein G Dynabeads and SDS-PAGE analysis.

Apoptosis

Apoptosis was measured using annexin V and 7-aminoactiomycin D (7-AAD) kit (BD biosciences). Cells positive for annexin V or both annexin V and 7-AAD were counted as apoptotic cells.

Cell proliferation

Cells were seeded in a 24-well plate and incubated for 48 hours. Living cells were stained with trypan blue at 24 h and 48 h and counted with a Countess cell counter.

Colony formation assay

Around 500 cells were seeded in semisolid methylcellulose medium (Stem Cell Technologies). Cells were cultured for seven days before counting colonies.

Exome sequencing

Total genomic DNA was extracted from cell lines using DNeasy Blood and Tissue kits (Qiagen). Human All Exon enrichment (Agilent SureSelectXT) library was used to read 100 bp paired-end sequencing on a Genome Sequencer Illumina HiSeq2500.

Microarray analysis

Triplicate samples from MV4-11 and MOLM-13 cells were used. Cells were cultured normally using standard growth medium as mentioned above. Total RNA was extracted from cells using RNeasy mini kit (Qiagen). Illumina bead array technology was used to analyze mRNA expression using Illumina HumanHT-12 v4 Expression BeadChip. Gene expression was compared using significance analysis of microarrays (SAM) tools [39] and gene set enrichment analysis (GSEA) [40]. SCIBLU facility at Lund University was used for microarray analysis.

Confocal microscopy

COS-1 cells were transiently transfected with either pcDNA3-Flt3-ITD, pCMV-BEX1-Myc-FLAG or both using Lipofectamine 2000. Sorafenib were added to some samples (50 nM), and cells were incubated overnight. Cells were then fixed in 4% para-formaldehyde in PBS and incubated for 30 min. Blocking and permeabilization were done by adding a mixture of 0.5% Triton-X100 in PBS and 5% goat serum. Finally cells were stained and washed before examination with confocal microscopy.

Mouse xenograft

Briefly, 0.1 ml PBS and Matrigel (1:1) containing 2×10^6 control or BEX1 expressing BaF3 or 32D cells were injected subcutaneously into 4-week old male BALB/c nude mice, 5 mice in each group. Animals were monitored for weight change and tumor size. Afterward the mice were maintained for 24 days before the tumors were collected.

Quantification of western blots and statistical analysis

Western blots were quantified using ImageJ. Target signals were normalized against loading control β-actin. One-way ANOVA was used for statistical analysis. In statistical significance tests, "ns" represents not significant, "*" represents $p < 0.05$, "**" represents $p < 0.01$, and "***" represents $p < 0.001$.

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CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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Paper III

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The role of HOXB2 and HOXB3 in acute myeloid leukemia

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ABSTRACT

Acute myeloid leukemia (AML) is a heterogeneous aggressive disease and the most common form of adult leukemia. Mutations in the type III receptor tyrosine kinase FLT3 are found in more than 30% of AML patients. Drugs against FLT3 have been developed for the treatment of AML, but they lack specificity, show poor response and lead to the development of a resistant phenotype upon treatment. Therefore, a deeper understanding of FLT3 signaling will facilitate identification of additional pharmacological targets in FLT3-driven AML. In this report, we identify HOXB2 and HOXB3 as novel regulators of oncogenic FLT3-ITD-driven AML. We show that HOXB2 and HOXB3 expression is upregulated in a group of AML patients carrying FLT3-ITD. Overexpression of HOXB2 or HOXB3 in mouse pro-B cells resulted in decreased FLT3-ITD-dependent cell proliferation as well as colony formation and increased apoptosis. Expression of HOXB2 or HOXB3 resulted in a significant decrease in FLT3-ITD-induced AKT, ERK, p38 and STAT5 phosphorylation. Our data suggest that HOXB2 and HOXB3 act as tumor suppressors in FLT3-ITD driven AML.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous aggressive hematopoietic disorder which is the most common adult acute leukemia accounting for around 80% of cases. Genetic alterations lead to abrogated differentiation of hematopoietic cells. Therefore, the self-renewal ability of those cells is increased, and regulation of normal cell proliferation is disturbed. The major genetic changes include mutation in genes affecting cell proliferation (FLT3, KIT, NRAS/KRAS, JAK/STAT and PTPN11), myeloid differentiation (RUNX1/AML1 and CEBPA), cell cycle regulation or apoptosis (TP53, NPM1), and up-regulation of genes involved in stem-cell maintenance (HOXA, HOXB) [1–4].

The homeobox (HOX) genes are a family of homeodomain-containing transcription factors mainly involved in development. The human HOX genes are clustered in four different chromosomes, 7p15 (HOXA), 17q21 (HOXB), 12q13 (HOXC) and 2q31 (HOXD). HOXA family genes have been thoroughly studied in respect to AML [5–7]. While some studies suggest that HOXB family genes are up-regulated in certain types of AML, their roles in AML have not yet been defined [8–13]. A recent report suggests that HOXB4 expression is elevated in a group of AML patients and higher HOXB4 expression correlated with better prognosis [8]. Another report suggests that over-expression of HOXB6 in NB4 cells or in HL60 cells caused inhibition of the granulocytic and monocytic maturation, respectively [12].

The type III receptor tyrosine kinase FLT3 is expressed in almost all AML, and about 35% of AML patients carry an oncogenic *FLT3* mutation [14]. Among the several mutations that have been found, the internal tandem duplication (ITD) of the sequence that encodes the juxtamembrane domain is the most common mutation in FLT3. The presence of FLT3-ITD mutation portends a poor prognosis in AML. FLT3 mutations also occur in other types of leukemia to a lesser extent, including acute lymphoblastic leukemia [15]. While wild-type FLT3 requires its ligand FL for activation, oncogenic mutants are constitutively active. Activated FLT3 recruits SH2 domain-containing protein through phosphotyrosine residues

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resulting in activation of AKT, ERK, p38, and STAT5 [16–20].

In this report, we show that expression of HOXB2 and HOXB3 genes is upregulated in AML patients carrying the FLT3-ITD mutations. Loss of HOXB2 or HOXB3 expression in patients carrying FLT3-ITD mutations results in enrichment of oncogenic pathways. Overexpression of HOXB2 or HOXB3 significantly inhibits FLT3-ITD-induced cell proliferation and colony formation and further increases apoptosis.

2. Materials and methods

2.1. Plasmids and antibodies

Plasmids expressing human HOXB2 and HOXB3 were generated by ligating the open reading frame (ORF) of the corresponding gene into the retroviral vector pMSCVneo. FLT3-ITD plasmid was described previously [21]. Anti-FLT3 antibody was also described previously [22]. Anti-phosphotyrosine antibody 4G10 was purchased from Millipore (Life Technologies, Carlsbad, CA) and Anti-phospho p38 and anti-p38 antibodies were from BD Biosciences (Franklin Lakes, New Jersey). Anti-phospho-ERK1/2, anti-ERK2, anti-STAT5 and anti-AKT antibodies were from Santa-Cruz Biotechnology (Dallas, Texas) and anti-phospho AKT was from Epitomics (Abcam, Cambridge, UK). Anti-β-actin antibody was from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture and transfection

The murine pro-B cell line Ba/F3 was cultured in RPMI-1640 medium (Hyclone, Thermo Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), 10 ng/ml murine interleukin 3 (IL3) and 1% penicillin and streptomycin. Generation of Ba/F3-FLT3-ITD cells was described previously [23]. FLT3-ITD-transfected Ba/F3 cells were then further transfected with the pMSCV-neo-HOXB2 or pMSCV-neo-HOXB3 construct or empty pMSCV-neo vector. Cells were selected with 0.8 mg/ml G-418 for 2 weeks. Transfected cells were maintained in Ba/F3 medium as previously described [24]. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Immunoprecipitation and western blotting

Cells were starved of serum and cytokines for 4 h and were washed once with cold PBS before lysis with Triton X-100 based lysis buffer. Cell lysates were mixed with SDS and DTT containing loading buffer in a 1:1 ratio and boiled before separation by SDS-PAGE. For immunoprecipitation 1 μg anti-STAT5 antibody was added to cell lysates and was kept for 1 h on ice followed by purification on protein G Dynabeads and SDS-PAGE analysis.

2.4. Apoptosis, cell proliferation, and colony formation assay

Cells were washed three times to remove cytokine before all experiments. Annexin V and 7-aminoactinomycin D (7-AAD) apoptosis kit (BD Biosciences) was used to measure apoptosis in cytokine-depleted cells. Cells positive for annexin V and both annexin V/7-AAD were counted as apoptotic cells. To measure cell proliferation, 10,000 cells were seeded in each well of a 96-well plate and incubated for 48 h. AlamarBlue (Molecular Probe) was used to measure cell viability. Semisolid methylcellulose medium (Stem Cell Technologies) was used for colony formation assay. Around 500 cells were seeded and cultured for seven days before counting colonies.

2.5. Microarray data analysis

The data set GSE14468 was used which was generated from a cohort of 598 newly diagnosed AML patients [25]. Gene expression was compared using significance analysis of microarrays (SAM) tools [26] and gene set enrichment analysis (GSEA) [27]. One-way ANOVA was used for statistical analysis. In statistical significance tests, "ns" represents not significant, "*" represents $p < 0.05$, "**" represents $p < 0.01$, and "***" represents $p < 0.001$.

3. Results

3.1. HOXB family proteins are upregulated in FLT3-ITD positive AML

To understand the molecular difference between oncogenic FLT3-ITD positive and negative AML, we analyzed gene expression data of AML patients. We used expression data from bone marrow aspirates or peripheral blood samples of 598 cases of de novo AML. Using SAM tool we checked the differential gene expression. We observed that several HOXB family genes were upregulated in FLT3-ITD positive patients (Table S1). HOXB2 displayed 2.3-fold upregulation, HOXB3 4.4-fold, HOXB5 1.4-fold and HOXB6 2.2 fold upregulation (Fig. S1A). Therefore, our data suggest that expression of several HOXB genes is deregulated in FLT3-ITD-driven AML.

