Dedicated to

Mom and Dad

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The Role of the Juxtamembrane Domain of FLT3-ITDs in Acute Myeloid Leukemia

Thesis Submitted for a Doctoral degree in Human Biology
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Submitted by

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From

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Die rolle der Juxtaembranösen Domaine für FLT3-ITDs in der Akuten Myeloischen Leukämie

Dissertation zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der Ludwig-Maximilians-
Universität zu München, Germany

Vorgelegt von

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myeloische Leukemia
APL	Acute promyelocytic leukaemia
AUL	Acute unidentified leukemia
AA	Amino acid
ATP	Adenosinetriphosphate
ATRA	All-trans-retinoic acid
BSA	Bovine serum albumin
BP	Base pair
FMS	Macrophage colony-stimulating factor receptor
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosidetriphosphate
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
FAB	French-American-British
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FL	FLT3-Ligand
FLT3	FMS-like tyrosine kinase 3
GFP	green fluorescent protein
GIST	gastrointestinal stromal tumor
GRB2	growth factor receptor-bound protein 2
HRP	horseradish peroxidase
IC50	The concentration of inhibitor required to induce a growth reduction of 50% compared to the cells grown in the absence of inhibitor
IL-3	interleukin-3
ITD	internal tandem duplication
JM	juxtamembrane
KI	Kinase insert
KIT	stem cell factor receptor

Abbreviations

LB	Luria Bertani
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
MDS	myelodysplastic Syndrome
MSCV	murine stem cell virus
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PE	phycoerythrin
PI3K	phosphatidyl inositol-3 kinase
PKC	protein kinase C
PTK	proteintyrosienkinase
R	arginine
RT	room temperature
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SH2	SRC-homology 2
STAT5	signal transducer and activator of transcription 5
TKB	tyrosine kinase binding
TKD	tyrosine kinase domain
U	unit
UV	ultraviolet
VEGFR	vascular endothelial growth factor receptor
WHO	World Health Organization
YFP	yellow fluorescent protein

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1 INTRODUCTION

1.1 Acute myeloid leukemia

Acute myeloid leukemia (AML), is characterized by the uncontrolled proliferation of malignant hematopoietic precursor cells which accumulate in the bone marrow and interfere with normal hematopoiesis. AML is the most common acute leukemia affecting adults, 80 to 85% of cases of acute leukemia diagnosed in individuals over the age of 20 years. AML is a relatively rare disease overall (1-2 cases/100,000), its incidence is expected to increase as the population ages (Schoch et al., 2001).

1.1.1 Pathophysiology and classification of AML

AML is caused by a block of differentiation at the state of myeloblast. The differentiation block is caused by accumulation of genetic changes (mutations) in the genes controlling the differentiation and cell proliferation. The clinical signs and symptoms of AML result from the fact that, as the leukemic clone of cells grows, it tends to displace or interfere with the development of normal blood cells in the bone marrow. This leads to neutropenia, anemia, and thrombocytopenia. The clinical symptoms of AML are in turn often due to the low numbers of these normal blood elements.

Much of the diversity and heterogeneity of AML stems from the fact that leukemic transformation can occur at different stages of differentiation pathway. The two most commonly used classification schemes for AML, are the older French-American-British (FAB) system and the newer World Health Organization (WHO) system.

French-American-British Classification:

The French-American-British (FAB) classification system was proposed in 1976 (Bennett et al., 1976) and divides AML into 8 subtypes, M0-M7 (Table 1), based on the type of cell from which the leukemia developed and its degree of maturity. This is done by examining the appearance of the malignant cells under light microscopy and/or by using cytogenetics to characterize any underlying chromosomal abnormalities. The subtypes have varying prognoses and responses to therapy.

FAB-Typ	Monoclonals for precursor cells			Myeloid markers			Monocyte markers			Cytogenetics
	Tdt	HLA-DR	CD34 (My10)	CD13 (My7)	CD33 (My9)	CD15 (Leu Mi)	CD11 (My01)	CD14 (My4)	Other	
M0 (minimally differentiated AML)	±	+	+	+	±	±	-	-		11q13
M1 (myeloid leukemia without maturation)	±	+	+	+	+	-	±	-		-5,-7,-17 del 3p+21,+8
M2 (myeloid leukemia with maturation)	-	+	-	+	+	+	±	-		t(8;21) del 3p or inv 3 -5,-7 t(6;9),+8
M3 (promyelocytic APL)	-	-	-	+	+	±	-	-		t (15;17)
M4 (myelomonocytic)	-	+	-	+	+	+	+	+		inv (16) or -16q t (16;16)
M5 (monocytic leukemia)	-	+	-	±	+	+	+	+		occ t(8;21), -5,-7 t(6,9) t(9;11)(p21;p23)+8
M6 (erythroid)	-	±	-	±	±	-	±	-	Glyco-protein A	-5q,-5,-7,-3,+8
M7 (megacaryocytic)	-	+	+	-	±	-	-	-	Platelet glyco-protein	inv or del 3 +8,+21

Table 1.1.1: French-American-British classification of AML

World Health Organization classification

The World Health Organization (WHO) classification of acute myeloid leukemia (AML) attempts to be more clinically useful and to produce more meaningful prognostic information than the FAB criteria. The WHO classification designates a patient into the category of AML if the patients have 20% or above myeloblasts. The WHO subtypes of AML are

AML with characteristic genetic abnormalities, which includes AML with translocations between chromosome 8 and 21 [t(8;21)], inversions in chromosome 16 [inv(16)], or translocations between chromosome 15 and 17 [t(15;17)]. Patients with AML in this category generally have a high rate of remission and a better prognosis compared to other types of AML.

AML with multilineage dysplasia. This category includes patients who have had a prior myelodysplastic syndrome (MDS) or myeloproliferative disease (MPD) that transforms into AML. This category of AML occurs most often in elderly patients and often has a worse prognosis.

AML and MDS, therapy-related. This category includes patients who have had prior chemotherapy and/or radiation and subsequently develop AML or MDS. These leukemias

may be characterized by specific chromosomal abnormalities, and often carry a worse prognosis.

AML not otherwise categorized. Includes subtypes of AML that do not fall into the above categories.

Acute leukemias of ambiguous lineage. Acute leukemias of ambiguous lineage (also known as mixed phenotype or biphenotypic acute leukemia) occur when the leukemic cells can not be classified as either myeloid or lymphoid cells, or where both types of cells are present.

1.2 Two-Hit model of AML

AML is not caused by a single genetic alteration, but it requires a minimum of two genetic alterations for the preleukemia to develop into AML. This hypothesis is called the two-hit model of AML, based on the studies carried out in mouse models with genetic alterations found in patients with AML (Grisolano et al., 1997; He et al., 1997; Kelly et al., 2002a; Schessl et al., 2005). The model hypothesizes that there are two classes of mutations that are required for development of AML; (i) Class I mutations which involves the mutations in the genes responsible for cell proliferation leading to survival and proliferative advantage to the cells. This class includes genes such as FLT3, RAS, c-KIT etc. (Figure 1.2). (ii) Class II mutation involves the mutations in the genes responsible for the differentiation. These mutations block the differentiation and apoptosis of cells, examples of this class are the chromosomal translocations like AML1/ETO, PML/RARa, inv(16) etc. or transcription factors like C/EBP alpha or p53 (Figure 1.2).

In support of this hypothesis, FLT3 mutations frequently occur in considering with other gene rearrangements and point mutations. For example, FLT3-ITDs have been reported in patients with t(8;21), inv(16), t(15;17), 11q23 gene rearrangements involving mixed lineage leukemia (MLL) gene, and MLL internal tandem duplications (Jamal et al., 2001; Kiyoi et al., 1997; Kottaridis et al., 2001; Nomdedeu et al., 2001; Thiede et al., 2002).

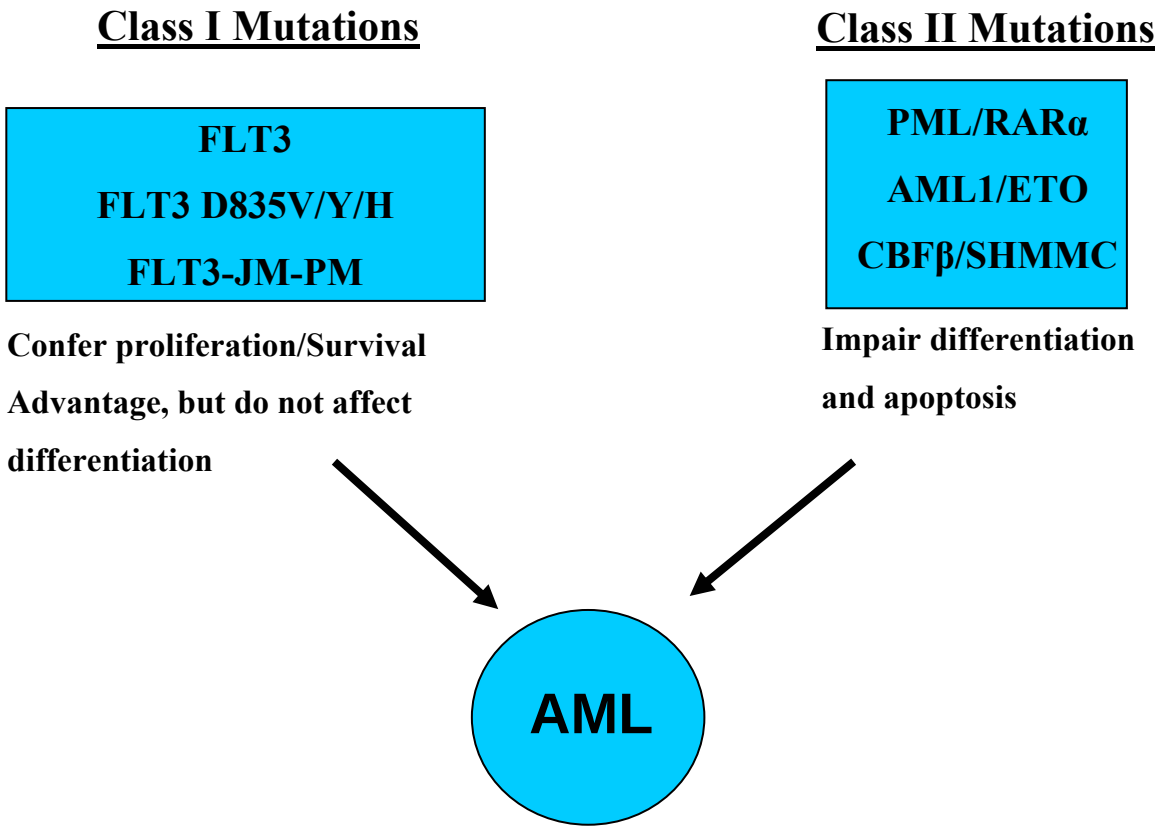

Figure1.2: Two-Hit model for AML: The model states that two classes of mutations of are required for development of AML; class I mutations that confers proliferative advantage to cells and class II mutation that blocks differentiation.

1.3 Receptor tyrosine kinases

The extracellular signal proteins that act through receptor tyrosine kinases consist of a large variety of secreted growth factors and hormones. The human genome as currently sequenced, is thought to contain 90 tyrosine kinase genes, of which 58 are of the receptor type. Receptor tyrosine kinases can be classified into more than 20 structural families, each dedicated to its complementary family of protein ligands. One such family is the Class III receptor tyrosine kinases, members of which have been have been found to be mutated or overexpressed in the patients with AML. This class includes FMS, KIT, FLT3, PDGFRα and PDGFRβ (Blume-Jensen and Hunter, 2001). All the members of this class have an extracellular five-fold immunoglobulin like domain, a transmembrane, and cytoplasmic juxtamebrane and tyrosine kinase domain.

1.3.1 Class III receptor tyrosine kinases: role in leukemogenesis

All the class III RTKs play an important role in normal hematopoiesis with the exception of PDGFR. FMS, the receptor for the macrophage colony-stimulating factor (M-CSF), is crucial for the growth and differentiation of the monocyte-macrophage-osteoclast lineage (Sherr, 1990). FLT3 and KIT are both required for the survival, proliferation and differentiation of hematopoietic progenitor cells, while c-kit is also important for the growth of mast cells, melanocytes, primordial germ cells and interstitial cells of Cajal (Drexler, 1996; Lyman and Jacobsen, 1998). The hematopoietic functions of PDGFRβ are less well defined, although the receptor and its ligand probably play a significant role in megakaryopoiesis (Yang et al., 1997).

The class III RTKs have recently been linked to the pathogenesis of an increasing number of hematological malignancies. KIT mutations, for example, have been shown to be causative in adult-type mastocytosis, as well as being associated with acute myeloid leukaemia and sinonasal lymphomas. (Longley et al., 2001), have proposed a classification of mast cell disease based on the nature of the c-kit mutations. A recent study has implicated the PDGFR alpha mutations in childhood AML, which generally is not expressed in hematopoietic cells (Hiwatari et al., 2005). The role of PDGFRβ-fusion genes in bcr-abl-negative chronic myeloproliferative disorders is still unfolding, but at least eight partner genes have been identified. FLT3 is a gene most commonly mutated in AML and the presence of FLT3-ITD is a strong independent prognostic factor (Kiyoi et al., 1999; Nakano et al., 1999).

1.4 Fms-like Tyrosine kinase (FLT3)

The FLT3 (also known as fetal liver kinase 2, Flk2) gene is located on chromosome 13q12, and and encodes an RTK of 993 amino acids in length. Two isoforms of human FLT3 are reported; the glycosylated 158–160-kDa membrane-bound protein and an cytoplasmic unglycosylated 130–143- KDa.