3.2. HOXB2 and HOXB3 are independent prognostic markers in AML

The HOXB family includes 10 genes, HOXB1-9 and 13. Since several HOXB-family genes were deregulated in FLT3-ITD driven AML, we checked whether expression of HOXB-family genes has any prognostic significance in AML. We transformed relative expression values to the Z-score and divided patients into two groups depending on higher or lower HOXB genes expression. We observed that higher expression of either HOXB2 (Fig. 1A) or HOXB3 (Fig. 1B) but not HOXB5 (Fig. S1B) correlated with poor prognosis compared to lower HOXB2 ($P = 0.0053$) and HOXB3 ($P = 0.0147$) expression. This also holds true for higher expression of HOXB6 (Fig. S1C), HOXB7 (Fig. S1D), HOXB8 (Fig. S1E) and HOXB9 (Fig. S1F). Lower HOXB2 (Fig. 1C) and HOXB3 (Fig. 1D) expression further correlated with better event-free survival ($P = 0.0234$ and $P = 0.0432$ respectively). Although HOXB2 and HOXB3 expression levels displayed prognostic significance in the total patient group independent of FLT3 mutations, we were unable to show any prognostic significance in only FLT3-ITD-dependent AML (data not shown) probably due to limited number of patient samples in each group. Since both HOXB2 and HOXB3 genes expression were upregulated in FLT3-ITD-dependent AML and since both genes expression profiles displayed independent prognostic significance, we checked whether HOXB2 and HOXB3 expression levels correlate with each other. We observed a strong correlation in between expression of the two genes ($r^2 = 0.8633$) suggesting that patients having higher HOXB2 expression will also have a higher HOXB3 expression and *vice versa* (Fig. 1E). In addition to FLT3-ITD positive AML patients (Fig. 1F), HOXB2 and HOXB3 expression was upregulated in patients carrying the NPM1 mutation (Fig. 1G). Although HOXB2 and HOXB3 expression correlated with FLT3-ITD and NPM1 mutations, expression neither correlated with patients age (Fig. S1G and S1H) nor with the patients sex (Fig. S1I). However, expression of both HOXB2 (Fig. S1J) and HOXB3 (Fig. S1K) was significantly downregulated in the FAB M3 group patients.

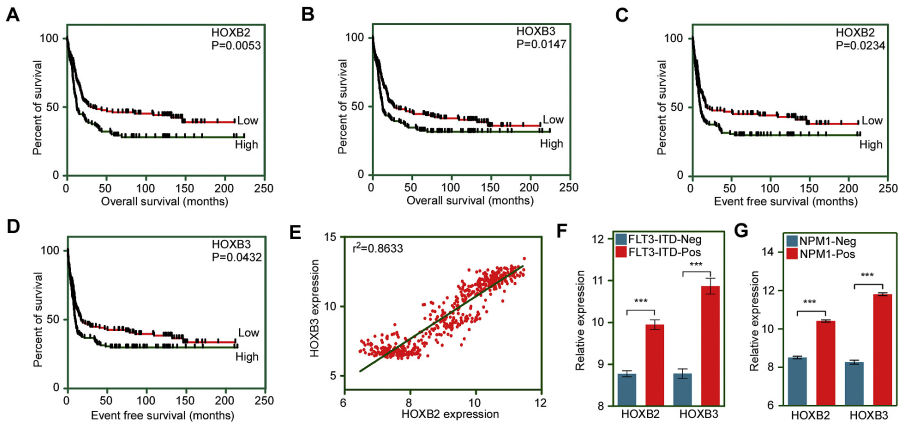

Fig. 1. Overall survival of AML patients with higher and lower HOXB2 or HOXB3 expression: Z-score was used to divide higher and lower HOXB2 or HOXB3 expressing AML patients. (A) Overall survival of AML patients with higher or lower HOXB2 expression. (B) Overall survival of AML patients with higher or lower HOXB3 expression. (C) The event-free survival of AML patients with higher or lower HOXB2 expression. (D) The event-free survival of AML patients with higher or lower HOXB3 expression. (E) Correlation in between HOXB2 and HOXB3 expression. (F) Expression of HOXB2 and HOXB3 in FLT3-ITD positive and negative AML patients. (G) Expression of HOXB2 and HOXB3 in NPM1 mutation-positive and negative AML patients.

3.3. Loss of HOXB2 or HOXB3 expression correlates with enrichment of oncogenic pathways

Since HOXB2 and HOXB3 expression levels were upregulated in FLT3-ITD positive patients, we wanted to analyze whether the loss of HOXB2 or HOXB3 expression results in the enrichment of any oncogenic pathways. Therefore, we analyzed enrichment of oncogenic pathways using gene set enrichment analysis (GSEA). We observed enrichment of several oncogenic pathways including loss of RB and p107 function, loss of SNF function and E2F3 pathways in FLT3-ITD positive AML patients with lower HOXB2 (Fig. 2A) or HOXB3 (Fig. 2B) expression. These results indicate a possible link between the loss of HOXB2 or HOXB3 expression and enhancement of oncogenic signaling in FLT3-ITD-driven AML.

3.4. HOXB2 or HOXB3 over-expression inhibits colony formation and cell proliferation and induces apoptosis

Results from GSEA suggest that HOXB2 or HOXB3 plays a role in FLT3-ITD-dependent AML patients. To determine the role of HOXB2 and HOXB3 in FLT3-ITD signaling we generated two cell lines by stably transfecting the pro-B cell line Ba/F3 with FLT3-ITD together

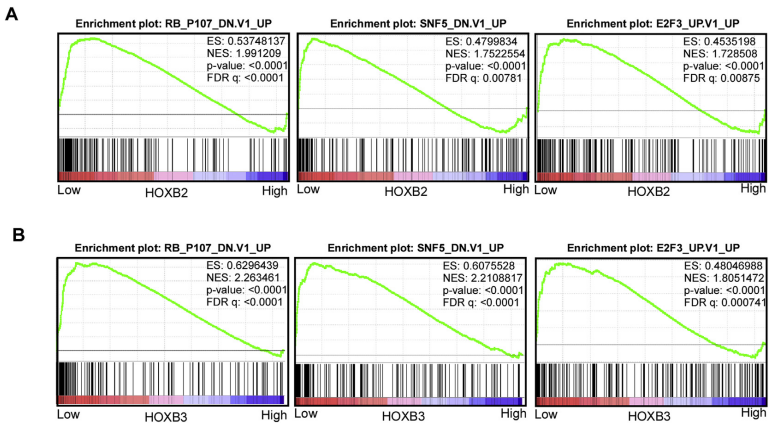

Fig. 2. GSEA shows enrichment of oncogenic pathways in lower HOXB2 or HOXB3 expressing AML patients: Z-score was used to divide higher and lower HOXB2 or HOXB3 expressing FLT3-ITD positive AML patients. (A) Loss of HOXB2 expression correlated with enrichment of several oncogenic pathways. (B) AML patients with lower HOXB3 expression showed enrichment of several oncogenic pathways compared to patients with higher HOXB3 expression.

with HOXB2 or HOXB3, or empty control vector. Expression of FLT3-ITD and HOXB2 or HOXB3 was verified by western blotting (data not shown). Expression of HOXB2 or HOXB3 significantly reduced the size (Fig. 3A) and number (Fig. 3B) of colonies in semi-solid medium. Furthermore, cells expressing HOXB2 or HOXB3 displayed reduced FLT3-ITD-dependent cell proliferation (Fig. 3C) and significantly enhanced apoptosis (Fig. 4D) compared to the empty vector. These findings suggest that expression of HOXB2 and HOXB3 is essential for controlling FLT3-ITD-induced biological events.

3.5. *HOXB2 or HOXB3 over-expression inhibits FLT3-ITD-induced phosphorylation of AKT, ERK1/2, p38, and STAT5*

Since we observed that HOXB2 and HOXB3 altered FLT3-ITD-dependent cell proliferation, apoptosis and colony formation, we analyzed FLT3 downstream signaling using phospho-specific antibodies. We observed that HOXB2 and HOXB3 overexpression blocked FLT3-ITD-dependent AKT (Fig. 4A), ERK1/2 (Fig. 4B), p38 (Fig. 4C) and STAT5 (Fig. 4D) phosphorylation in Ba/F3 cells. Thus, we suggest that overexpression of HOXB2 or HOXB3 initiates a transcriptional program that inhibits FLT3-ITD downstream signaling.

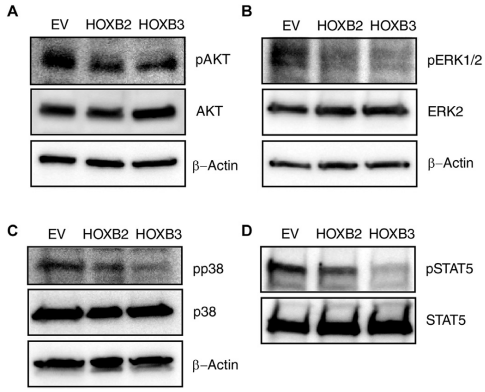

Fig. 4. Expression of HOXB2 or HOXB3 decreases FLT3-ITD-induced downstream signaling: Total cell lysates were used for SDS-PAGE and Western blotting analysis with AKT (A), ERK (B) and p38 (C) antibodies. (D) Cell lysates were immunoprecipitated with an anti-STAT5 antibody followed by western blotting analysis.