FLT3 plays an important in role in proliferation, differentiation and apoptosis of normal hematopoietic cells. In normal cells, expression of FLT3 occurs mainly in early myeloid and lymphoid progenitors (Rosnet et al., 1996), and not in erythroid cells (Gabbianelli et al., 1995), megakaryocytes (Ratajczak et al., 1996) or mast cells (Hjertson et al., 1996). Since FLT3 belongs to RTKs, its activation and signalling occurs in the same manner as the other

RTKs. In all cases of RTKs, the binding of the signal protein to the ligand-binding domain on the outside of the cell activates the intracellular tyrosine kinase domain (Figure 2). Upon binding of ligand, transphosphorylation of the selected tyrosine side chains of TKD happens. The tyrosine phosphorylated receptor serves as a docking site for an array of intracellular signalling molecules, including the GTPase-activating protein (GAP), the p85 subunit of phosphatidyl-inositol 3'-kinase (PI3K), phospholipase C-ɣ (PLC-ɣ), the protein tyrosine phosphatase SHP1, Grb2 and Src-like non-receptor kinase (Rosnet et al., 1996). These activated proteins then initiate serine/threonine phosphorylation cascades resulting in activation of transcription factors that determine a variety of cell responses, including cell maintenance, mitogenesis, migration and differentiation (Claesson-Welsh, 1994). Activation of FLT3 happens upon the binding of Flt3 ligand (FL), which leads to downstream signalling and promotes growth of early progenitor cells (Ray et al., 1996; Rusten et al., 1996; Veiby et al., 1996).

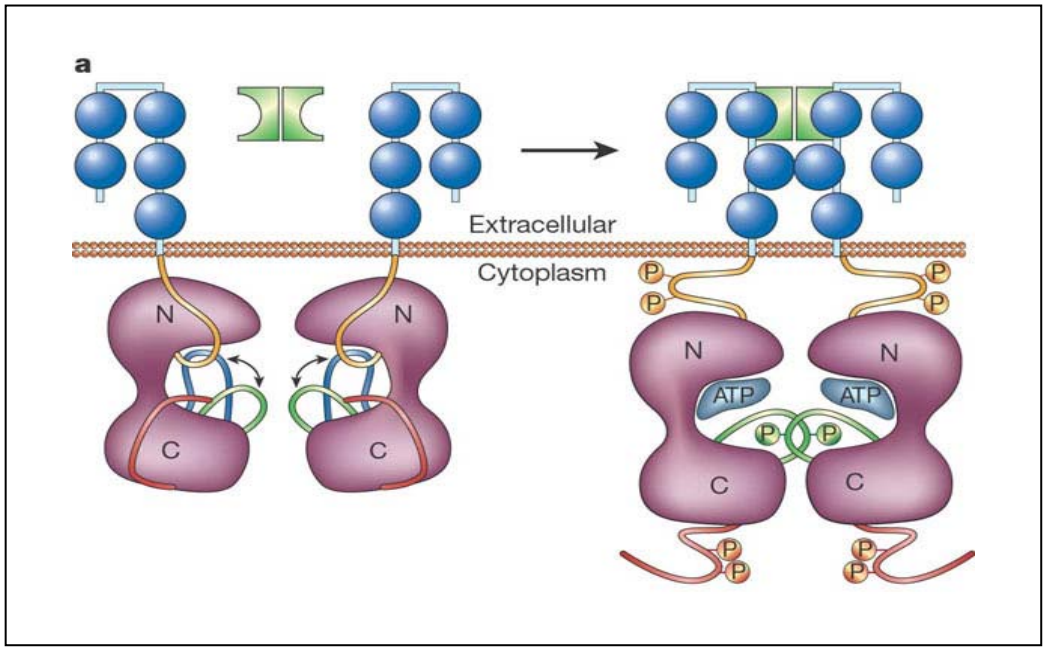

Figure 1.4: Protein tyrosine kinase activation mechanisms; Left: inactive conformation of RPTK. Right: ligand-induced receptor dimerization and tyrosine autophosphorylation (From P. Blume-Jensen and T. Hunter, Nature 411:355-365, 2001).

1.4.1 FLT3 crystal structure

Griffith et al., 2004, crystallised the inactive or unphosphorylated form of the cytoplasmidc domain of FLT3. The principal features of the autoinhibited FLT3 structure include the bilobal kinase fold, the activation loop, and the JM domain (Figure 1.4.1). The kinase fold in FLT3 is that typically found in most protein kinases and consists of N- and C-terminal domains (N and C lobes) (Knighton et al., 1991). Kinase N and C lobes are typically connected by a single flexible polypeptide stretch that allows considerable rotational movement of the two domains relative to each other. This conformational diversity is observed in the multitude of available kinase structures (Huse and Kuriyan, 2002). When the N lobe is rotated away from the C-terminal domain, the kinase is in the catalytically "inactive" form. Conversely, if the N lobe is rotated toward the C lobe, allowing key catalytic residues from both lobes to align, the kinase adopts the catalytically "active" conformation. The crystal structure of autoinhibited FLT3 conforms to the prototypical conformation common to other inactive kinases that have a "closed" activation loop folded between the two lobes of the inactive kinase fold. Activation of FLT3 requires at least phosphorylation of the three tyrosines residues in the activation loop. When these tyrosines are unphosphorylated, the activation loop of FLT3, typically assumes the closed conformation blocking access to the peptide substrate and ATP binding sites.

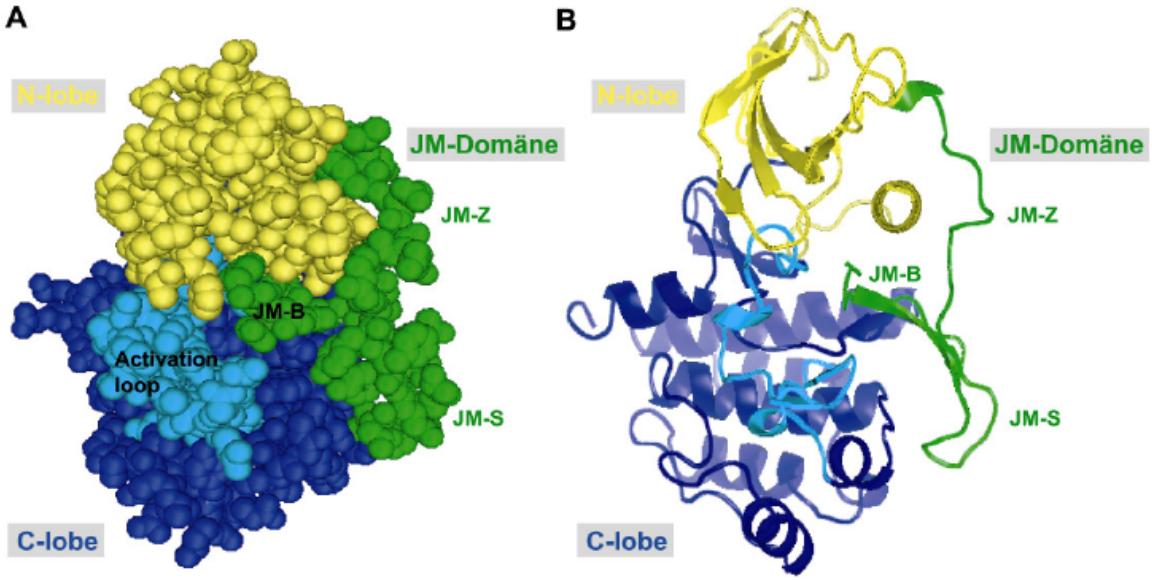

Figure 1.4.1: Crystal structure of inactive conformation of intracellular FLT3 protein (' protein data bank (PDB) accession No:' 1RJB). ' space fill; (A) and ' Ribbon ' (B) model of the JM domain and TKD. The JM domain (green) with the three regions JM-B, JM-S and JM-Z as well as the TKD is shown consisting of ' N lobe '

(yellow), ' C lobe ' (blue) and the activation loop (light blue). The JM domain is the most important autoinhibitory element of the inactive FLT3-Kinase. (Figure taken from the thesis of Dr. Carola Reindl, University hospital, University of Munich, Germany).

A distinct feature of the FLT3 structure is the presence of a Juxtamembrane domain that adopts its autoinhibited conformation and interacts with all key features of FLT3. Topologically the JM can be divided into three parts: the JM binding motif (JM-B), the JM switch motif (JM-S), and the zipper or linker peptide segment (JM-Z). The JM binding motif (JM-B), acts as a autoinhibitory domain, by preventing the rotation of N lobe towards the C lobe of the tyrosine kinase domain (TKD) to generate the activated kinase fold. The JM switch motif (JM-S) that lies next to JM-B provides a rigid and properly oriented framework for the interposition of tyrosines 589 and 591 between the JM-S and the C lobe of the kinase.

1.4.2 FLT3 mutations in AML

FLT3 is expressed at high levels in a spectrum of hematologic malignancies including 70% to 100% of acute myelogenous leukemia (AML) of all FAB subtypes, B-precursor cell acute lymphoblastic leukemia (ALL), a fraction of T-cell ALL, and chronic myelogenous leukemia (CML) in lymphoid blast crisis. These data indicate that FLT3 expression may play a role in the survival or proliferation of leukemic blasts (Carow et al., 1996; Drexler, 1996). Both FLT3 overexpression and activating mutations in the FLT3 gene can be found in patients with AML. Three distinct activating mutations of FLT3 in haematological malignancies have been reported: point mutations (FLT3-JM-PM) and FLT3-internal tandem duplications (FLT3-ITD) found in the JM domain are present in 2% and 20-25% of AML patients respectively, whereas, mutations in the tyrosine-kinase domain (FLT3-TKD) represent 7-10% of AML patients (Figure 1.4.2) (Abu-Duhier et al., 2001; Abu-Duhier et al., 2000; Kindler et al., 2005; Kottaridis et al., 2001; Reindl et al., 2006; Stirewalt et al., 2004; Thiede et al., 2002; Yamamoto et al., 2001).

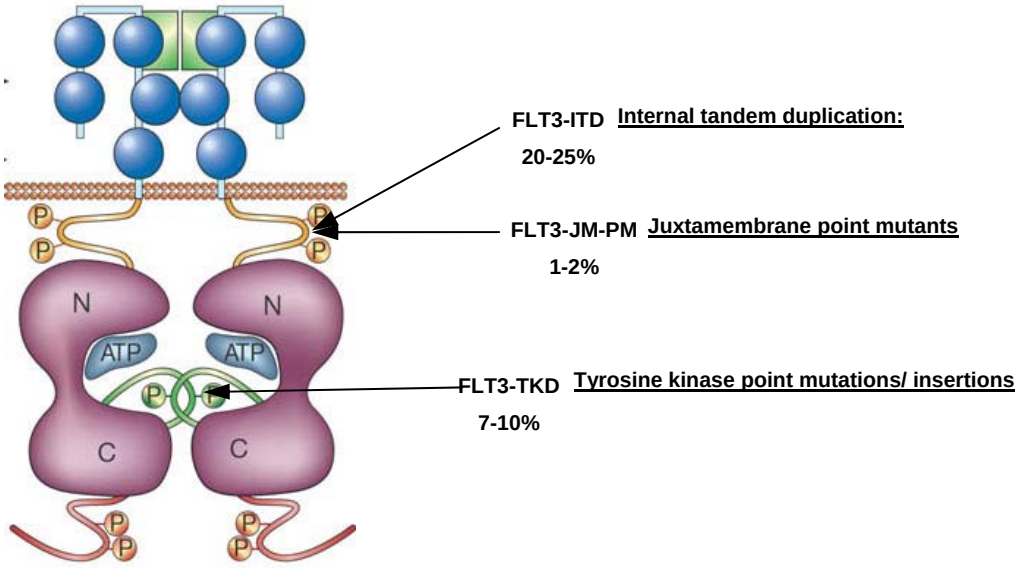

Figure 1.4.2: Flt3 activating mutations found in AML patients: FLT3-ITD and FLT3-JM-PM mutations are found in the juxtamembrane, where as FLT3-TKD mutations are found in the tyrosine kinase domain of FLT3.

1.4.3 FLT3-Internal tandem duplications

Recent advances in genetics have shown that not only chromosome abnormalities but also molecular alterations are useful to characterize and subclassify acute myeloid leukemia (AML). For example, partial tandem duplication within the MLL gene (MLL-PTD) has been shown to define a subgroup of AML patients with unfavourable clinical outcome (Schnittger et al., 2000). FT3-ITDs are another class of muations which defines a poor prognostic subgroup in the AML patients.

FLT3-ITD have been detected in all FAB subtypes of AML, with the highest reported frequency in the M3 and M5 subtype, and less frequently in the M2 subtype. FLT3-ITD are associated with leukocytosis and poor prognosis in most, (Kiyoi et al., 1999; Rombouts et al., 2000; Thiede et al., 2002; Whitman et al., 2001; Xu et al., 1999; Yamamoto et al., 2001) except few studies (Schnittger et al., 2002; Thiede et al., 2002). Recent data indicate that FLT3-ITD is not present in systemic mast cell disease or in a spectrum of solid tumors. In addition to length mutations in one allele of FLT3 several studies have demonstrated biallelic mutations in FLT3, as well as patients in whom the residual wild-type allele is lost (Kottaridis et al., 2001; Schnittger et al., 2002; Thiede et al., 2002; Whitman et al., 2001).

FLT3-ITDs are in-frame duplications of a fragment of the JM domain. FLT3-ITDs are highly heterogeneous and vary in length from 2 to 68 AA. These duplications are thought to disrupt the autoinhibitory mechanism of FLT3 and result in constitutive activation of the tyrosine kinase function of FLT3. Recent studies have shown that FLT3-ITDs are found in the leukemic stem cells of the AML patients (Levis et al., 2005). The presence of an FLT3-ITD has been recognized as an independent poor prognostic factor in AML and is associated with a decreased survival due to an increased relapse rate (Boissel et al., 2002; Kiyoi et al., 1999; Kottaridis et al., 2001; Moreno et al., 2003; Pollard et al., 2006; Preudhomme et al., 2002; Schnittger et al., 2002; Thiede et al., 2002). Several factors influence the poor prognosis seen in AML patients harboring the FLT3-ITDs, e.g., a high FLT3-ITD/wild type ratio is one of the reasons for poor prognosis in AML patients.(Thiede et al., 2002; Whitman et al., 2001) A recent study has reported that the detection of FLT3-ITD mutation in less mature progenitor populations, e.g. $CD34^{+}/CD33^{-}$, might be associated with disease resistance (Pollard et al., 2006).