4. Discussion

In this study, we aimed to address the role of HOXB2 and HOXB3 in FLT3-ITD-dependent AML. Expression of HOXB2 and HOXB3 was upregulated in FLT3-ITD-dependent patients, and both HOXB2 and HOXB3 can be used as an independent prognostic marker in AML. Patients carrying lower HOXB2 or HOXB3 have enriched oncogenic

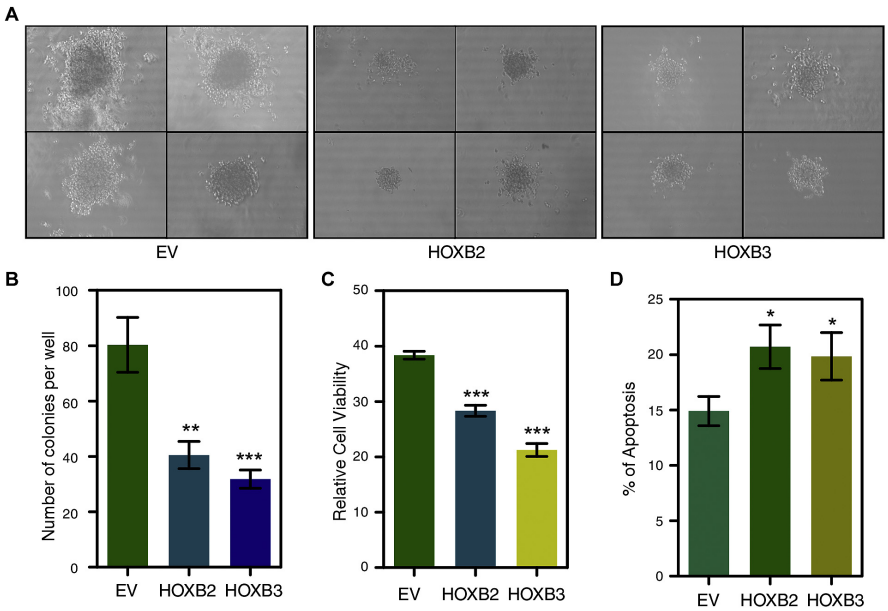

Fig. 3. Expression of HOXB2 or HOXB3 inhibits cell proliferation, colony formation, and enhanced apoptosis: (A) About 500 stably transfected Ba/F3 cells were used to determine colony formation potential in the semi-solid medium. (B) The number of colonies in each well of 24 well plate. (C) FLT3-ITD dependent cell viability in presence and absence of HOXB2 or HOXB3 expression was measured after 48 h using AlamarBlue. (D) Apoptosis induced by overexpression of HOXB2 or HOXB3 was measured using Annexin-V and 7-AAD kit in cytokine depleted cells.

pathways enrichment and overexpression of HOXB2 or HOXB3 resulted in inhibition of FLT3-ITD signaling as well as of biological effects.

Although aberrant expression of homeobox genes is quite common in many cancers, the role of HOXB2 and HOXB3 in human cancer has not been thoroughly studied [28]. HOXB2 has been shown to be overexpressed in breast cancer, and overexpression of HOXB2 was correlated with better prognostic outcome [29]. HOXB2 acts as a tumor suppressor in breast cancer cells. Knockdown of HOXB2 using shRNA resulted in increased tumor growth [29]. We observed that overexpression of HOXB2 in Ba/F3 cells expressing FLT3-ITD resulted in negative regulation of FLT3-ITD-induced signaling as well as the corresponding biological outcomes such as cell proliferation and colony formation and enhancement of apoptosis. Although our observations in Ba/F3 cells are in line with the observation in breast cancer cell lines [29], other studies in cervical cancer [30], lung adenocarcinomas [31] and pancreatic cancer [32] suggest that overexpression of HOXB2 is associated with cancer progression. Therefore, we suggest that the role of HOXB2 in cancer is context dependent.

HOXB3 expression correlated with HOXB2 expression in AML patients suggesting that HOXB3 displays a similar function as HOXB2 in AML. Although expression of HOXB3 increased cell proliferation in several tumors [33–35], we observed that overexpression of HOXB3 resulted in reduced cell proliferation and colony formation as well as increased apoptosis suggesting a similar function as HOXB2 in AML.

Taken together, our study suggests that HOXB2 and HOXB3 have a tumor suppressor role in FLT3-driven AML. Since HOXB2 and HOXB3 are capable of limiting cell proliferation and colony formation as well as inducing apoptosis, overexpression of HOXB2 and HOXB3 keeps the oncogenic FLT3-ITD signaling in balance [36,37]. It is well-known from a number of cell types that too strong oncogenic signaling can lead to induction of apoptosis [38,39], so the tumors needs to keep the oncogenic signals at a moderate level in order to be transforming. Future studies are aiming at elucidating the downstream effectors of HOXB2 and HOXB3 and their role in oncogenic transformation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.10.071.

Transparency document

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Paper IV

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ORIGINAL ARTICLE

Aberrant activation of the PI3K/mTOR pathway promotes resistance to sorafenib in AML

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Therapy directed against oncogenic FLT3 has been shown to induce response in patients with acute myeloid leukemia (AML), but these responses are almost always transient. To address the mechanism of FLT3 inhibitor resistance, we generated two resistant AML cell lines by sustained treatment with the FLT3 inhibitor sorafenib. Parental cell lines carry the FLT3-ITD (tandem duplication) mutation and are highly responsive to FLT3 inhibitors, whereas resistant cell lines display resistance to multiple FLT3 inhibitors. Sanger sequencing and protein mass-spectrometry did not identify any acquired mutations in FLT3 in the resistant cells. Moreover, sorafenib treatment effectively blocked FLT3 activation in resistant cells, whereas it was unable to block colony formation or cell survival, suggesting that the resistant cells are no longer FLT3 dependent. Gene expression analysis of sensitive and resistant cell lines, as well as of blasts from patients with sorafenib-resistant AML, suggested an enrichment of the PI3K/mTOR pathway in the resistant phenotype, which was further supported by next-generation sequencing and phospho-specific-antibody array analysis. Furthermore, a selective PI3K/mTOR inhibitor, gedatolisib, efficiently blocked proliferation, colony and tumor formation, and induced apoptosis in resistant cell lines. Gedatolisib significantly extended survival of mice in a sorafenib-resistant AML patient-derived xenograft model. Taken together, our data suggest that aberrant activation of the PI3K/mTOR pathway in FLT3-ITD-dependent AML results in resistance to drugs targeting FLT3.

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INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease of the blood originating in the bone marrow. Although overall survival of childhood AML has increased in the past decade, it still remains poor compared with that of childhood acute lymphoblastic leukemia. Moreover, survival rates in adults are quite poor and remain virtually unchanged over the last decade.[1] The molecular genetics of AML has been extensively studied. AML with normal cytogenetics accounts for ~50% of all AML, and this subtype of AML is notable for recurrent mutations in several genes: NPM1, CEBPA, TET2, IDH, DNMT3A and FLT3. The receptor tyrosine kinase FLT3 is expressed at high levels in almost all AML, and >30% of AML bears an oncogenic FLT3 mutation.[2] The most common FLT3 mutation is an internal tandem duplication (ITD) of the sequence that encodes the juxtamembrane domain, which portends a poor prognosis. Other mutations include point mutations in the kinase domain.

Wild-type FLT3 requires its ligand FL for activation, whereas oncogenic mutants are constitutively active. The key feature of FLT3 activation is phosphorylation of a number of tyrosine residues in the cytoplasmic domain. Phosphotyrosine residues facilitate association with multiple SH2 domain-containing proteins, including cytosolic tyrosine kinases, ubiquitin ligases, adaptor proteins and phosphatases.[3] Interacting proteins either potentiate receptor signaling by activating multiple pathways, including PI3K-AKT, RAS-RAF-ERK and the p38 pathways, or block receptor signaling by destabilizing the receptor through recruitment of ubiquitin ligases. Oncogenic FLT3 displays equal affinity for the interacting proteins, and thus regulates similar signaling pathways as wild-type FLT3, except for potent activation of STAT5 signaling by FLT3-ITD.[4]

Clinically, FLT3-ITD mutations frequently occur in AML with normal karyotype, t (6:9), t (15:17), and trisomy 8.[5,6] The presence of FLT3-ITD does not appear to affect the complete remission rates, but it significantly increases the risk of relapse.[7] Therefore, expression of FLT3-ITD limits disease-free and overall survival.[8] FLT3-ITD mutations occur in frame with duplications of 3–400 base pairs in the juxtamembrane domain, and the length of the ITD correlates with overall survival.[9] Thus, inhibition of FLT3 should be beneficial for patients with AML with constitutively active FLT3 mutants. To date, >20 small molecule FLT3 inhibitors have been developed, 8 of which have been evaluated in clinical trials.[10] These inhibitors compete with ATP and can efficiently block FLT3 activation as well as downstream signaling. However, none of them has displayed a convincing advancement in AML treatment as a single drug.[10] Responses were mostly limited to transient reductions in peripheral blood blasts, and bone marrow responses were very rare.[11,12] Limited response to the FLT3 inhibitors could

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be due to several reasons. First, it is possible that FLT3 is efficiently inhibited in cell and animal models by these inhibitors but not in AML in human patients. The use of plasma inhibitory activity assays have addressed this question.[13] It is also possible that inhibition of FLT3 alone is not sufficient to achieve complete remissions. Another possibility is that primary and secondary mutations in FLT3 make the receptor resistant to these inhibitors.[14] Earlier studies suggested that acquired mutations in the second part of the kinase domain resulted in a resistant phenotype.[15] Expression of several survival genes in resistant cells also led to FLT3 inhibitor resistance.[16] Recently, a second-generation FLT3 inhibitor, AC220 (quizartinib), has been used in a phase II clinical trial for patients with relapsed and chemotherapy-refractory AML and induced a composite complete remission rate of 44–54%. Response was much better than that observed with any other prior FLT3 inhibitor. Later studies suggest, however, that this drug also suffers from secondary resistance.[17] Another study suggest that the multi-kinase inhibitor midostaurin prolongs survival when used in combination with chemotherapy.[18] Bone marrow blasts from eight patients with AML treated with quizartinib, who achieved a complete remission and then later relapsed, were studied. All eight were found to have new mutations in the kinase domain of FLT3-ITD.[19] Thus, the discovery of novel drugs targeting FLT3 or FLT3 downstream or parallel pathways will be useful.

The PI3K-mTOR signaling pathway has been studied extensively in human disease. This pathway has key functions in regulating cell growth, survival and metabolism, and is aberrantly activated in a number of malignant or non-malignant diseases. Components of this pathway have become attractive drug targets and several drugs are in pre-clinical studies or in clinical trials.[20] The deregulation of the PI3K/mTOR signaling cascade can be specific to the signaling pathway or a consequence of mutations in other pathways that can activate the PI3K/mTOR pathway aberrantly. For instance activating mutations in FLT3, NRAS, KRAS, KIT, the regulatory subunit of PI3K, or loss-of-function mutations of PTEN, can affect this pathway.[3,21,22] Recently, we have shown that, in addition to mutations in these genes, activation of other downstream kinases, such as SYK and p110, contribute to hematopoietic malignancies by activating the PI3K/mTOR pathway.[23,24] Several PI3K/mTOR inhibitors have been used in the treatment of solid tumors and hematological malignancies. The majority of these inhibitors target mTOR, whereas others target AKT and PI3K.[20] Many of them, however, have displayed limited efficacy due to poor specificity and/or poor solubility or bioavailability. Although the PI3K/mTOR pathway is over-represented in hematopoietic malignancies, and mTORC1 is involved in drug resistance, use of drugs targeting this pathway is still limited in the clinic.[25] A recent study suggests that treatment with a PI3K or AKT inhibitor leads to a differential apoptotic response in 32D cells transfected with FLT3-ITD compared with those expressing FLT3-TKD.[26] Activation of the STAT5 pathway is partially responsible for the differential effects as FLT3-ITD, but not FLT3-TKD, is a potent activator of this pathway.[4]

In this study, we propose a novel mechanism of FLT3 inhibitor resistance. Using patient-derived cell lines we show that sustained treatment with sorafenib abrogates FLT3 dependency, even though the inhibitor still effectively blocks FLT3 activation in the resistant cells. Exome sequencing of the resistant cell lines revealed that resistant cell lines acquired novel mutations in different signaling proteins and transcription factors. Gene expression and phospho-specific antibody array experiments revealed an enrichment of the PI3K/mTOR signaling pathway in resistant cells. Furthermore, resistant cells responded to PI3K/mTOR inhibitors in cell lines and in mouse xenograft models of AML.

RESULTS

Sorafenib-resistant MV4-11 and MOLM-13 cell lines display resistance to AC220

To identify alternative resistance mechanisms to FLT3 inhibitors in the treatment of FLT3-ITD-driven AML,[10] we used two patient-derived cell lines, MV4-11 and MOLM-13. MV4-11 cells express only the FLT3-ITD, whereas MOLM-13 cells express wild-type FLT3 and FLT3-ITD. Both cell lines are dependent on FLT3 activity as sorafenib, PKC-412, and AC220, but not imatinib, dasatinib, nilotinib or bosutinib, inhibit cell survival in both cell lines (Figure 1a). After treatment of these cell lines with sorafenib for 90 days, we observed that both cell lines displayed resistance to sorafenib as well as to AC220 (Figure 1b) suggesting that sustained treatment with an FLT3 inhibitor results in acquired resistance. To test whether sorafenib was still effective in FLT3 inhibition, we treated sensitive and resistant cells with sorafenib or dimethyl sulfoxide and then stimulated with FLT3 ligand (FL). Although dimethyl sulfoxide-treated cells responded to FL as expected, sorafenib-treated cells displayed poor FLT3 activation (Figure 1c), suggesting that sorafenib is still capable of inhibiting FLT3 activation in these resistant cells. Surprisingly, we observed that the resistant cells treated with dimethyl sulfoxide had a much more robust response to ligand in terms of FLT3 activation. Similar results were observed with AKT and ERK activation, as sorafenib-treated cells poorly respond to FL stimulation (Figure 1d). Furthermore, resistant cells treated with sorafenib could still form colonies similar to dimethyl sulfoxide-treated cells (Figure 1e), indicating that these cells were no longer dependent on FLT3 activity, although sorafenib could partially block FLT3 activity. Although both MV4-11- and MOLM-13-resistant cell lines displayed similar patterns of FLT3 activity and downstream signaling, MV4-11-resistant cells were more sensitive to sorafenib than MOLM-13-resistant cells, suggesting a complex mechanism behind the resistant phenotype. To determine whether secondary mutations occurred in FLT3, we sequenced the whole coding region using Sanger sequencing. Except for a mutation in the extracellular domain, which was present in all four cell lines (sensitive as well as resistant), we were unable to detect any mutations in the inhibitor-binding site (data not shown). Similar to Sanger sequencing, mass-spectrometric analysis of affinity-enriched FLT3 indicated no differences in FLT3 among the sensitive versus resistant cells in the intracellular part of FLT3 (Supplementary Figure S1). Because we observed an unexpected activation of FLT3 in resistant cells stimulated with FL (Figure 1c), we hypothesized that certain FLT3 residues remain hyper-tyrosine phosphorylated. To test our hypothesis, we used phospho-specific antibodies against known FLT3 residues. Although we observed an increase in total FLT3 phosphorylation, we were unable to identify a single site that was selectively hyper-phosphorylated. Instead, all sites remained slightly more phosphorylated (Supplementary Figure S2) compared with control cells. Therefore, we suggest that sustained treatment with a FLT3 inhibitor abolished FLT3 dependency of cells for survival without the occurrence of any additional secondary mutations in FLT3.

Resistant cells carry novel mutations compared with sensitive cells

As we were unable to identify any secondary mutation in the inhibitor-binding site of FLT3, we hypothesized that acquired resistance could be due to mutations in genes encoding parallel signaling proteins, leading to hyperactivation of the PI3K/mTOR pathway. Therefore, we sequenced the whole coding region of all genes using a next-generation sequencing approach. FLT3 inhibitor-sensitive and -resistant MV4-11 and MOLM-13 cells were sequenced with an average of a 60-fold coverage and $>92\%$ of bases were covered with at least 10 reads. We observed a similar mutational burden in sensitive as well as in resistant cell lines (Figure 2a). In previously reported next-generation sequencing of

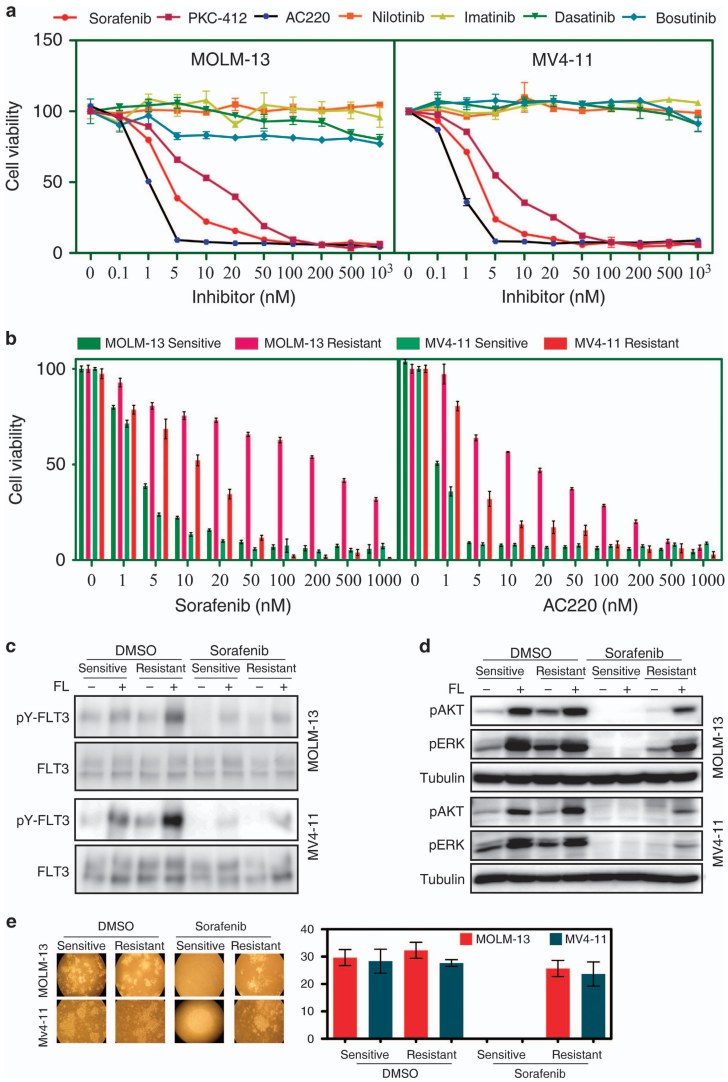


Figure 1. Sorafenib-resistant MV4-11 and MOLM-13 cell lines display resistance to multiple FLT3 inhibitors. (**a**) MOLM-13 and MV4-11 cell lines were treated with an increasing concentration (from 0 to 1000 nM) of multiple tyrosine kinase inhibitors. Cells were cultured with inhibitors for 46 h followed by PrestoBlue viability analysis. (**b**) Sorafenib-sensitive and -resistant cell lines were treated with increasing concentrations of AC220 and sorafenib for 46 h before processing for PrestoBlue viability assays. (**c**) Sorafenib-sensitive and -resistant MOLM-13 and MV4-11 cells were serum-starved for 4 h in the presence or absence of 100 nM sorafenib before 100 ng/ml FL stimulation for 5 min. Cells were then lysed and immunoprecipitated with an anti-FLT3 antibody. The 4G10 (anti-phospho-tyrosine) and anti-FLT3 antibodies were used to probe the blots. (**d**) Cell lysates from the experiment described in **c** were resolved by SDS–PAGE and analyzed by western blotting using anti-phospho AKT, anti-phospho ERK and anti-Tubulin antibodies. (**e**) MOLM-13 and MV4-11 cells were seeded with or without 100 nM sorafenib in semisolid medium and cultured for 7 days.