FLT3-ITDs constructs are constitutively autophosphorylated on tyrosine residues, causing activation of signal transducer and activator of transcription (STAT5) and mitogen-activated protein (MAP) kinases and AKT (Brandts et al., 2005; Hayakawa et al., 2000; Mizuki et al., 2000; Spiekermann et al., 2003; Tse et al., 2000; Tse et al., 2001). In addition, transduction of FLT3-ITD and TKD mutants in murine IL-3-dependent cell lines, such as Ba/F3 and 32D induces IL-3 independent growth (Hayakawa et al., 2000; Tse et al., 2000) *in vitro*. 32D or Ba/F3 cells stably transfected with constitutively activated FLT3 when injected into syngeneic recipient mice results in the development of a leukemic phenotype (Mizuki et al., 2000). Furthermore, retroviral transduction of FLT3-ITD constructs in primary murine bone marrow cells induces in a myeloproliferative phenotype in a mouse bone marrow transplantant model (Kelly et al., 2002a). These data demonstrate that, although FLT3-ITDs have been associated primarily with AML in humans, FLT3-ITD alone is not sufficient to induce AML in primary hematopoietic cells. Furthermore, a kinase-dead mutant of FLT3 in the context of FLT3-ITD abrogates the myeloproliferative disease, indicating an absolute requirement of FLT3 kinase activity for the myeloproliferative phenotype in this model. No difference in biologic activity of FLT3-ITD mutants have been found in cell culture or murine models, despite considerable variation in repeat length that ranges from several to more than 50 amino acids.

Thus, FLT3-ITDs mutations in the FLT3 gene result in constitutive activation of the kinase activity and downstream targets including STAT5, RAS/MAPK, and PI3K/AKT.

1.5 RTK inhibitors

Although activating FLT3 mutations are not sufficient to cause an AML phenotype they represent a potential therapeutical target. Targeted inhibition of aberrant kinase signalling can be an effective therapeutic intervention in hematologic malignancies, as evidenced by hematologic and cytogenetic responses in chronic myelogenous leukemia (CML) and CML blast crisis patients treated with the BCR-ABL kinase inhibitor imatinib mesylat (STI571, Gleevec®) (Druker et al., 2001a; Druker et al., 2001b). An analogous kinase inhibitor strategy might have therapeutical potential in AML patients with activating mutations in the FLT3 gene.

In the past decade, many laboratories embarked on projects aimed at generating compounds that specifically inhibit the activity of the signalling cascades triggered by tyrosine kinases. Compounds with selective activity to class III RTK *in vitro* include AG1295 (Levis et al., 2001), SU5416 (Fong et al., 1999; O'Farrell et al., 2004; Spiekermann et al., 2003; Spiekermann et al., 2002), SU11248 (Yee et al., 2004) and CT53518 (Kelly et al., 2002b). Three compounds (CEP-701, SU11248 and PKC412) with in vivo activity are currently being evaluated in phase I/II clinical trials in patients with AML and have shown promising results (O'Farrell et al., 2003a; O'Farrell et al., 2003b; Smith et al., 2004).

PKC412 (Novartis), an inhibitor initially discovered as an inhibitor of protein kinase C, was found to block the phosphorylation and activity of FLT3-WT and mutant FLT3-receptors (Stone et al., 2005; Weisberg et al., 2002). This inhibitor blocks the FLT3 kinase activity thereby inducing apoptosis in FLT3-expressing cell lines and cause cytotoxicity in primary ALL and AML blasts (Spiekermann et al., 2003; Tse et al., 2001)

2 AIM OF THE STUDY

Activating mutations in the juxtamembrane domain of FLT3 (FLT3-ITDs, internal tandem duplications) represent the most frequent genetic alterations in acute myeloid leukemia and define a distinct molecular entity in AML. FLT3-internal tandem duplications (FLT3-ITDs) are a heterogenous group of mutations in patients with acute leukemias that are prognostically important. Crystal structure of the inactive FLT3-WT has shown that the juxtamambrane domain acts as an autoinhibitory domain and two tyrosine residues 589 and 591 has been postulated as possible phosphorylation sites. No study so far has looked for any common signature in the FLT3-ITDs or the role of tyrosine residues in juxtamembrane on FLT3-ITDs. Hence we wanted to study the role of the Juxtamabrane domain of FLT3-ITDs in two directions.

1. To look for a common motif in juxtamembrane domain that is duplicated in all the patients harboring FLT3-ITDs and influences the transforming potential of FLT3-ITDs.
2. To look for the role of tyrosine residues in the juxtamebrane domain on the transforming potential of the FLT3-ITDs

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Antibodies

Primary antibodies

α-AKT (#9272)	Rabbit, polyclonal	Cell signalling, NEB Frankfurt
α-FLT3 (S18, sc-480)	Rabbit, polyclonal	Santa Cruz, CA, USA
α-FLT3 (C20, sc-479)	Rabbit, polyclonal	Santa Cruz, CA, USA
α-p44/42MAP-Kinase (#9102)	Rabbit, polyclonal	Cell Signaling, NEB, Frankfurt
α-STAT5 (C17, sc-835)	Rabbit, polyclonal	Santa Cruz, CA, USA
normal IgG (sc-2027)	Rabbit	Santa Cruz, CA, USA
normal IgG (sc-2025)	Mouse	Santa Cruz, CA, USA

Phosphospecific primary antibodies

α-pTyr(PY99) (sc-7020)	Mouse, Monoclonal	Santa Cruz, CA, USA
α-pSTAT5 (Tyr-694) (#9351)	Rabbit, polyclonal	Cell Signaling, NEB, Frankfurt
α-p44/p42 MAP Kinase (#9101)	Rabbit, polyclonal	Cell Signaling, NEB, Frankfurt
α-pAKT (Ser473) (#9271)	Rabbit, polyclonal	Cell Signaling, NEB, Frankfurt

Secondary antibodies

α-Mouse IgG-HRP conjugated	Santa Cruz, CA, USA
α-Rabbit IgG-HRP conjugated	Sigma, Taufkirchen

FACS antibodies

CD135-PE IgG1 (2234) Mouse	Immunotech, Marseille, France
IgG1-PE contol (A07796) Mouse	Immunotech, Marseille, France

3.1.2 Plasmids

Retroviral expression Vector

pMSCV-eYFP-IRES (MIY)	A murine stem cell virus vector containing and IRES site and YFP Tag, was kindly provided by Dr. R. K. Humphries (Terry Fox Laboratory, Vancouver, Canada)

RTK Plasmid constructs

MIY-FLT3-WT	Prepared in K. Spiekermann group, CCG-Leukemia, GSF National Research Center for Environment and Health, Munich, Germany. Sub cloned from pCDNA6-FLT3-WT, provided by Dr. D. G. Gilliland (Harvard Medical School, Boston, MA, USA).

FLT3-ITD constructs

MIY-FLT3-NPOS MIY-FLT3-W51	Prepared in K. Spiekermann group, CCG-Leukemia, GSF National Research Center for Environment and Health, Munich, Germany. Sub cloned from pCDNA6-FLT3-W51/NPOS, provided by Dr. D. G. Gilliland (Harvard Medical School, Boston, MA, USA).

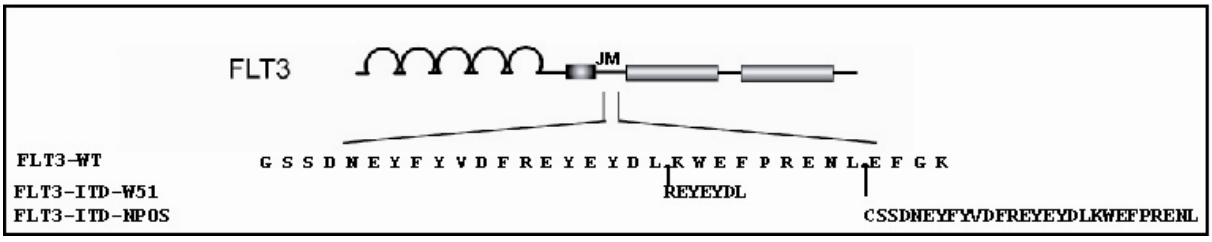

Figure 3.1.2: Amino acid sequence of the FLT3-ITD constructs. FLT3-W51 has a duplication of 7 AA (REYEYDL), between AA 601/602, whereas, FLT3-NPOS has duplication of 28 AA (CSSDNEYFYVDFREYEYDLKWEFPRENL), inserted between AA 611/612 in the human FLT3-WT.

FLT3-WT- mutant constructs

FLT3-WT-ins595R

FLT3-WT-ins596RE

FLT3-WT-ins597EY Constructs made for this study

FLT3-WT-ins597REY

FLT3-WT-ΔR595

FLT3-ITD- mutant constructs

FLT3-W51-R602A

FLT3-W51-R602E

FLT3-W51-R602K

FLT3-W51-ΔR602 Constructs made for this study

FLT3-W51-ΔR595

FLT3-W51-ΔE603

FLT3-W51-ΔY604

FLT3-NPOS-ΔR623

FLT3-W51-Y589F

FLT3-W51-Y591F

FLT3-W51-Y597F

FLT3-W51-YY589/591FF

FLT3-W51-YY589/597FF

FLT3-W51-YY589/599FF

FLT3-W51-YY591/597FF

FLT3-W51-YY597/599FF

3.1.3 Primer oligonucleotides

All the primer oligonucleotides were synthesized from Metabion International, Martinsreid, Germany.

Primers for FLT3 sequencing

The primers for sequencing was designed using the FLT3 sequence (gene bank accession number-NM_004119) taken from NCBI.

pMSCV

5'- cccttgaacctcctcgttcg-3' 1-260 bp of FLT3

FLT3-2

5’-aagacctcgggtgtgcgttg-3’	260-600 bp
FLT3-3	
5’-acgccctggtctgcatatc-3’	600-940 bp
FLT3-4	
5’- cgggctcacctgggaattag-3’	940-1280 bp
FLT3-5	
5’-tttgcaatcataagcaccagc- 3’	1280-1620 bp
FLT3-6	
5’-atacaattcccttggcacatc-3’	1620-1960 bp
FLT3-7	
5’-aacggagtctcaatccagg-3’	1960-2300 bp
FLT3-8	
5’-cagcatgcctggttcaagag-3’	2300-2640 bp
FLT3-9	
5’-aggcatctacaccattaagag-3’	2640-2980 bp

Primers for mutagnesis of FLT3-WT and FLT3-ITDs

FLT3-WT-ins595R sense

5'-gagtacttctacgttgatttcagaagagaatatgaatatgatctcaaatgg-3'

FLT3-WT-ins595R antisense

5'-ccatttgagatcatattcatattctcttctgaaatcaacgtagaagtactc-3'

FLT3-WT-ins597EY sense

5'-ctacgttgatttcagagaatatgagtacgaatatgatctcaaatggg-3'

FLT3-WT-ins597EY antisense

5'-cccatttgagatcatattcgtactcatattctctgaaatcaacgtag-3'

FLT3-WT-ins597REY sense

5'-ctacgttgatttcagacgggaatatgagtacgaatatgatctcaaatg gg-3'

FLT3-WT-ins597REY antisense-

5'-ccc atttgagatcatattcgtactcatattcccgtctgaaatcaacgt ag-3'

FLT3-W51- R602E sense

5'-gatttcagagaatatgaatatgatctcgaagaatatgaatatgatctcaaatgggag-3'

FLT3-W51- R602E antisense

5'-ctcccatttgagatcatattcatattcttcgagatcatattcatattctctgaaatc-3'

FLT3-W51- R602K sense

5'-gatttcagagaatatgaatatgatctcaaagaatatgaatatgatctcaaatgggag-3'

FLT3-W51- R602K antisense

5'-ctcccatttgagatcatattcatattctttgagatcatattcatattctctgaaatc-3'

FLT3-W51- ΔR602 sense

5'-gatttcagagaatatgaatatgatctcgaatatgaatatgatctcaaatgggag-3'

FLT3-W51- ΔR602 antisense

5'-ctcccatttgagatcatattcatattcgagatcatattcatattctctgaaatc-3'

FLT3-W51- ΔR595 sense

5'-ttctacgttgatttcgaatatgaatatgatctcaga-3'

FLT3-W51- ΔR595 antisense

5'-tctgagatcatattcatattcgaaatcaacgtagaa-3'

FLT3-W51- ΔE603 sense

5'-gatttcagagaatatgaatatgatctcagatatgaatatgatctcaaatgggag-3'

FLT3-W51- ΔE603 antisense

5'-ctcccatttgagatcatattcatatctgagatcatattcatattctctgaaatc-3'

FLT3-W51- ΔY604 sense

5'- gatttcagagaatatgaatatgatctcagagaagaatatgatctcaaactcaaatgggag-3'

FLT3-W51- ΔY604 antisense

5'- ctcccatttgagatcatattcttctctgagatcatattcatattctctgaaatc-3'

FLT3-NPOS- ΔR623 sense

5'-gagtacttctacgttgatttcgaatatgaatatgatctcaaatggg-3'

FLT3-NPOS- ΔR623 antisense

5'-cccatttgagatcatattcatattcgaaatcaacgtagaagtactc-3'

FLT3-Y589F sense

5'-ggctcctcagataatgagttcttctacgttgatttcagagaa-3'

FLT3-Y589F antisense

5'-ttctctgaaatcaacgtagaagaactcattatctgaggagcc-3'

FLT3-Y591F sense

5'-ggctcctcagataatgagtacttcttcgttgatttcagagaatat-3'

FLT3-Y591F antisense

5'-atattctctgaaatcaacgaagaagtactcattatctgaggagcc-3'

W51-Y589/Y591F sense

5'-gataatgagttcttctacgttgatttcagagaatatgaatttgatctcaga-3'

W51-Y589/Y591F antisense

5'-tctgagatcaaattcatattctctgaaatcaacgtagaagaactcattatc-3'

FLT3-WT-Y589/Y597FF sense

5'-gctcctcagataatgagttcttctttgatttcagagaatatgaatatg-3'

FLT3-WT-Y589/Y597FF antisense

5'-catattcatattctctgaaatcaacaaagaagaactcattatctgaggagc-3'

3.1.4 Cell lines

Human

HEK-293	Hunam embryonic kidney cells (Graham, 1977)

Murine

Ba/F3	IL-3-dependent pro-B cell line
WEHI-3B	myelomonocytic Leukemia-cell line (Warner, 1969); established from macrophage cells of BALB/c-Mice; cells produce the haematopoietic growth factor IL-3

3.1.5 Chemicals, enzymes, inhibitors and cytokines

Chemicals

Acrylamide/Bisacrylamide 30%	Roth, Karlsruhe
Agarose	Sigma, Taufkirchen

Ammonium peroxidesulfate (APS)	BioRad, Munich
Aprotinin	Sigma, Taufkirchen
Bio-Rad Protein Assay Dye Reagent	BioRad, Munich
Bovineserum albumin (BSA)	Fluka, Buch, Switzerland
Bromphenolbue	Sigma, Taufkirchen
Dimethylsulfoxide	Sigma, Taufkirchen
1,4-Dithiothretiol	Merck, Darmstadt
DNA Ladder	Promega, Mannheim
dNTPs	Roche, Mannheim
ECL Detection Reagent	Amersham Pharmacia, Freiburg
EGTA(ethylen glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid)	Sigma, Taufkirchen
Acetic acid	Merck, Darmstadt
Ethanol	Merck, Darmstadt
Fetal calf serum (FCS)	Sigma, Taufkirchen
Glycerin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl) piperazine-N´-(2-ethanesulfonic acid))	Sigma, Taufkirchen
High Performance Chemiluminescence Film	Amersham Pharmacia, Freiburg
Leupeptin A	Sigma, Taufkirchen
2-Mercaptoethanol	Sigma, Taufkirchen
Methanol	Merck, Darmstadt
Sodium acetate	Sigma, Taufkirchen
Sodium fluoride	Sigma, Taufkirchen
Sodium vanadate	Sigma, Taufkirchen
Nitrocellulose membrane	Schleicher&Schüll, Dassel
Pepstatin A	Sigma, Taufkirchen
Phenylmethylsulfonylfluoride (PMSF)	Sigma, Taufkirchen
Phosphatase Inhibitor Cocktail 2	Sigma, Taufkirchen
Ponceau	Sigma, Taufkirchen
Polyfect Transfection Reagent	Qiagen, Hilden
Polybrene	Sigma, Taufkirchen
Proteinase Inhibitor Cocktail-(P8340)	Sigma, Taufkirchen
Protein G-Agarose	Roche, Mannheim

Protein A-Agarose	Roche, Mannheim
Propidium iodide	Sigma, Taufkirchen
Rainbow Marker	Amersham, Freiburg
Sodiumdodecylsulfate (SDS)	BioRad, Munich
Tetramethylethylendiamin (TEMED)	Serva, Heidelberg
Tris(hydroxymethyl)aminomethan (Tris)	Merck, Darmstadt
Triton-X-100	Sigma, Taufkirchen
Trypan Blue	Invitrogen, Karlsruhe
Tween 20	Merck, Darmstadt

Enzyme

Taq DNA Polymerase	Roche, Mannheim
Pfu Turbo DNA Polymerase	Stratagene, Heidelberg
T4-DNA-Ligase	Roche, Mannheim
T4-DNA-Ligase	Fermentas, St.Leon-Rot
Restriction enzymes (NEB, Frankfurt)	

Inhibitor

PKC412 (CGP 41251)	Novartis, Basel, Switzerland

Cytokine

FLT3-Ligand (FL), recombinant human	Promocell, Heidelberg
Interleukin-3 (IL-3), recombinant murine	Biosource, Camarillo, USA

The lyophilised cytokines were reconstituted in 0.1% BSA solution, aliquoted and stored at –-20°C.

3.1.6 Kits

BigDye Terminator Cycle Sequencing Kit	Appl. Biosystems, ForsterCity, USA
PCR Purification Kit	Qiagen, Hilden
Qiagen Endofree Plasmid Maxi Kit	Qiagen, Hilden
QIAprep-spin Miniprep Kit	Qiagen, Hilden
QuikChange Site-Directed Mutagenesis Kit	Stratagene, Heidelberg

3.1.7 Laboratory equipment

Blotting chamber	Bio-rad, Munich
Cell culture CO_2 incubator	Haereus, Rodenbach
Cell culture hood	Bio Flow Technik, Meckenhein
Centrifuges ROTIXA/P	Hettich, Tuttlingen
Developing machine M35X-OMAT Processor	Kodak AG, Stuttgart
Eppendorf ultracentrifuge 2K15	Sigma-Aldrich Chemie GmbH, Taufkirchen
FACS scan	Beckton Dickinson, Mountain View, CA, USA
Fridge (4°C, -20°C)	Siemens AG
Fridge (-80°C) UF80-450S	Colora Messtechnik GmBH, Lorch
Gel electrophoresis systems	Bio-rad, Munich
Heating block BT 130-2	HLC, Haep Labor Consult, Bovenenden
Liquid nitrogen tank	Cryoson, Schöllkrippen
Microscope	Carl Zeiss Jena
pH-meter 766	VWR International, Ismaning
Rotor Ti 75	Beckman, Palo Alto, CA
Shaker	Edmund Bühler, Tübingen
Sequencer (ABI Prism 310 Genetic Analyzer)	Appl. Biosys, ForsterCity, USA
Spectophotometer Smartspec ^{TM} 3000	Bio-rad, Munich
Ultracentrifuge L7-65	Beckman, Palo Alto
Vortex	Cenco, Breda, The Netherlands
Water bath	HAAKE, Karlsruhe

3.1.8 Software

Adobe Illustrator	Adobe Systems, Unterschleißheim
Adobe Photoshop	Adobe Systems, Unterschleißheim
BioEdit 7.0	ISIS Pharmaceutica, Carlsbad, USA
Cellquest 3.3	Beckton Dickinson, Heidelberg
EndNote 6.0.2	Thompson ISI, Carlsbad, CA, USA
Microsoft Office 2003	Microsoft, Redmond, WA, USA
SigmaPlot 6.0	SPSS Incorporated, Chicago, USA
TINA 2.0	Raytest, Straubenhardt
WinMDI 2.8	Joseph Trotter

3.2 Methods

3.2.1 Bacterial cultures

3.2.1.1 Preparation of competent *E.coli* cells

For the preparation of competent cells using $CaCl_2$ method, *E.coli* (XL-10) was cultured overnight in 50 ml LB medium without antibiotics. Next morning the cells were split 1:10 in LB medium and grown until the OD_{600} = 0.4-0.9. The cells were cooled on ice for 15 min and then centrifuged at 3000 rpm for 5 min. The supernatant was discarded, the cells resuspended in 50 ml of ice cold TbI buffer and again incubated on ice for 15 min. After centrifugation the cells were resuspended in 2-4 ml of ice-cold TbII buffer. 100 µl aliquots of cells were transferred in Eppendorf- tubes and frozen down immediately in dry ice for 1 hour. The aliquots were stored at -80°C.

Tfb I:	30 mM KOAc, 100 mM KCl, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 15 % (v/v) Glycerin (Sterilfiltration, store at 4°C)
Tfb II:	5 ml 1 M NaMOPS (pH 7,0), 75 mM $CaCl_2$, 10 mM KCl, 15 % (v/v) Glycerin (Sterilfiltration, store at 4°C)

3.2.1.2 Transformation of competent E.coli

The competent cells were thawed on ice and 25 µl of the cells were transferred in a tube. 1-10 µl DNA,10 µl 5xKCM (500 mM KCl, 150 mM $CaCl_2$, 250 mM $MgCl_2$) and remaining water were added to make up the final volume to 50 µl. The mix was incubated on ice for 20 min and then for 10 min at room temperature. After the incubations 250 µl of LB medium was added, the tubes were incubated at 37°C for one hour to let cells recover from the shock. After 1 hr incubation 150 µl of the cells were spread on LB agar plate with containing appropriate antibiotics and incubated at 37° overnight.

3.2.1.3 Preparation of plasmid DNA

Mini-Prep

Pasmid Mini-Kit (Qiagen, Hilden)

MaxiPrep

Plasmid Maxi-Kit (Qiagen, Hilden)

3.2.2 Molecular biology techniques

3.2.2.1 Digestion of DNA with restriction enzymes

All the mini and maxi-Plasmid preparations were checked for the correct DNA identity using enzymatic restriction enzyme digestion. Restriction enzymes from New England Biolabs (Frankfurt, Germany) were used and the conditions were selected corresponding to the statements of the manufacturer. All the digested products were checked in 1% agarose gels containing ethidium bromide to detect the DNA.

3.2.2.2 Mutagenesis

To introduce point mutants, deletions, insertions or substitutions, site directed mutagenesis was done using the ´QuikChange site Directed Mutagenesis Kit (Stratagene, La Jolla, USA). For mutagenesis, the primers were designed with a length between 40 and 60 bases, with a melting temperature of ≥75°C and containing the desired mutation in the middle. Optimum primers had a minimum GC content of 40% and terminated on one or more C and G bases at the 3'-end. Each reaction contained 35 µl H_2O, 5 µl 10xPCR buffer, 3 µl Quick Solution, primers. Then template DNA, 1µl dNTPs (10mM) and water were added up to a final volume

of 50 µl. PCR conditions and further steps were followed as directed by the manufacturer. Sequencing of the constructs was performed to confirm the correct positioning of the mutations and no unwanted additional mutations.

3.2.2.3 Agarose Gel Electrophoresis

To determine the size of DNA fragments, to estimate DNA quantity and to separate and isolate the DNA fragments Agarose gel electrophoresis was used. Concentration of the agarose gels (0.8-2%) was adjusted according to the size of the DNA fragments, with smaller fragments needing higher concentration of agarose. The correct amount of powdered agarose (Gibco BRL, Germany) was added to a measured quantity of electrophoresis buffer (1xTBE), then the mix was heated slurry in a microwave oven until the agarose was dissolved. After cooling of this solution to 50°C, ethidium bromide solution [0,5 µg/ml] (Roth, Germany) was added and the warm solution was poured into a chamber. After polymerization (ca. 30-40 min at room temperature), the gel was placed in an electrophoeresis chamber with 1xTBE buffer. The samples were mixed with 6x loading buffer (Promega) and loaded into the slots of the submerged gel. Then the DNA fragments were visualised under a UV light. To determine the size of the DNA fragments, a marker with defined fragment sizes was applied.

TBE-buffer (5x): 54,0 g Tris, 27,5 g boric acid , 20 ml 0,5 M EDTA,and make up to 1000 ml

3.2.2.4 Sequencing

For sequencing of the plasmids, Big Dye terminator sequencing kit was used. For 1 PCR reaction, 2 µl of Big dye, 1 µl water, 1 µl primer (10pmol) and 1 µl DNA (75ng-200ng) were used. Following PCR conditions were used: 30 cycles at 94°C 10 sec, 50°C 5 sec, 60°C 4 min; 4°C. The PCR products were purified in a two step manner. In the first step 25µl of 1:24, 3M NaAc:ethanol was added and centrifuged at 4000 rpm for 20 min. Supernatant was discarded and 100 µl of 70% ethanol was added and centrifuged at 4000 rpm for 10 min. Then the supernatant was discarded and the pellet was kept till dry. DNA was dissolved in 10 µl of water and transferred into sequencing tubes and subsequently analysed in Prism 310 Genetic Analyzer.

3.2.3 Cell culture

3.2.3.1 Cell culture conditions

All cell lines used in this study were cultivated in an incubator set at 37°C with 95% relative air humidity in the presence of 5% CO2 (for buffering). All cell culture mediums, RPMI-1640 and Dulbecco's modified Eagle Medium (DMEM), were supplemented with 10% FCS and 5U/ml of pencillin and streptomycin each respectively.

3.2.3.2 Culture of adherent cell lines

The human embryonic kidney cell line 293 was maintained in DMEM medium. These cells grow as monolayers which are held together to the substratum by mucoproteins and sometimes by collagens. For the subculturing of the cells, the confluent cells were washed once with DPBS and then treated with trypsin supplemented with 0,5M EDTA for 5 mins to release the cells from monolayers. Each 2 to 3 days the cells were split in 1:5 ratios into new flasks.

The murine myelomonocytic leukemic cell line WEHI-3B was grown in RPMI-1640 medium. These cells are trypsin sensitive and were therefore incubated with DPBS (without Ca^{2+} or Mg^{2+}) for 15 min and cells scrapped using cell scrapers. The cells were then split at a ratio of 1:5 into a fresh culture flask. The supernatant was used as a source of murine IL-3 supplement to cultivate the IL-3 dependent Ba/F3 cells. It was filtered and stored at -20°C until use.

3.2.3.3 Culture of suspension cell lines

The IL-3 dependent murine proB cell line Ba/F3 cells was grown in RPMI-1640 medium supplemented with 10% WEHI conditioning medium as a source of murine IL-3. The cells were split every two or three days at 1:20 ratio.

3.2.3.4 Freezing and thawing of cells

Cell stocks can maintain for years by storing them in liquid nitrogen. To freeze the cells, 1 x 10^7 cells were pelleted, resuspendended in 1 ml of freezing medium and transferred to a 1.5 ml cryo ampoules (Nunc, Roskilde, Denmark). In order to minimize the cellular injury induced by freezing and thawing procedures (intracellular ice crystals and osmotic effects), 10% of cryoprotective agent: dimethyl sulphoxide (DMSO) is added to the freezing medium.

The ampoules are cooled up before and every step is performed on ice. While short-term preservation of cell lines using mechanical freezers (-80°C) is possible, storage in liquid nitrogen (-196°C) or its vapor (-120°C) is much preferred.
For optimal recovery rapid thawing of the cells is essential. Therefore the cells were thawed in 37°C water bath and, as DMSO in the freezing medium are toxic to the cells, subsequently diluted with DPBS or culture medium at a ratio of 1:20 and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet resuspended in culture medium. All the thawed cell lines were cultured for 3 days before being used for experiments.