primary AML samples, recurrent mutations in >20 different genes were demonstrated.[27] In this subset of genes, we observed mutations in TP53 (P72R), TET2 (V218M and I1762V) and FLT3 (T227M) in all four cell lines (Figure 2b). The FLT3-D835Y mutation was identified in only the MOLM-13-resistant cell line with an allele depth of 67:37, suggesting that the FLT3-D835Y mutation is subclonal, explaining the fact that Sanger sequencing was unable to identify this mutation in FLT3. Probably the FLT3-D835Y mutation also partially contributed to the stronger resistant phenotype observed in MOLM-13 cells (Figures 1b–e) as FLT3-D835Y can render drug resistance. We also observed many novel mutational events in the resistant cell lines compared with the sensitive cells. In MV4-11-resistant cells 52 novel indels (Supplementary Table S1) and 336 novel point mutations (other than synonymous mutations) (Supplementary Table S2) were identified in 50 and 240 genes, respectively. Similar to resistant MV4-11 cells, MOLM-13-resistant cells carried 44 novel indels (Supplementary Table S3) in 42 genes and 279 novel point mutations (Supplementary Table S4) in 192 genes. Novel mutations in 73 genes were common in both resistant cell lines (Figure 2c). According to TCGA data and the Cosmic database, 51 out of 73 genes were found to be mutated in at least one patient. Mutations occurred in several transcriptional regulators, such as E2F4, NOTCH2, ATF5, MKL1, MESP2, MEF2A, MLL3, SPEN, TSHZ1, ZNF587, ZNF717 and KRTAP1-1, and cell surface receptor signaling proteins, such as GPR153, GAB2, NOTCH2, MESP2, NPVF, OR8U1, SPEN and TAS2R43. Furthermore, we observed novel mutations in PIK3C2G (MOLM-13 sorafenib-resistant cells, Supplementary Table S3) and mTOR (MV4-11 sorafenib-resistant cells, Supplementary Table S4) genes in each set of cell lines. The frame-shift mutation observed in the regulatory domain of PI3K has not been reported before. Although several activating mTOR mutations have been described, MV4-11 cells carry E2536A and this mutation has not been previously reported.[28,29] Although the finding of mutations in genes involved in the PI3K/mTOR pathway is intriguing, follow-up studies will be needed to determine whether these or the other mutations identified actually contribute to drug resistance.

PI3K/mTOR pathways are upregulated in resistant cell lines

We next attempted to identify gene expression profiles of resistant cell lines. We analyzed mRNA expression of all cell lines

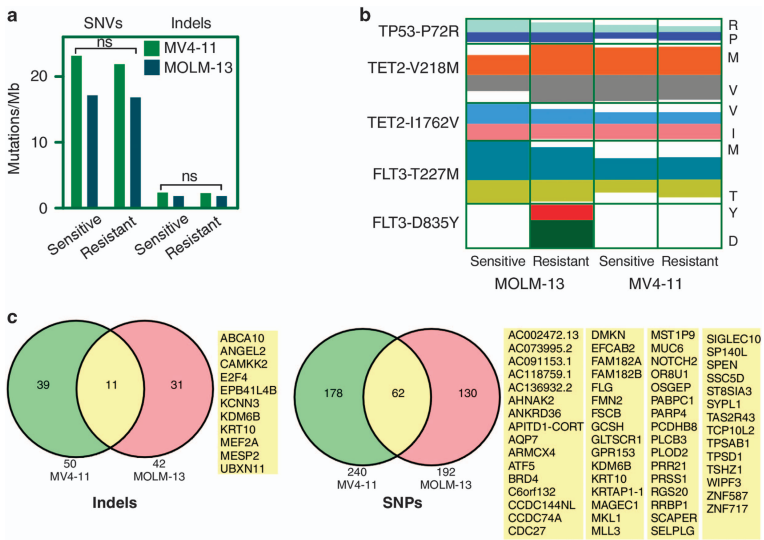
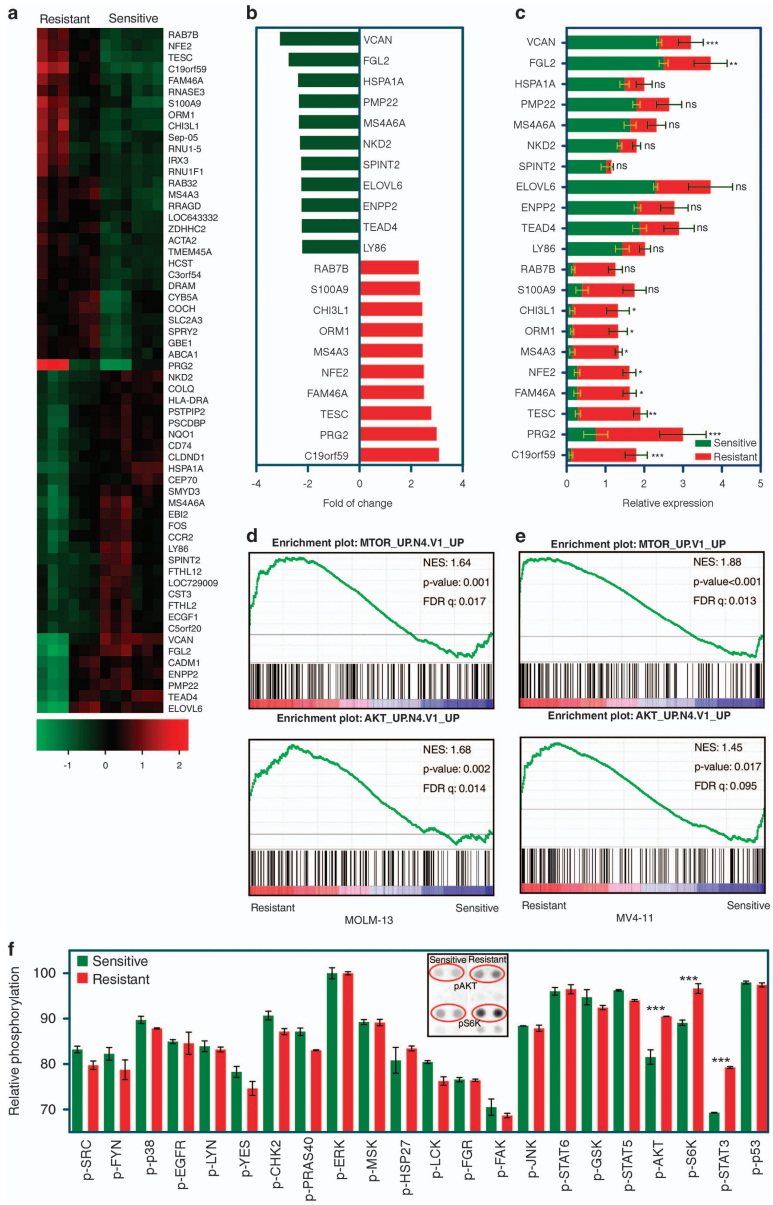


Figure 2. Sorafenib-resistant cell lines carry novel mutations. Genomic DNA from FLT3 inhibitor-sensitive and resistant MV4-11 and MOLM-13 cell lines was extracted using standard protocols. DNA was processed for exome sequencing. (**a**) Comparison of total SNVs per mega bases DNA. (**b**) Known mutations identified in different cell lines. Color code indicates observed allele depth. (**c**) Indels and point mutations were identified by comparing the generated sequence with that from the human reference genome. Genes with novel (not present in sorafenib-sensitive cells) indels and point mutations in both sorafenib-resistant MOLM-13 and MV4-11 cells are reported.

Figure 3. Gene expression profiling suggests an enrichment of the PI3K/mTOR pathways in sorafenib-resistant cells. (**a**) Heatmap of upregulated and downregulated genes in sorafenib-sensitive versus -resistant cells. (**b**) Upregulated and downregulated genes in sorafenib-resistant cells compared with sensitive cells. (**c**) Significantly upregulated and downregulated genes in resistant cells. ANOVA was used to measure the significance. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ and ns, $P > 0.05$. (**d**, **e**) GSEA was performed using MOLM-13-sensitive cells and resistant cells. GSEA was applied to compare pathways significantly enriched between sorafenib-sensitive and -resistant MOLM-13 (**d**) and MV4-11 (**e**) cells. (**f**) Sorafenib-sensitive and -resistant MOLM-13 cells were serum-starved 4 h before lysis. Lysates were the processed for phospho-specific protein array using manufacturer's protocol. Spots intensities were measured using ImageJ. ***$P < 0.001$. Total phosphorylation was normalized against a loading control.

using Illumina HumanHT-12 v4 Expression BeadChips that provides coverage of >47 000 transcripts. We observed that a group of genes responsible for cell survival and proliferation were upregulated in resistant cells, whereas expression of pro-apoptotic genes was downregulated (Figure 3a). To enable prioritization of candidate genes, we analyzed the expression data using ANOVA



(Figure 3b) and significance analysis of microarrays (Figure 3c). This analysis further showed that genes previously known to be associated with cell proliferation or cell cycle progression, such as TESC,[30] FAM46A,[31] NFE2[32] and MS4A3[33] were significantly upregulated. Because gene expression is regulated by sustained expression of signaling cascades, we examined the enrichment of signaling pathways in resistant cell lines. We observed an enrichment of the mTOR and AKT pathways in both MOLM-13- (Figure 3d) and MV4-11- (Figure 3e) resistant cell lines. In addition to pathway enrichment, using a phospho-protein antibody array, we showed that the phosphorylation of the mTOR substrates S6K and AKT were selectively increased in resistant cells (Figure 3f). We also observed an increase in STAT3 phosphorylation. Elevated STAT3 phosphorylation was probably due to the previously described upregulation of JAK3 expression in sorafenib-resistant AML.[34] Thus, we suggest that the activation of the PI3K/mTOR pathway leads to aberrant expression of survival proteins that further contributes resistance to multiple FLT3 inhibitors in MV4-11- and MOLM-13-resistant cell lines.