Freezing medium:	90 % FCS, 10 % DMSO

3.2.3.5 Transfection of the 293 cells

The 293 cell line can be transfected with high efficiency. Hence this cell line is used for protein overexpression and transient transfections for producing high titre retroviral supernatant. For transient transfection, cells were seeded at a density $5x10^5$/ml. The next day the cells were transfected with 2µg plasmid (cloned in MSCV-IRES-YFP) mixed with 2µg of EcoPack (Retroviral packaging genes) and transfected with Polyfect transfection Reagent (Stratagene, Hiedelberg) according to the manufacturers instructions. 48 hours after transfection cells were either lysed to analyse protein expression or the supernatant was collected and filtered through a 0.45 m filter to stably tranduce BaF3 cells.

3.2.3.6 Stable transduction of Ba/F3 cells

$2x10^5$/ml Ba/F3 cells were seeded in 200µl of growth medium RPMI-1640 and subsequently transduced once with 200µl of retroviral supernatant collected from the transient transfection of 293 cells in the presence of polybrene (8µg/ml). After 4-12 hours 1 ml of medium was added to avoid the toxic action of the polybrene on the cells. The cells were expanded and sorted after 5 days.

3.2.3.7 Cell sorting by facs

Five days after the transduction of the Ba/F3 cells, positive cells expressing YFP/GFP were sorted using the Vintage SE Flowcytometer equipped with a turbo-sort DEVICE (assortment of 20.000 cells/s), and the Cellquest 3,3 software. For the cell sorting, cells were centrifuged 1000 rpm for 5 mins and resuspended in 1ml of FACs buffer (RPMI-1640 with 1µg/ul of

Propidium Iodide). The sorted YFP/GFP positive cells were expanded and examined after 3 days in the FACS Calibur Flowcytometer (Beckton Dickinson, Heidelberg) for the purity of the population. Cell populations with > 95 % YFP/GFP positive cells are taken for further experiments or they are resorted to attain > 95 % purity

3.2.3.8 Proliferation assays

Determination of viable cell number is important aspect of cell culture especially for seeding the cells and proliferation experiments. In proliferation experiments, viable cells are counted at the background of various factors such as in the presence or absence of cytokines or inhibitors.

For the proliferation experiments with Ba/F3 cell lines, cells were seeded at a density of 4 x 10^4 cells/ml in the presence of IL-3, or inhibitors as indicated. Viable cells were counted at 24, 48 and 72 hours in a standard hemacytometer after staining with trypan blue. The IC_{50} was defined as the concentration of inhibitor at which 50% of cells were viable compared to cells grown in the absence of inhibitor.

3.2.3.9 Trypan blue exclusion method

Trypan blue does not enter viable cells but dead cells, so that they appear blue and can be distinguished from viable cells under the microscope. For determination of the viable cells, cells were mixed with a ratio of 9:1 (cells:typan blue) and counted under an optical microscope using a standard neubauer chamber.The formula for the calculation of viable cells is: cells /ml = number of viable cells /4 x dilution factor x 10,000.

3.2.3.10 Cell starving for cell lysis

To analyse the phosphorylation of specific signalling pathways it is necessary to starve the cells before cell lysis, to reduce the background of phosphorylated proteins. The cells were washed twice with PBS and cultivated subsequently in starving medium. The maximum time the cells could be starved without effecting the morphology of cells was determined. For Ba/F3 cells a maximum starvation time of 24 hr was applied.

Starving medium:	RPMI-1640 or DMEM + 0.3 % FCS + 50 U/ml Penicillin + 50 U/ml Streptomycin

3.2.3.11 Stimulation of cells with cytokine/ligand before cell lysis

Cells were treated frequently with cytokine/ligand before cell lysis. Cell lines expressing RTKs are stimulated upon binding of respective ligands/cytokines and show upregulation of signalling pathways. The cytokine/ligand stimulated cells serve as positive controls for signalling pathways when the wild type RTKs is compared with mutant RTKs. For stimulation of cells with cytokines/ligand, cells were incubated with a defined quantity of cytokine/ligand in 1 ml of starving medium for 5 mins. Then the cells were centrifuged at 1000 rpm at 4°C to stop the reaction. The cell pellet was lysed in lysis buffer.

3.2.4 Protein Biochemistry

3.2.4.1 Cell lysis

For cell lysis the starved/cytokine/ligand induced cells were centrifuged at 1000rpm for 5 min at 4^{o}C. Then the supernatant was discarded and 1ml of lysis buffer (for 3 X 10^6 cells) was added and cells were transferred to 1.5ml Eppendorf tubes. Then the tubes were rotated at 10 rpm at 4°C on a rotator for 30 min, then the tubes were centrifuged at 14,000 rpm for 20 min. Supernatants from the tubes were transferred into fresh eppendorf tubes and stored at -20°C until further use.

Lysis Buffer:	50 mM Hepes pH 7,5, 150 mM NaCl, 1 mM EGTA, 10 % glycerol, 1 % Triton X-100, 100 mM NaF, 10 mM $Na4P_2O_7$ Add fresh for 10 ml: 50 µl Aprotinin (200x), 100 µl 0,1 M PMSF, 100 µl 0,1 M orthovanadate

3.2.4.1 Protein Quantification

The determination of the protein concentration in cell lysates was done by the Bradford method (Bradford, 1976). The Bradford dye contains Coomassie Brilliant Blue G-250, whose absorption maximum increases upon binding to basic and aromatic groups of proteins from 495 to 595 nm. The intensity of the dye correlates directly with the concentration of protein in the lysate and can be measured in a spectral photometer. The dye has to be diluted at 1:5 with water before use. 5µl of the lysate and 995µl of the BioRad-Dyes were mixed and incubated for 10 min in RT. Subsequently the absorption was measured in 595 nm in the spectral photometer against 1ml of 1:5 diluted Bio-Rad dye. To determine the absolute concentration of the samples, standards made from different concentrations of BSA (1, 5, 10, 15, 20 and 25ug) was measured in parallel and concentrations of the samples calculated.

3.2.5 3.2.4.2 SDS-Polyacrylamidegelelectrophoresis

SDS-PAGE was performed as described by Laemmli (Laemmli, 1970). In SDS-PAGE proteins were separated according to their molecular weight. A gradient gel (range 7.5- 12.5% polyacrylamide) was used for standard applications. For SDS page protein samples (whole cell lysates) were first denaturized by boiling at 95°C for 5 minutes in SDS laemmli buffer; 40 to 100 µg protein was loaded into the gel and run at 150 volts in running buffer to separate electrophoretically for 8-12 hours time. A marker (Rainbow marker) with well-characterized proteins allows identifying the molecular weight of unknown proteins.

Laemmli-Buffer (2x):	187.5 mM TRIS, 6 % SDS, 30 % Glycerol
7,5 % separting gel:	5.65 ml 1.5 M Tris-Buffer (pH 8,8), 6,25 ml Polyacrylamide (30 %), 150 µl 10 % SDS, 50 µl 10 % APS, 2.9 ml H_2O, 1.5 µl TEMED
12,5 % separating gel:	5.65 ml 1.5 M Tris-Buffer (pH 8,8), 3,75 ml Polyacrylamide (30 %), 2 g Sucrose, 150 µl 10 % SDS, 50 µl 10 % APS, 5.4 ml H2O, 1.5 µl TEMED
4 % stacking gel:	2.5 ml 1.5 M Tris-Buffer (pH 6.8), 1.3 ml Polyacrylamide (30 %), 100 µl 10 % SDS, 50 µl 10 % APS, 6.1 ml H2O, 10 µl TEMED
Elektrophoreses buffer (10x):	151.4 g Tris, 720,65 g glycin, 50 g SDS, ad 5000 ml, pH 8.3 with HCl

3.2.6 3.2.4.3 Western Blot analysis

After electrophoresis, proteins are transferred onto a nitrocellulose membrane in a blotting chamber using a semidry method (Towbin, 1979, Kyhse-differently, 1984). Blotting was performed for 3 h at 250mA. To confirm the protein transfer, the membrane was stained shortly with Ponceau dye, a transient red staining which binds every protein. Afterwards the proteins on the membrane were blocked by incubating with 0.25% gelatin in NET buffer (1xG-NET) for 1 h (3x 20min) and then incubated overnight with appropriate antibody dilutions. For analysis, the membrane was washed 3 times for 15 minutes in TBST and then incubated for 1 hour at room temperature in secondary antibody, a species-specific horseradish peroxidase (HRP) conjugated antibody diluted in the G-Net solution. To wash away unspecifically bound antibody the membrane was washed 3 times for 15 minutes in TBST. Protein/antibody complexes were detected using chemiluminisense (ECL-Enhanced Luminol Reagent, Amersham) system. This western blot chemiluminescence reagent is a non-radioactive light emitting system, which detects proteins immobilized on a membrane, from

the oxidation of luminol, which results in light emission at a wave length of 428 nm, captured by a autoradiograph film

Transblot without Methanol(10x):	72.75 g Tris, 36,63 g Glycine, 0.475 g SDS, ad 5000 ml
Transblot-Buffer:	80 ml Transblot without Methanol, 200 ml Methanol, make upto 1000 ml
Ponceau:	0.5 g Ponceau S, make upto 100 ml
Net (10x):	438.3 g NaCl, 93.6 g Na2EDTA, 302.85 g Tris, 25 g Triton-X-100, ad 5000ml, pH 7,7 with HCl
G-Net:	2.5 g Gelatin, 100 ml Net (10x), make upto 1000 ml
TBS (10x):	12.11 g Tris, 87.66 g NaCl, ad 1000 ml (pH 8,8)
TBST:	100 ml TBS (10x), 1 ml Tween 20, make upto 1000 ml

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3.2.4.4 Stripping of Nitrocellulose-Membrane

The nitrocellulose membrane with proteins can be used for detection of multiple proteins, but the membrane should be devoid of previous antibody. Hence, the nitrocellulose membrane was stripped with stripping solution (warmed till 56^{o}C). the membrane was kept in stripping solution for 40 min at 56^{o}C, washed 3X 20min with TBST, blocked in GNET and used for subsequent analysis as described above..

Stripping-solution:	62.5 mM Tris pH 6.8, 0.1 M β-Mercaptoethanol, 2 % SDS

4 RESULTS

4.1 Arginine 595 is duplicated in patients with acute leukemias carrying internal tandem duplications of FLT3 and is critical for its transforming potential

4.1.1 Clinical and laboratory data of the patients taken for this study.

Two hundred and eighty four patients diagnosed with acute leukemias and carrying FLT3-ITD were taken for this study from the patient data bank of the Laboratory for Leukemia Diagnostics, University Clinic of Grosshadern, Munich. All patients gave informed consent before entering the study. The study design adhered to the principles of the Declaration of Helsinki and was approved by the ethics committees of the participating institutions. Clinical and laboratory data of patients analyzed in this study are given in Table 4.1. Ten patients included in this study had no clinical data.

Number of patients = 284		
Age	Range	18-89
	Median	60.5
Gender	Female	163
	Male	121
FAB class	AUL	2
	Biphenotypic	3
	ALL	4
	AML M0	6
	AML M1	63
	AML M2	49
	AML M3	28
	AML M4	39
	AML M5	21
	AML M6	4
	MDS	3
	AML with unknown FAB	52
Patients without clinical data	------	10
WBC count	Median	50000
	Range	12000-67500
Cytogenetic abnormality	Favourable	29
	Intermediate	185
	Adverse	11
	Unknown	49

Table 4.1.1: Clinical and laboratory data of the patients analyzed in this study

4.1.2 FLT3-ITDs are a higly heterogenous group of mutations

Sequencing of cDNA of 284 patients carrying FLT3-ITDs was done using patients specific primers at the Laboratory of leukaemia diagnostics, Grosshadern, munich. DNA from these patients used for sequencing was previously checked for the FLT3-ITDs using a PCR method and then doing an agarose gel electrophoeresis. Then sequence of the JM region of ITDS was the translated into amino acids. Amino acid analysis revealed that 111 patients carried pure tandem duplication whereas 163 patients had additional amino acid insertions at the start of duplication. The length of of duplication varied from 2AA to 42 AA with a median length of 17 AA (Table 4.1.2).

Number of patients = 284	
Pure tandem duplications	118
Tandem duplications + additional insertions	166
Length of AA duplications	2-42 AA
Median of AA duplications	17 AA

Table 4. 1.2: Characteristics of the duplications found in this study.

4.1.3 Internal tandem duplications are located in the common motif YVDFREYEY and include R595 in 77% of patients

Analysis of the frequency of single AA in the duplicated region revealed that arginine 595 was the most frequently duplicated single AA in 77%, followed by Y597 in 74%, F594 and E596 in 73% of all patients, respectively (Figure 4.1.3.1). In 95% of the patients at least one AA within the stretch Y591 to Y599 (YVDFREYEY) was duplicated. Next, we analyzed the frequency of AA combinations statistically, ranging in length from 1 to 30 AA within the duplicated region. As shown in Figure 4.1.3.2A the single R595 is subsequently followed by the combination of amino acids EY (AA596-597) and REY (AA595-597) in 70% of all patients.

These findings point to a commonly duplicated motif that centers around R595 within the Y-rich stretch from AA 591 to 599 (YVDFREYEY). We hypothesized that this region might play an important role for the transforming activity of FLT3-ITDs. To confirm this

hypothesis, we analyzed the patients that carried the shortest ITD. Figure 4.1.3.2B shows the duplicated sequences of 25 patients ranging in length from 2 to 8 AA. The patient with the shortest duplication (2 AA) showed insertion of R595 and E596. All but 4 patients (21/25=84%) had duplications of R595. Moreover, all patients had duplications of at least one amino acid of the protein stretch REYEY (AA 595 to 599).