PI3K/mTOR inhibitors are equally effective in inhibiting growth of sensitive and resistant cell lines

Because we observed that the PI3K/mTOR pathway is upregulated in resistant cells, we treated cells with three PI3K/mTOR inhibitors: gedatolisib (also known as PF 05212384 or PKI-587),[35] PI 103[36] and WYE 687.[37] Although all three inhibitors could inhibit cell viability, gedatolisib, a dual PI3K/mTOR inhibitor that has been studied in solid tumors,[35,38] was the most potent inhibitor (IC_{50} 23 nM) in the resistant cell lines (Figure 4a). In addition, we observed that addition of 5 or 10 nM sorafenib in combination with gedatolisib potentiated inhibition of the growth of sorafenib-sensitive cells, but it did not potentiate the effect of gedatolisib on sorafenib-resistant cells (Figure 4b). We also observed enrichment of an mTOR signature in primary blasts from eight samples from patients with sorafenib-resistant AML (GSE35907) (Figure 4c) and in primary patients AML blasts expressing an FLT3-ITD compared with those lacking the FLT3-ITD (525 AML samples, GSE14468) (Figure 4d). Taken together, these data suggest that aberrant activation of the PI3K/mTOR pathway can lead to acquired resistance to sorafenib in AML cells.

Gedatolisib is a specific PI3K/mTOR inhibitor in AML

Gedatolisib has been shown to be a highly selective inhibitor of PI3K/mTOR.[35] The inhibitor has been used in cell models, animal models and clinical trials for solid tumors.[38–40] To test the specificity of gedatolisib in our cell line model, we ran a phospho-specific antibody array. Treatment of cells with a higher concentration of gedatolisib did not alter the phosphorylation of other signaling proteins, except for AKT and S6K (Figure 5a). The array data were further verified with western blotting using phospho-specific antibodies against AKT, ERK1/2, p38 and S6K (Figure 5b). These results suggest that gedatolisib efficiently blocks the downstream effectors PI3K/mTOR without affecting other signaling pathways. Thus, we suggest that gedatolisib is a specific PI3K/mTOR inhibitor in AML that can be used to block cell growth.

Gedatolisib inhibits colony formation, cell proliferation and induces apoptosis

To assess the biological outcomes of gedatolisib treatment, we performed several biological assays including colony formation, cell proliferation and apoptosis studies. Dose-dependent impairment of colony formation suggested that the inhibitor efficiently reduced the number of colonies as well as size of colonies in both MOLM-13- (Figure 6a) and MV4-11- (Figure 6b) resistant cell lines. Furthermore, increasing concentrations of the inhibitor gradually reduced Edu incorporation indicating that the gedatolisib was capable of reducing cell proliferation (Figure 6c). The compound also induced apoptosis in the same cell lines (Figure 6d).

Gedatolisib delays tumor formation in mouse xenograft

To test whether gedatolisib is also effective in animal models, we developed mouse AML xenograft models by injecting MV4-11- and MOLM-13-resistant cells subcutaneously. Mice were treated with 12.5 mg/kg of gedatolisib or vehicle for 25 days. As expected, the tumors of vehicle-treated animals grew rapidly. In sharp contrast, tumors developed with a significant delay in mice treated with gedatolisib (Figures 7a and b). Furthermore, gedatolisib treatment significantly reduced tumor weight in both cell lines (Figures 7c–e).

Gedatolisib significantly extended survival of mice in a sorafenib-resistant patient-derived xenograft model

To further address the efficacy of gedatolisib in FLT3 inhibitor-resistant AML *in vivo*, we generated a patient-derived xenograft model using an AML sample from a patient with sorafenib-resistant AML. A methylcellulose colony-formation assay demonstrated that gedatolosib effectively reduced colony-formation potential of primary AML cells, and the addition of sorafenib did not significantly increase the inhibitory potential (Figure 8a). Mice treated with gedatolosib displayed a lower number of circulating CD45-positive cells compared with vehicle-treated mice (Figure 8b). Furthermore, gedatolosib significantly extended survival of mice engrafted with sorafenib-resistant AML patient cells (Figure 8c).

DISCUSSION

Despite ongoing progress in understanding the biological mechanisms of the pathogenesis of AML, patients still suffer from poor outcomes with current therapies. Second-generation FLT3 inhibitors have displayed promising results in some clinical trials. However, development of drug resistance has become a major challenge in targeting this oncogene. In this report, we have shown that aberrant activation of the PI3K/mTOR pathway leads to FLT3 inhibitor resistance in AML, and that a dual PI3K/mTOR pathway inhibitor effectively blocks the growth of these resistant cells *in vitro* and *in vivo*.

Several mechanisms have been described to explain secondary resistance in FLT3-ITD-mutated AML. FLT3-ITD-positive patients harboring D835Y or F961L mutations frequently show reduced response to FLT3 inhibitors.[19,41] Moreover, aberrant activation of parallel signaling pathways, such as STAT5 and MAPK, can lead to FLT3 inhibitor resistance even though FLT3 inhibition is maintained.[11] Our results suggest another mechanism of acquired resistance to sorafenib: activation of the PI3K/mTOR pathway. Hyper-activation of the PI3K/mTOR pathway is known to be involved in leukemia and drug resistance.[25] The gene expression data suggested an enrichment of the PI3K/mTOR pathway in the resistant cell lines, as well as in primary patient AML blasts, which are resistant to sorafenib therapy. These data were further supported by the phospho-specific-antibody array profiling where we observed that AKT and S6K were selectively activated. Furthermore, an mTOR inhibitor, rapamycin, did not display a growth inhibitory effect (data not shown) suggesting that resistance cells have an upregulation of PI3K/mTOR signaling. The PI3K/mTOR pathway can be activated through several different mechanisms. Amplification or mutations in upstream receptors or signaling proteins frequently activate downstream signaling cascades. With next-generation sequencing we observed novel, non-recurrent mutations in mTOR and PIK3C2G in sorafenib-resistant cell lines and also many overlapping mutations in both

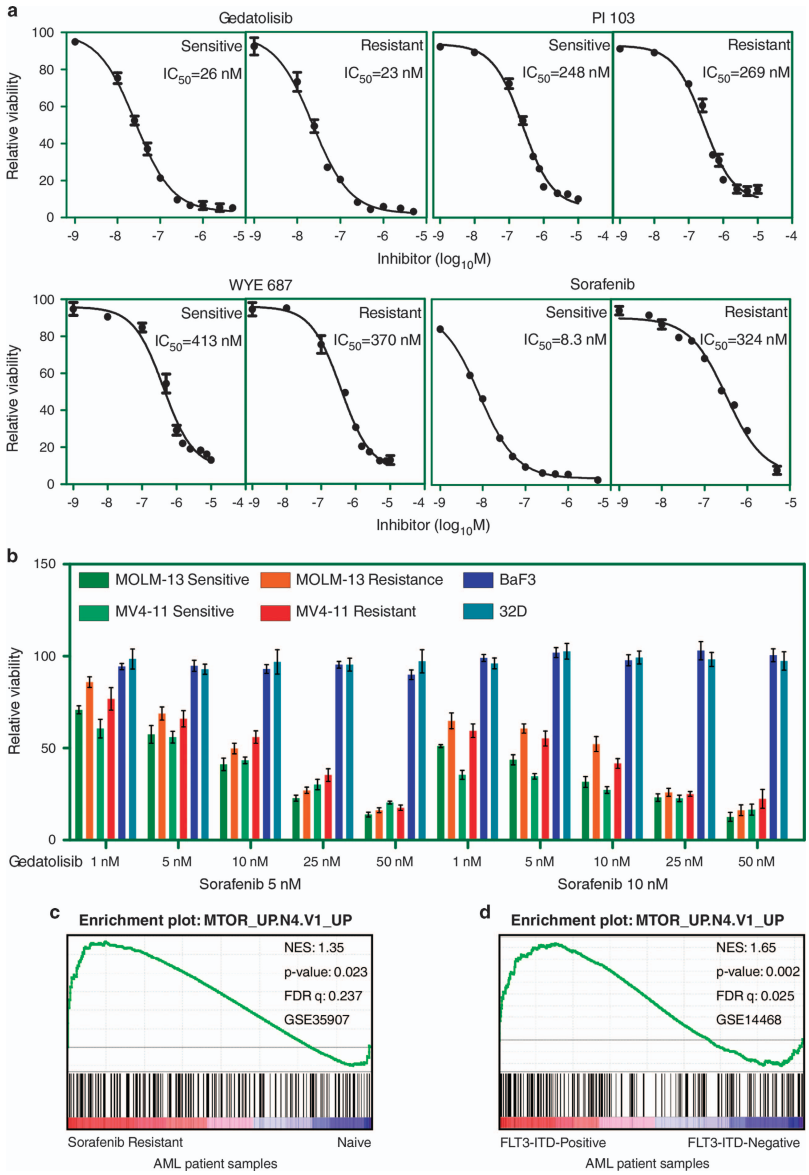


Figure 4. PI3K/mTOR inhibitor effectively reduces cell viability. (**a**) Sorafenib-sensitive and -resistant MOLM-13 cells were treated with different concentrations of inhibitors for 46 h. Cell viability was measured using PrestoBlue cell viability assay. IC_{50} was calculated using GraphPad prism 5.0. (**b**) Sorafenib-sensitive and -resistant MOLM-13 and MV4-11 cells were treated with 5 or 10 nM sorafenib along with increasing concentrations of gedatolisib. Ba/F3 and 32D cells were used as control. (**c**) GSEA was performed using gene expression data from AML patient samples carrying a FLT3-ITD mutation before and after sorafenib treatment. The data set GSE35907 was used for analysis. (**d**) GSEA was performed using gene expression data from AML patient samples carrying FLT3-ITD mutations or not. The data set GSE14468 was used for analysis.