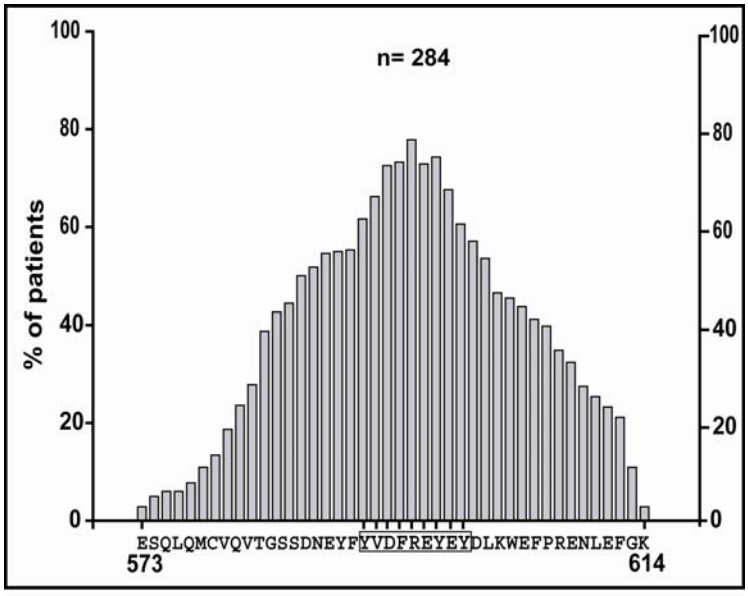


Figure 4.1.3.1: Analysis of the AA composition in the duplicated region in FLT3-ITDs: Frequency of single AAs by position in the duplicated region is shown. For each position the most frequent single AA was selected.

A

No: of AA	570 580 590 600 610	% of patients
	FRYESQLQMV QVTGSSDNEY FYVDFREYEY DLKWEFPREN LEFGKVLGSG	
1	R	77
2	EY	70
3	REY	70
4	FREY	63
5	DFREY	60
6	VDFREY	52
7	EY FYVDF	48
8	EY FYVDFR	46
9	DNEY FYVDF	44
10	DNEY FYVDFR	42
11	SDNEY FYVDFR	38
12	SSDNEY FYVDFR	36
13	SSDNEY FYVDFRE	33
14	SSDNEY FYVDFREY	32
15	GSSDNEY FYVDFREY	28
16	GSSDNEY FYVDFREYE	25
17	SSDNEY FYVDFREYEY D	19
18	NEY FYVDFREYEY DLKWE	16
19	EY FYVDFREYEY DLKWEFP	16
20	NEY FYVDFREYEY DLKWEFP	15
21	DNEY FYVDFREYEY DLKWEFP	14
22	SDNEY FYVDFREYEY DLKWEFP	13
23	SSDNEY FYVDFREYEY DLKWEFP	11
24	SDNEY FYVDFREYEY DLKWEFPRE	10
25	SSDNEY FYVDFREYEY DLKWEFPRE	9
26	GSSDNEY FYVDFREYEY DLKWEFPRE	8
27	SSDNEY FYVDFREYEY DLKWEFPREN L	6
28	GSSDNEY FYVDFREYEY DLKWEFPREN L	5
29	GSSDNEY FYVDFREYEY DLKWEFPREN LE	3
30	VTGSSDNEY FYVDFREYEY DLKWEFPREN L	3

B

580 590 600	no of AA
QVTGSSDNEY FYVDFREYEY DLKWEFPREN	
RE	2
FREYE	5
REYEY D	6
DYVDFR	6
GDFREY	7
HVDFREY	7
YEY DLKW	7
REYEY DL	7
DVDFREY	7
FDFREYE	7
VDFREYE	7
REYEY DL	7
REYEY DL	7
DVDFREY	7
REYEY DL	7
FREYEY D	7
YEY DLKW	7
Y DLKWEFP	8
FREYEY DL	8
EY DLKWEF	8
YVDFREYE	8
DYVDFREY	8
NREYEY DL	8
XYVDFREY	8
DYVDFREY	8

Figure 4.1.3.2: Duplications locate in the motif YVDFREYEY and include R595 in 77% of patients: (A) Most frequent AA combinations within the duplicated region, sorted by length from 1 to 30 AA. **(B)** Panel showing the duplicated sequences of 25 patients ranging from 2 to 8 AA.

4.1.4 Insertion of a single arginine between AA 595 and 596 in FLT3-WT confers IL-3 independent growth to Ba/F3 cells

4.1.4.1 Generation of R595 insertion mutants

The acquisition of FLT3-ITD mutations in the FLT3 gene was shown to induce constitutive activation of the receptor and ligand-independent cell growth in different cell lines(Hayakawa et al., 2000; Kindler T, 2005; Kiyoi et al., 1998; Yamamoto et al., 2001). To validate our hypothesis that duplication of R595, which is found in 77% of patients with FLT-ITDs plays an important role for the transforming activity of the receptor, we introduced an arginine in the FLT3-WT cDNA between positions 595 and 596 (FLT3-ins595R). The second and third most frequent duplicated AA combinations E596/Y597 (FLT3-ins597EY) and R595/E596/Y597 (FLT3-ins597REY), and also the shortest duplication found in patients, i.e. AA combination R595/E596 (FLT3-ins597RE) were also generated by *in vitro* mutagenesis (Figure 4.1.4.1). The correct sequences of all mutants were confirmed by nucleotide sequencing.

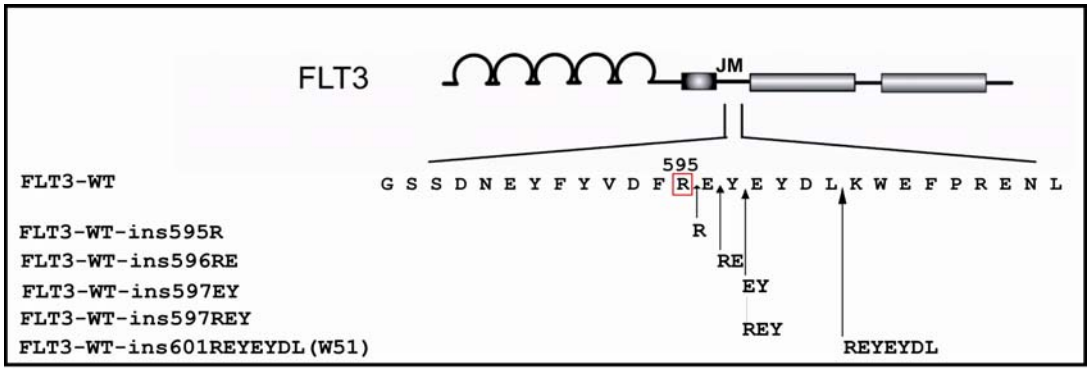


Figure 4.1.4.1: Map of insertion mutants generated in FLT3-WT: Localization of four insertion mutants of FLT3 generated by duplication of 1 to 3 AA of the stretch between AA 595 to 597

4.1.4.2 Generation of R595 insertion mutant cell lines

Activating mutations of FLT3 confer factor independent growth to IL-3 dependent Ba/F3 cells *in vitro*. Hence we transduced the Ba/F3 cells with the constructs FLT3-WT-ins595R, FLT3-WT-ins597EY, FLT3-WT-ins596RE, FLT3-WT-ins597REY. FLT3-WT, mock-expressing cells (MIY) as negative controls and FLT3-ITD-W51 as positive control were also transduced in the Ba/F3 cells. Transduced EYFP-positive cells were sorted by FACS on the basis of EYFP fluorescence and were expanded in the presence of IL-3. After expansion the positive cells were checked for YFP expression in the FACS calibur. 95% or above cells must be YFP positive for further studies or they are resorted. Then the cells are checked for FLT3 expression using PE- conjugated CD135, a FLT3 specific surface antibody in FACS caluber. Only the cells expressing the FLT3 show a shift in flouresence intensity (Figure 4.1.4.2).

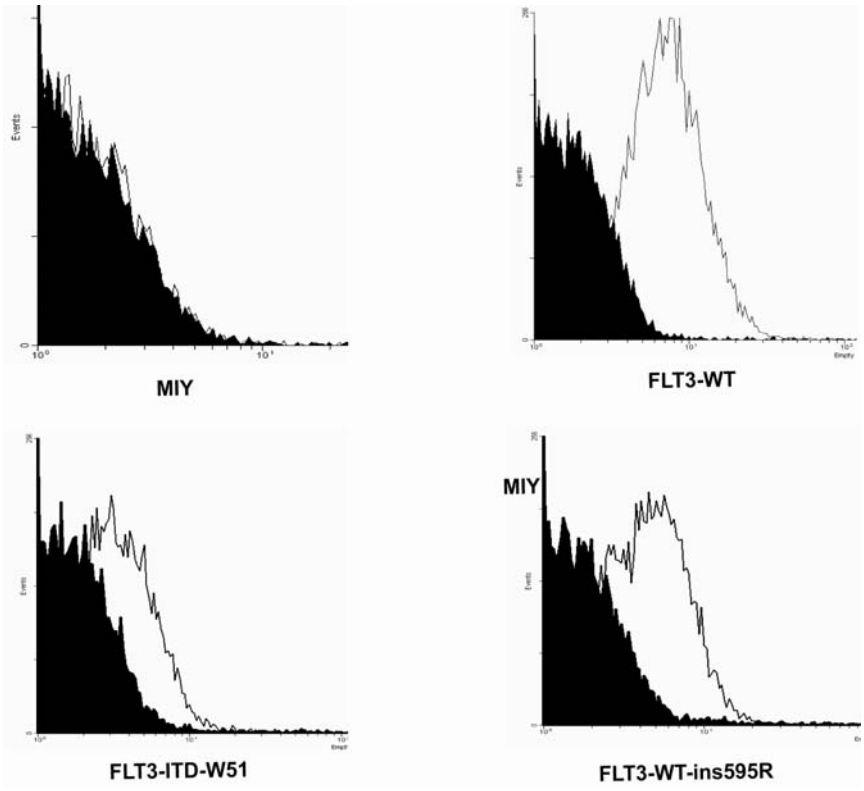


Figure 4.1.4.2: Overexpression of the FLT3 receptor. Example histograms for four of the many FLT3-WT and FLT3-ITD cell lines generated for this study are shown, whereby the cell number is represented against the fluorescence intensity in channel FL2. The black histogram represents the cells after colouring with IgG1-PE-antibody (control), the white histogram the cells after colouring with specific CD135-antibody.

4.1.4.3 Transforming potential of cells expressing a single R595 duplication

Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-ins595R and mock transduced cells were grown in the absence or presence of IL-3 for 72 hours. Viable cells were counted using trypan blue exclusion method. Overexpression of the point mutant FLT3-ins595R, but not FLT3-WT induced IL-3 independent growth in Ba/F3 cells (Figure 4.1.4.3a). In detail, the growth rate of FLT3-ins595R was 40% compared to cells expressing the FLT3-ITD-W51 construct, which served as a positive control.

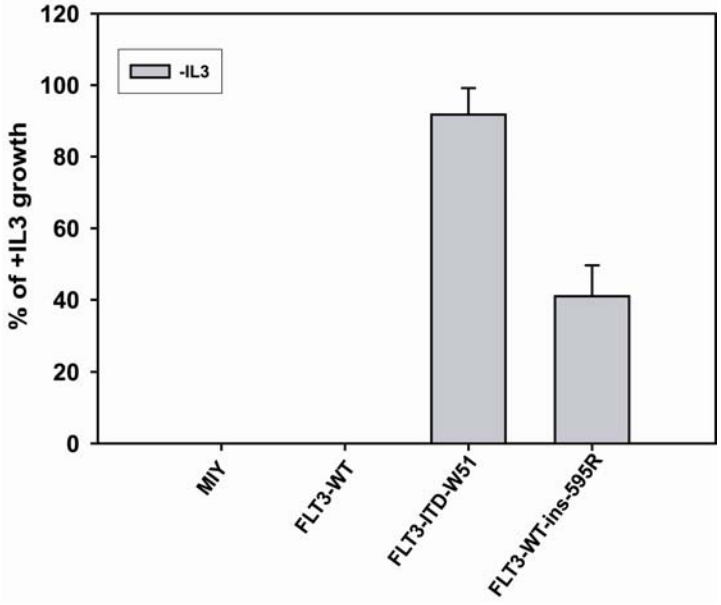


Figure 4.1.4.3a: Duplication of single arginine 595 in FLT3 induce IL-3 independent growth in Ba/F3 cells: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-ins595R or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3. Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is shown.

Similar results were obtained for cells expressing FLT3-ins597RE and FLT3-ins597REY. The mutants FLT3-ins597RE and FLT3-ins597REY showed a growth rate of 49% and 58% when compared to the growth rate of FLT3-ITD (Figure 4.1.4.3b), suggesting that duplication of additional amino acids along with arginine assist in increasing the transforming potential of FLT3-ITDs.

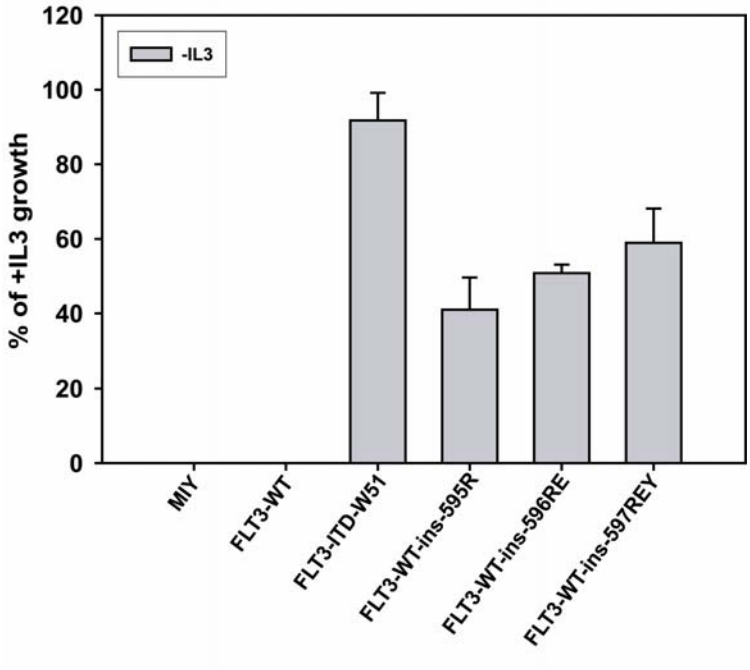


Figure 4.1.4.3b: Addition of AAs to duplicated R595 increases the transforming potential of FLT3 in Ba/F3 cells: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-WT-ins595R, FLT3-WT-ins596RE, FLT3-WT-ins587REY or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3 . Viable cells were counted after 72 h by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is shown.

Quite interestingly, the cells expressing the FLT3-insEY construct that do not have a duplication of arginine, showed a factor independent growth but it induced only a 15% growth rate compared to cells grown in the presence of IL-3 (Figure 4.1.4.3c).

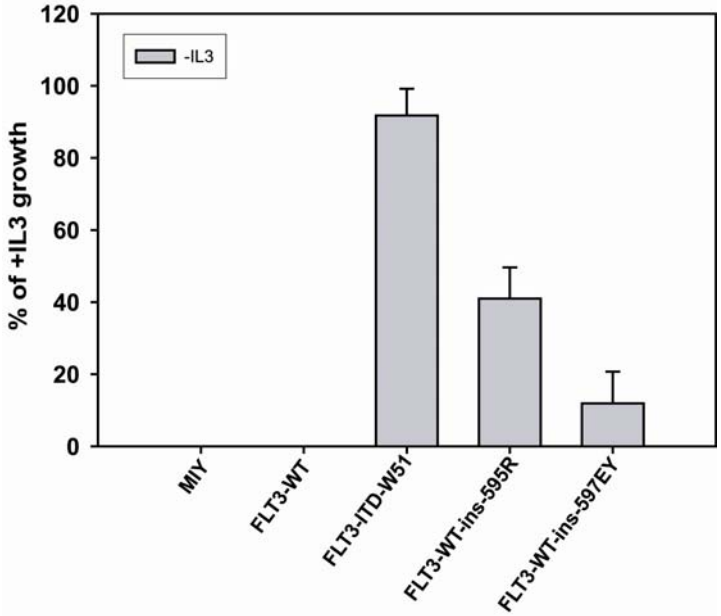


Figure 4.1.4.3c: Duplication of AAs next to R595 in FLT3 induces a very weak IL-3 independent growth in Ba/F3 cells: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-ins595R, FLT3-WT-ins597EY or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3. Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is shown.

4.1.4.4 Stimulation of FLT3 arginine insertion mutants show hyperproliferation upon stimulation with FL.

Progenitor cells expressing FLT3 have been shown to grow upon stimulation by FL *in vitro (Zheng et al., 2004)*. It has been shown in bone marrow and AML blasts that both FL and FLT3 are co-expressed. To check whether the autonomous growth of the FLT3 insertion mutants can be further stimulated by exogenous FL, Ba/F3 cells expressing FLT3-WT, FLT3-ITD-W51, FLT3-ins595R, FLT3-ins596RE, FLT3-ins597EY, FLT3-ins597REY or mock transduced cells were grown in the absence or presence of 60 ng of FL for 72 hours. All the mutants showed hyperresponsiveness in growth upon stimulation. The prolefaration of FLT3-ins595R was 54%, FLT3-ins596RE 65%, FLT3-ins597EY 35%, and FLT3-ins597REY 70% when compared to FLT3-WT which showed a growth of 22% (Figure 4.1.4.4).

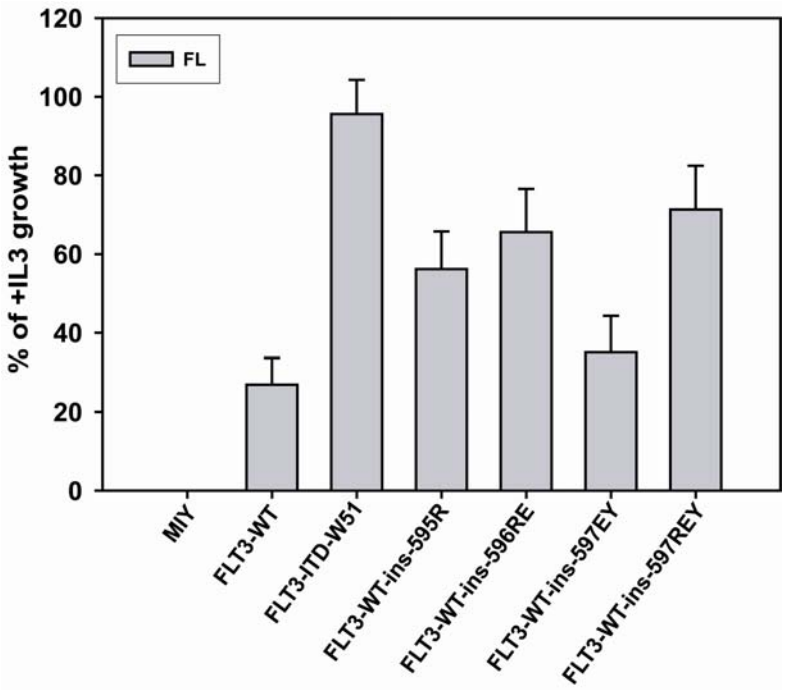


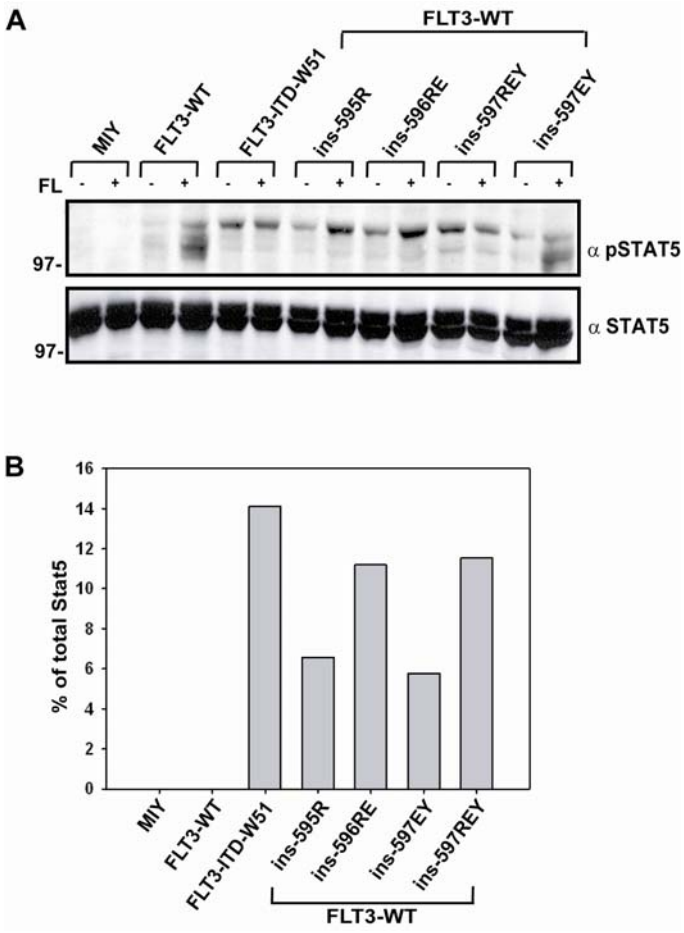
Figure 4.1.4.4. **Insertion mutants of AA595-597 show hyperproliferation upon stimulation with FL:** Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-ins595R, FLT3-WT-ins597EY or mock

transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of FL (60ng/ml). Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is shown.

4.1.4.5 A single R595 duplication causes the activation of STAT5

FLT3 has been shown to activate three important pathways upon stimulation with FL i.e. STAT5, AKT and MAPK pathway (Brandts et al., 2005; Hayakawa et al., 2000; Srinivasa and Doshi, 2002; Zhang and Broxmeyer, 1999; Zhang et al., 2000; Zhang et al., 1999). All these three pathways have been shown to be constitutively activated in FLT3-ITDs. All three pathways have been found to be responsible for the transforming potential, but STAT5 has been found to be the most important signaling pathwy in FLT3-ITD transformed cells. These pathways regulate the genes, which are responsible for apoptosis, proliferation and early cytokine signalling like CyclinD1, CyclinD2, BclXl, c-Myc, SHP2, and PIM-1 (Benekli et al., 2003; Brandts et al., 2005; Gouilleux-Gruart et al., 1996; Kim et al., 2005; Lilly and Kraft, 1997; Mizuki et al., 2000; Scheijen et al., 2004; Vander Heiden et al., 1997).

To investigate the activation of the STAT5 signaling pathway, we prepared crude cell lysatesof serum starved Ba/F3 cells transduced with either vector control(MIY), FLT3WT, FLT3-ITD-W51, FLT3-ins595R, FLT3-ins597EY, FLT3-ins597RE and FLT3-ins597REY. Lysates were analyzed by immunoblotting with a specific antibody against phospho-STAT5. We could clearly demonstrate that the expression of FLT3-ins595R, FLT3-ins597EY, FLT3-ins597RE and FLT3-ins597REY led to increased phosphorylation at position 694 of STAT5 compared to FLT3-WT (Figure 4.1.4.5A). In accordance with the proliferation data, the FLT3-ins597EY mutant showed a significantly weaker STAT5 activation (Figure 4.1.4.5A). Densitometric analysis of the western blots was done to check for the ratio of protein phosphorylated in comparision to total protein. This ratio revealed that all the mutants FLT3-ins595R, FLT3-ins597RE and FLT3-ins597REY showed a phosphorylation of 6-11% (Figure 4.1.4.5B) when compared to FLT3-ITD (14%) (Figure 4.1.4.5B). The mutant FLT3-ins597EY showed a phosphorylation of 4.8% (Figure4.4.5B) which was in accordance with the proliferation data. All the mutants showed activation of the MAPK and AKT but no significant difference was seen between the mutants.



4.1.4.5: A single R595 duplication causes the activation of STAT5: (A) Western blot showing the autoactivation of STAT5 in the mutants FLT3-ins595R, FLT3-ins597EY, and FLT3-ins597REY, FLT3-ITD-W51 when compared to FLT3-WT in the unstimulated cells. FLT3-WT-ins595R, FLT3-WT-ins597EY, and FLT3-WT-ins597REY, FLT3-WT-ins596RE, FLT3-ITD-W51, FLT3-WT or mock trandusced cell lines were starved for 24 hours in the presence of 0.3% FCS and stimulated with 60 ng FL/ml for 5 minutes. Crude cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoeresis (SDS-PAGE) and blotted on a nitrocellulose membrane. Blots were then incubated with anti-phospho-STAT5 antibody, stripped and reblotted with anti-STAT5 antibody. (B) Densitometric analysis of the film in panel C was used to quantify the ratio of phospho-STAT5 to total STAT5.

4.1.5 Deletions or substitutions of arginine 595 in the duplicated region of FLT3-ITD reduce transforming potential of FLT3-ITD

4.1.5.1 Generation of duplicated R595 deletion/Substitution mutants

Having shown that duplication of R595 can activate the transforming activity of FLT3 we asked whether duplicated R595 is necessary for the oncogenic potential of FLT3-ITD mutants. For this purpose, we substituted duplicated AA Arginine (R602) with the neutral AA alanine (FLT3-W51-R602A) and also with the negatively charged AA glutamic acid (FLT3-W51-R602E) (Figure 4.1.5.1) and positively charged amino acid Lysine (FLT3-W51-R602K). Furthermore, we generated a deletion mutant of duplicated R595 (FLT3-W51ΔR602) (Figure 4.1.5.1) and the deletion of neighbouring AAs glutamic acid (FLT3-W51-ΔE603) and AA tyrosine (FLT3-W51-ΔY604) (Figure 4.1.5.1).

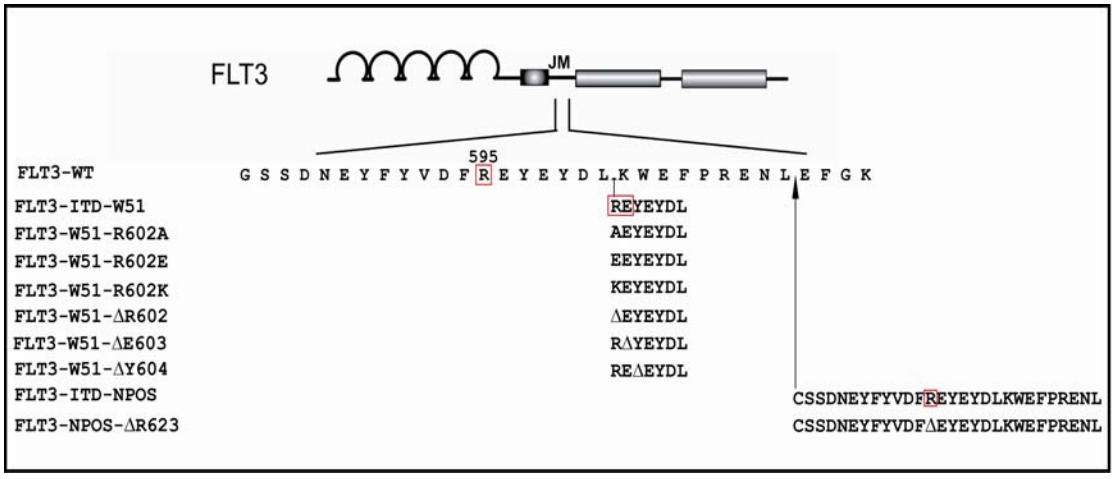


Figure 4.1.5.1: Map of sustitution/deletion mutants of duplicated R595: The Panel shows three different substitution and two deletion mutants of duplicated R595 generated in two different FLT3-ITD's (W51 and NPOS). The deletion mutants of duplicated E596 and Y597 in FLT3-ITD-W51 are also shown.