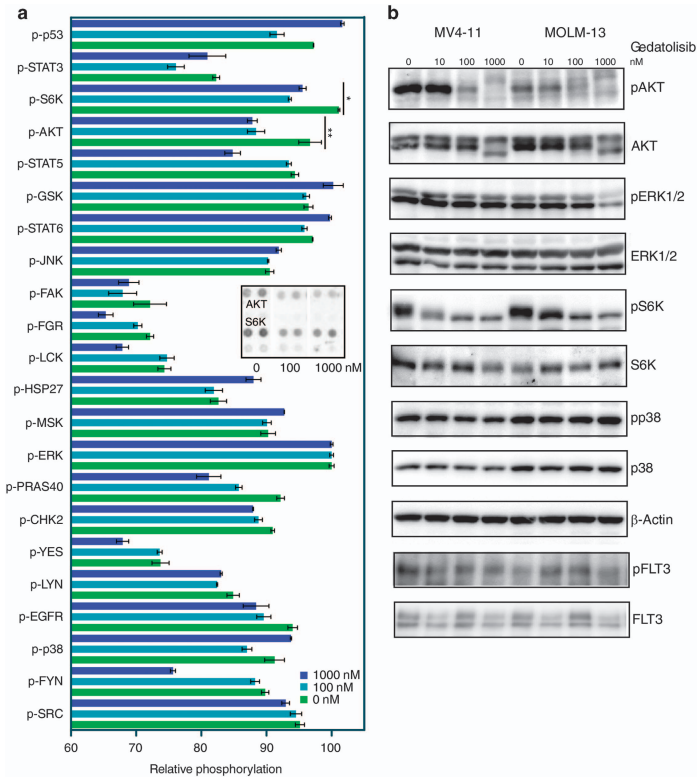


Figure 5. Gedatolisib is a selective PI3K/mTOR inhibitor in AML cell lines. (**a**) Sorafenib-resistant MOLM-13 cells were serum-starved and treated with 0, 100 and 1000 nM gedatolisib for 4 h before lysis. Lysates were then processed for phospho-specific protein array using the manufacturer's protocol. Spot intensities were measured using ImageJ. Total phosphorylation was normalized against a loading control. (**b**) Sorafenib-resistant MV4-11 and MOLM-13 cells were serum-starved and treated with increasing concentration of gedatolisib before lysis. Lysates were then analyzed by western blotting using anti-phospho-specific antibodies. ANOVA was used to measure the significance. $^{**}P < 0.01$; $^{*}P < 0.05$ and ns, $P > 0.05$.

cell lines, which might be involved in aberrant activation of the PI3K/mTOR pathway. Consequently, it would be of interest to identify a direct link between the mutations observed in resistant cells and hyperactivation of the PI3K/mTOR pathway. In addition to the alternative pathways, FLT3-D835Y mutation in MOLM-13 cells also contributed a resistant phenotype that was stronger than in the corresponding MV4-11 cells. As FLT3-D835Y occurred only in a small portion of the cells, Sanger sequencing was unable to identify the mutation.

Dual PI3K/mTOR inhibition has been shown to be effective in MLL-rearranged AML cell lines.[42] In our study, both FLT3 inhibitor-sensitive and resistant human AML cell lines responded to the potent PI3K/mTOR inhibitor gedatolisib.[35] This is not unexpected because patients carrying FLT3-ITD display upregulation of the PI3K/mTOR signaling pathway. The PI3K/mTOR inhibitor gedatolisib displayed selective target specificity, even at very high concentrations, suggesting that the drug should have few off-target effects. Nano-molar concentrations of the drug induced apoptosis, blocked cell proliferation, abolished colony formation in AML cell lines, delayed tumor formation in a xenograft model, and extended survival in a patient-derived xenograft model, supporting its activity in AML.

In conclusion, we propose an alternative mechanism of FLT3-drug resistance. We show that resistant cells lose FLT3 dependency even though FLT3 remains responsive to the inhibitor. Furthermore, resistant cells display hyperactivation of the PI3K/mTOR pathway and a highly selective inhibitor against this pathway can efficiently block colony formation, decrease cell proliferation, induce apoptosis and block tumor growth *in vivo*.

MATERIALS AND METHODS

Patient's data

Patient sample data were collected from two previously published studies. In GSE35907 data set, four AML patient samples carrying FLT3-ITD mutations were treated with sorafenib, and samples were collected before treatment and after developing resistance to the sorafenib.[34] The data set GSE14468 was generated from a cohort of 598 newly dioagonosed AML patients.[43]

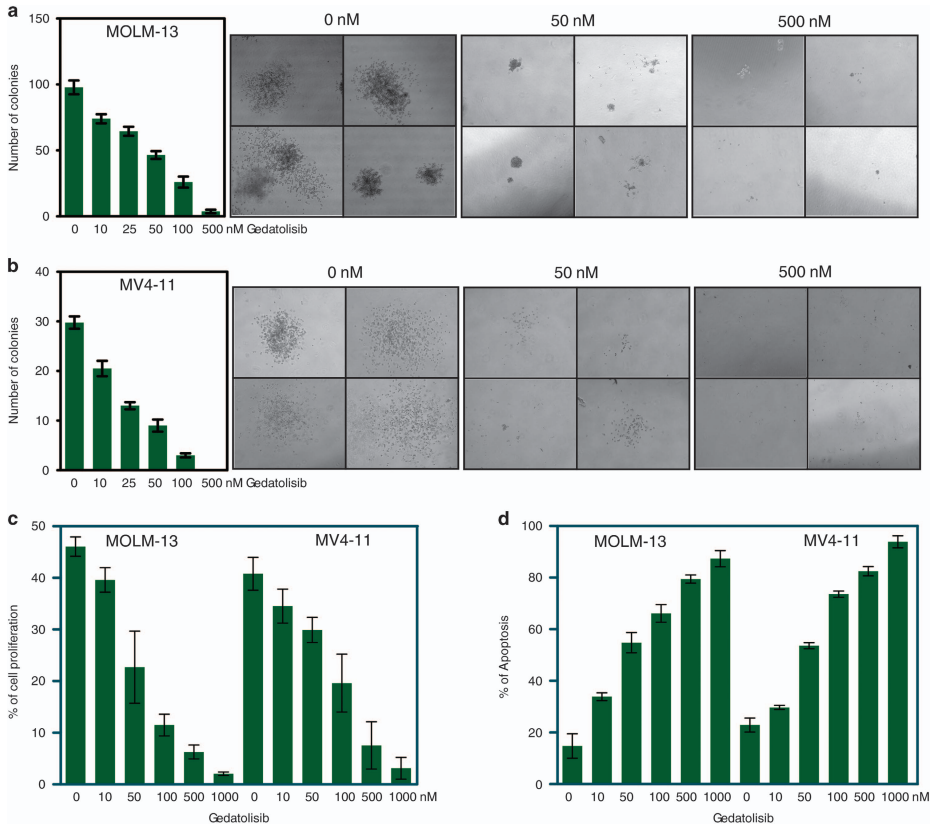


Figure 6. Gedatolisib reduces colony-formation potential, cell proliferation and induces apoptosis. (**a**, **b**) Sorafenib-resistant MOLM-13 (**a**) and MV4-11 (**b**) cells were seeded in semisolid medium containing increasing concentration of gedatolisib. Colonies were counted after 7 days of seeding. (**c**) Sorafenib-resistant cells were seeded with an increasing concentration of inhibitor and incubated for 46 h followed by 2 h of Edu incubation. Cells were then fixed and processed for proliferation assays. (**d**) Sorafenib-resistant cells were seeded with an increasing concentration of inhibitor and incubated for 48 h followed by annexin V and 7-AAD apoptosis assays.

Cell culture

The human AML cell lines, MV4-11 and MOLM-13 (obtained from DSMZ), were maintained in RPMI-1640 medium (Hyclone, South Logan, Utah) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Waltham, MA, USA, Australian origin), 100 μg/ml streptomycin and 100 units/ml penicillin. The murine hematopoietic cell line Ba/F3 and the myeloid cell line 32D were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 10 ng/ml murine interleukin 3, 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. All cells were routinely checked for mycoplasma contamination.

Generation of resistant cell lines

Initially, MV4-11 and MOLM-13 cell lines were treated with sorafenib starting at 1 nM concentration. Complete growth medium with sorafenib was changed twice a week. The concentration of sorafenib was increased to 50 nM within 90 days.

Antibodies and inhibitors

Anti-FLT3 and anti-phospho-specific FLT3 antibodies were described previously.[44] Additional antibodies include: anti-phosphotyrosine 4G10 (Millipore, Darmstadt, Germany), anti-phospho AKT (Epitomics, Burlingame, CA, USA), anti-phospho ERK (Santa-Cruz Biotechnology Inc., Dallas, TX, USA), anti-phospho S6K and anti-S6K (Abcam, Cambridge, UK), anti-phospho p38 and anti-p38 (BD biosciences, Sparks, MD, USA) and anti-tubulin and anti-β-actin (Sigma-Aldrich, St Louis, MO, USA). Inhibitors were obtained from TOCRIS chemical (Bristol, UK) and the human phospho-array was from R&D systems (Abingdon, UK).

Immunoprecipitation and western blotting

After ligand-stimulation and/or inhibitor treatment, cells were washed once with cold phosphate-buffered saline. Cells were then lysed using Triton X-100-based lysis buffer. Cell lysates were mixed with dithiothreitol and sodium dodecyl sulfate polyacrylamide gel electrophoresis containing loading buffer in a 1:1 ratio and boiled before analysis by sodium dodecyl

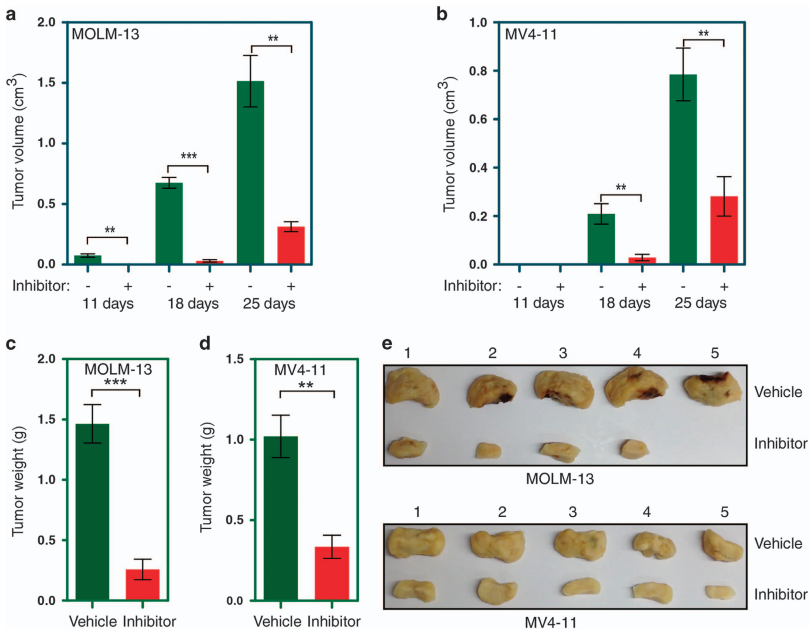


Figure 7. Gedatolisib delays tumor formation in xenograft mice: mice xenografts (five mice in a group) with sorafenib-resistant MOLM-13 (**a**) or MV4-11 (**b**) cells, were treated with gedatolisib or vehicle. Tumor volume was measured at different time points. (**c**–**e**) Tumor weight was measured after dissecting tumor from the inhibitor or vehicle-treated mice.

sulfate polyacrylamide gel electrophoresis. For immunoprecipitation, cell lysates were mixed with specific primary antibodies for 1 h on ice followed by protein-A based purification and sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis.

Cell viability

PrestoBlue cell viability assay (Molecular Probes, Eugene, OR, USA) was used to measure cell viability. Ten thousand cells were seeded per well in 96-well plates in 90 µl medium with or without drugs. Cells were incubated for 46 h. Then 10 µl of PrestoBlue was added to each well. Cells were further incubated for 2 h. Changes of color of PrestoBlue were measured by determining absorbance at 570 and 600 nm. Relative cell viability was calculated as per the manufacturer's instructions.

Apoptosis

Apoptosis was measured using an annexin V and 7-aminoactinomycin D kit (BD biosciences). Cells positive for annexin V or both annexin V and 7-aminoactinomycin D were counted as apoptotic cells.

Cell proliferation

Click-iT Edu Alexa Fluor 647 kit was used to measure cell proliferation. Cells were seeded in a 24-well plate with different concentrations of inhibitors and incubated for 46 h. Edu was added to each well and incubated for 2 h before processing cells for staining using the manufacturer's protocols. Cells were then analyzed by flow cytometry.

Colony-formation assay

Approximately 100 cells were seeded in semisolid methylcellulose medium (Stemcell Technologies, Vancouver, BC, Canada) with different concentrations of inhibitors. Cells were cultured for 7 days before counting colonies.

Exome sequencing

Total genomic DNA was extracted from cell lines using a DNeasy Blood and Tissue kits (Qiagen, Copenhagen, Denmark). The Human All Exon enrichment (Agilent SureSelectXT) library was used to read 100 bp paired end sequencing on a Genome Sequencer Illumina HiSeq2500. Sequence reads are mapped to the reference sequence (human, hg19, GRCh37) using Burrows-Wheeler Aligner with the default parameters. The SNP and InDel calling is done using Genome Analysis Toolkit's Unified Genotyper, which use Bayesian genotype likelihood model to estimate simultaneously the most likely genotypes and allele frequency in a population of *N* samples.

Mass spectrometric analysis of FLT3

Immunoprecipitated FLT3 was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, reduced and alkylated before separate in-gel digestions using trypsin and LysC, respectively. The digests were separated by nano-LC (Eksigent) and analyzed online by liquid chromatography–mass-spectrometry (LC-MS)/MS on an Orbitrap XL ETD. MS/MS spectra were matched against the human part of Swissprot as of 2013-02, extended with an equal size reverse sequence database using Mascot v 2.4.1(www.matrixscience.com). For MS/MS matching a precursor tolerance of 7 ppm and a fragment tolerance of 0.5 Da were used. A label-free quantification workflow[45] was used for FLT3 sequence coverage comparisons, and peptides, which were

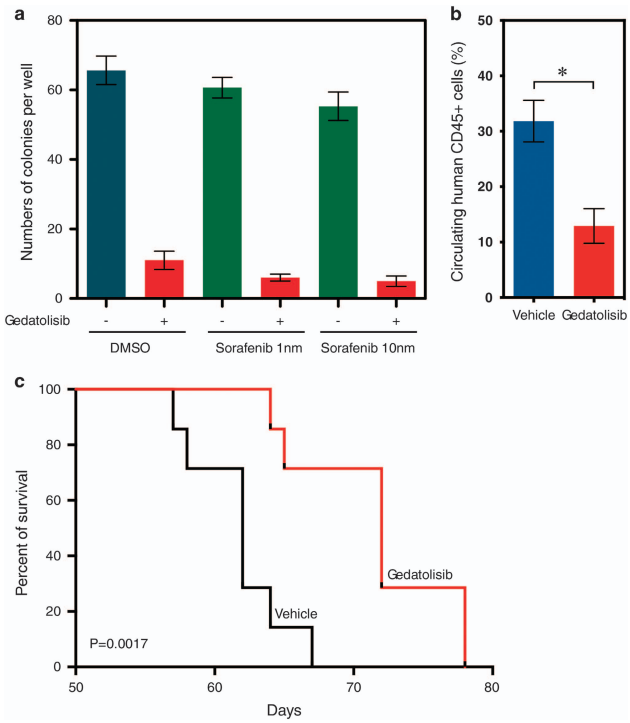


Figure 8. Gedatolisib increases survival of mice in a sorafenib-resistant PDX model. (**a**) Colony-forming units assay from sorafenib-resistant primary AML cells treated with either 1 or 10 nM sorafenib in combination with 50 nM gedatolisib. Results represent the average of triplicate assays. Error bars represent mean ± s.e.m. (**b**) Proportion of circulating human CD45-positive AML cells in peripheral blood from four mice per group 58 days post injection. *P*-value calculated using Mann–Whitney test. Error bars represent mean ± s.e.m. (**c**) Kaplan–Meier curves showing overall survival of mice ($n = 7$ for each group) transplanted with sorafenib-resistant primary cells harvested from a sorafenib-treated patient with AML. Statistical significance determined by log-rank (Mantel–Cox) test. **P*-value ⩽ 0.05 by comparison with vehicle-treated group.

detected in at least one file with a false discovery rate below 1% as determined in the Proteios Software Environment,[46] were included in the comparison.

Mouse xenograft model

NOD/SCID mice were injected with 200 000 cells subcutaneously and treated, from the subsequent day after injection, with twice weekly intravenous 12 mg/kg gedatolisib or vehicle (5% (278 mM) dextrose in water, 0.3% lactic acid pH 3.5) for 25 days. Experiment was performed following Swedish animal authority approved protocols.

Microarray analysis

Total RNA was extracted from cells using RNeasy mini kit (Qiagen). Illumina bead array technology was used to analyze mRNA expression using Illumina HumanHT-12 v4 Expression BeadChip. Gene expression was compared using significant microarray analysis tools and gene set enrichment analysis.

Patient-derived xenograft model

Blasts from a sorafenib-resistant patient with AML were collected from bone marrow aspirates after obtaining patient informed consent under a Dana-Farber Cancer Institute Internal Review Board-approved protocol. Mononuclear cells were isolated using Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, UK) and red blood cells were lysed before tail-vein injection into 250 cGy-irradiated NSG (Nod Scid Gamma) mice. After disease burden, human AML blasts were harvested and reinjected into secondary NSG recipients (1.5×10^6 cells per mouse) separated into two groups treated twice a week by tail-vein injection with 100 µl vehicle (5% dextrose in water, 0.3% lactic acid pH 3.5), or gedatolisib (12 mg/kg). Treatment started 3 weeks after cell injection. Disease burden was monitored by evaluation of circulating human CD45+ AML cells in peripheral blood using flow cytometry.

Colony-formation assay for primary AML cells

Colony–plating assays were performed in methylcellulose-based medium MethoCult GF M3434 (StemCell Technologies). In total, 1×10^4 sorafenib-resistant primary cells were plated in triplicate, treated with either 1 or 10 nM sorafenib in combination with 50 nM gedatolisib, and scored for colony formation 14 days later.

Statistical analysis

GraphPad Prism was used for statistical analysis. Data were shown as mean value and error bar represents s.e.m. All tests were two-sided.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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