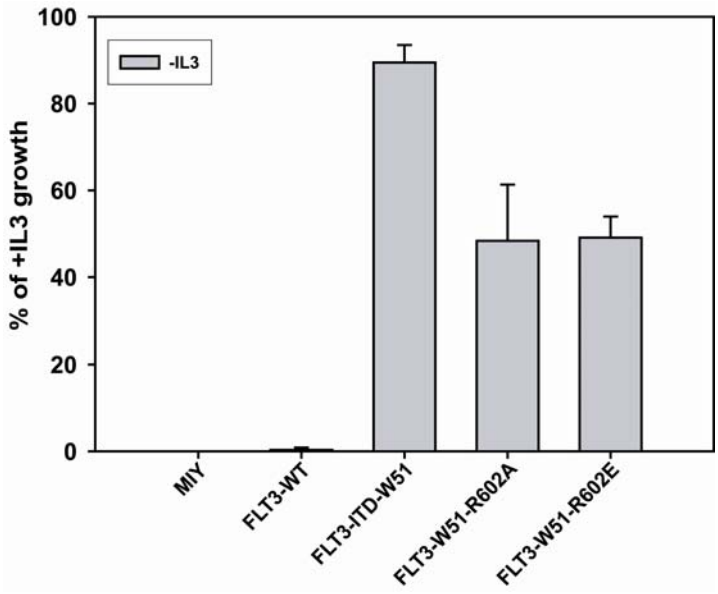
4.1.5.2 Generation of duplicated R595 substitution/deletion mutant cell lines

To check the overexpression of the duplicated R595 substitution and deletion mutants, Ba/F3 cells were stably transduced with these different FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51ΔR602, FLT3-W51-ΔE603, FLT3-W51-ΔY604, FLT3-NPOS-ΔR623 and with FLT3-WT, FLT3-ITD-W51 and FLT3-ITD-NPOS as controls. Transduced EYFP-positive cells were sorted by FACS on the basis of EYFP fluorescence and were expanded in the presence of IL-3. After expansion the positive cells were checked

for FLT3 expression using PE-conjugated CD135, a FLT3 specific surface antibody in FACS calibur.

4.1.5.3 Duplicated R595 substitution by alanine or glutamine reduces the transforming potential

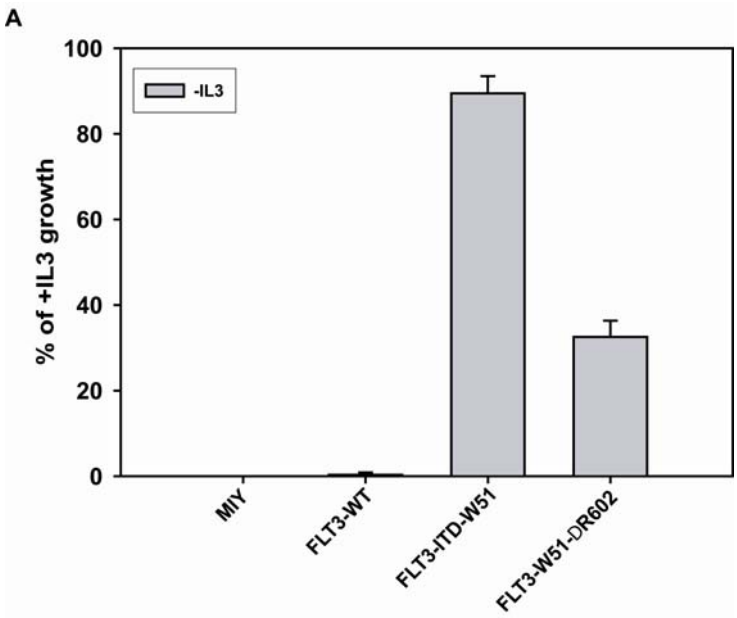
Since arginine is a positively charged AA, and to check role of positive charge in transforming potential of FLT3-ITDs, we generated substituted arginine with Alanine and Glutamine in the background of FLT3-ITD-W51 (Figure 4.1.5.1). Overxpression of the mutants FLT3-W51-R602E and FLT3-W51-R602A in the absence of IL3 medium showed a factor independent growth. The transforming potential of the FLT3-W51-R602A and FLT3-W51-R602E reduced when compared to the unmanipulated FLT3-ITD-W51 (Figure 4.1.5.3). In detail, growth rate of FLT3-W51-R602A and FLT3-W51-R602E was ~ 46% when compared to +IL3 growth. In contrast unmanipulated FLT3-ITD-w51 showed a growth rate 92% of +IL3 growth (Figure 4.1.5.3).

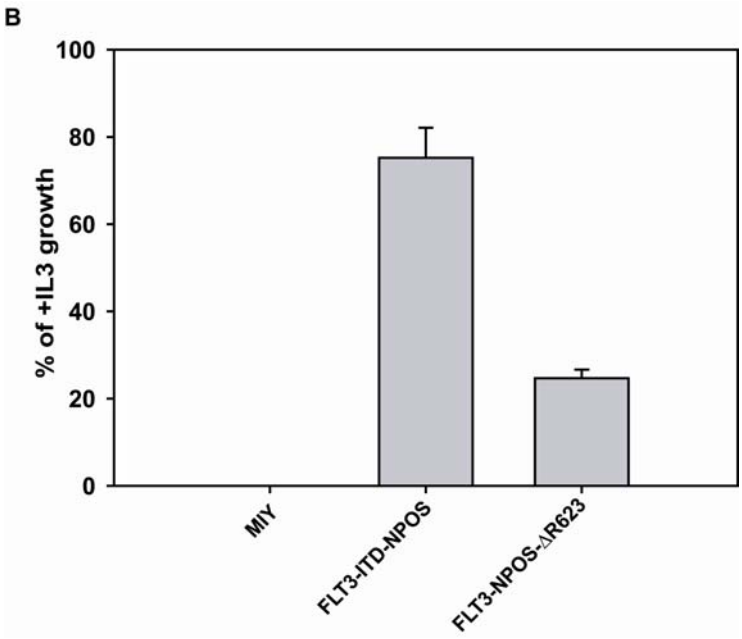


4.1.5.3: Substitution of duplicated R595 by alanine or glutamic acid in FLT3-ITD-W51 reduces the transforming potential: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51-R602A, FLT3-W51-R602E, or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3. Viable cells were counted after 72 hours. The growth of cells in the presence of IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

4.1.5.4 Deletion of R595 in FLT3-ITDs shows reduction in the transforming potential.

Since 23% of the patients with FLT3-ITDs don't have any R595 duplications. Hence, we wanted to check the transforming potential of FLT3-ITDs without Arginine. Overexpression of the deletion mutant FLT3-W51-ΔR602 in –IL3 medium showed factor independent growth of cells, but the growth of cells was drastically reduced by ~70% when compared to +IL3 growth and FLT3-ITD-W51 (Figure 4.1.5.4A). To further validate this finding, we generated a deletion mutant of duplicated R595 (FLT3-ITD-NPOSΔ623R) in a structurally different FLT3-ITD construct, FLT3-ITD-NPOS that contains a 28AA duplicated sequence (CSSDNEYFYDFREYEYDLKWEFPRENL) inserted between AA611/612 of FLT3-WT (Figure 4.5.1). Expression of the deletion mutant (FLT3-ITD-NPOSΔ623R) showed a similar phenotype compared to FLT3-W51ΔR602 with a reduction of ~70% of its proliferative capacity when compared to the FLT3-ITD-NPOS construct (Figure 4.1.5.4B).

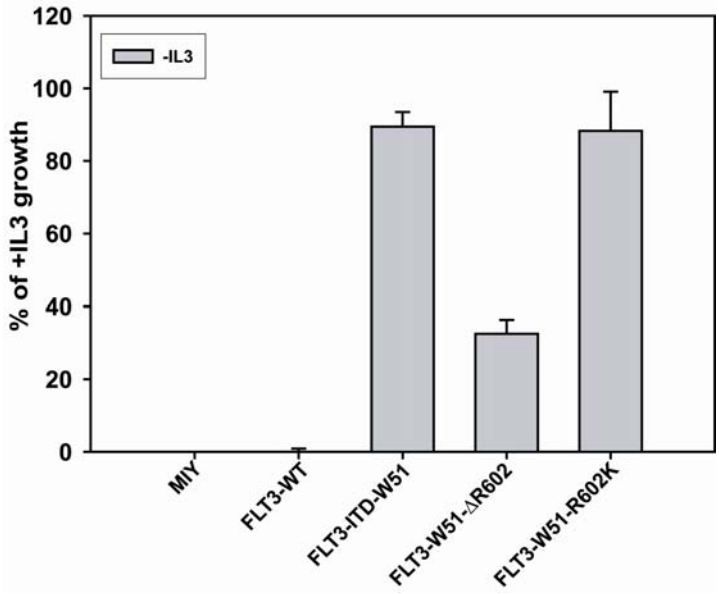




4.15.4: Deletion of duplicated R595 in two differnt FLT3-ITDs show significant reduction in the transforming potential: (A) Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51-ΔR602, or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3. Viable cells were counted after 72 h. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated. (B) Proliferation curves of deletion mutant of duplicated R595 made in a different FLT3-ITD (FLT3-ITD-NPOS).

4.1.5.5 Duplicated R595 substitution by lysine has no effect on the transforming potential

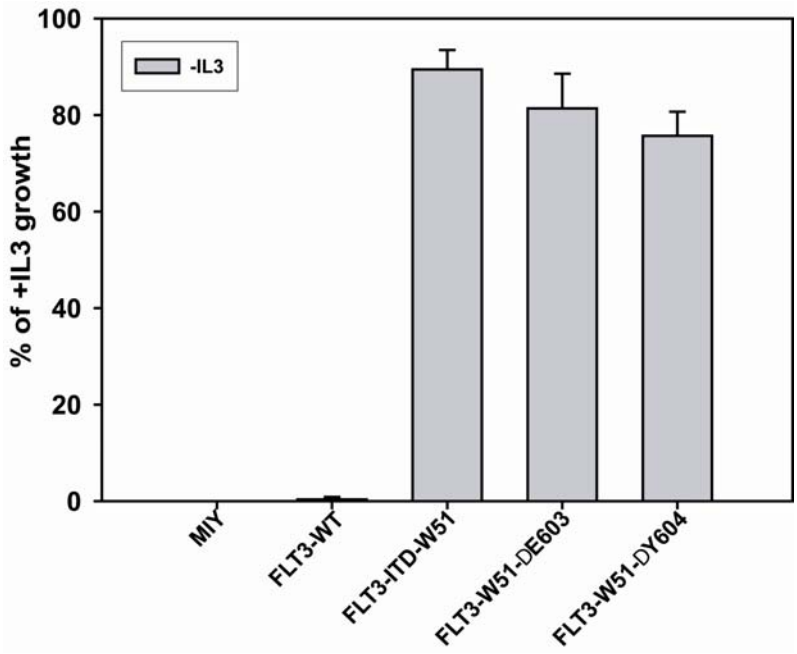
Having shown that the positively charged arginine plays an important role in the transforming potential of FLT3-ITDs, we asked if the duplicated arginine can be replaced with another positively charged amino acid. Hence, we substituted the duplicated arginine with the positively charged AA lysine (FLT3-W51-R602K) (Figure 4.1.5.1). Overexpression of FLT3-W51-R602K in Ba/F3 cells induced factor independent growth and did not show any reduction of the transforming potential when compared to FLT3-ITD-W51 (Figure 4.1.5.5). These data clearly show that the positive charge of the AA (arginine or lysine) plays a crucial role in the transforming potential of FLT3-ITD's.



4.1.5.5: Substitution of duplicated R595 with onother positively charged AA lysine shows no reduction in the transforming potential of FLT3-ITD-W51: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51-ΔR602, FLT3-W51-R602K or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3. Viable cells were counted after 72 hours. The growth of cells in the presence of IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

4.1.5.6 Deletion of duplicated AA E596 and Y597 has no effect on transforming potential of FLT3-ITDs.

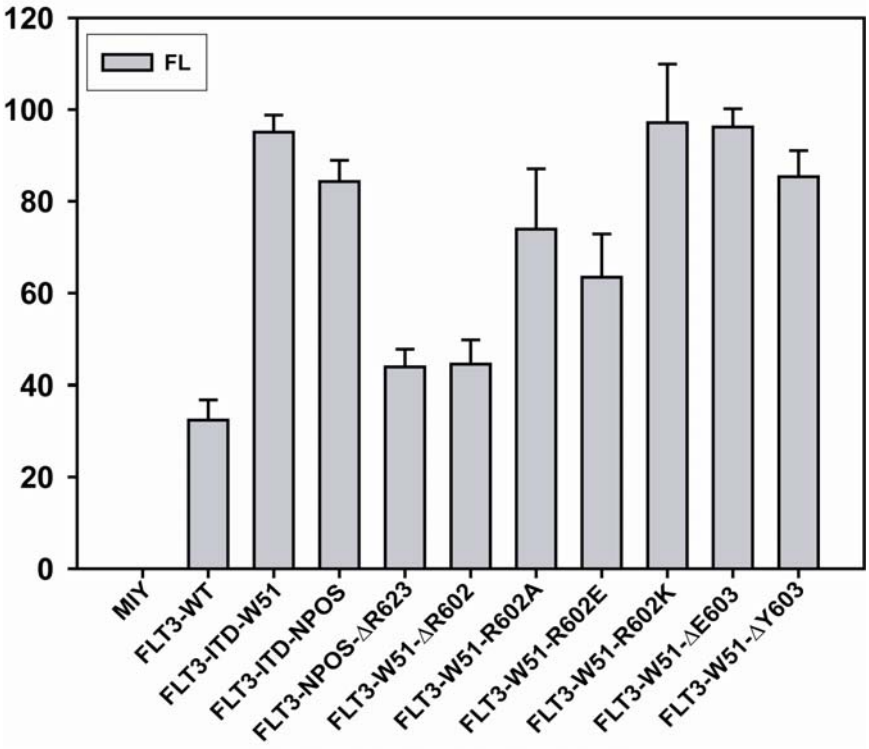
Patient data have shown that AAs E596 and Y597 are the next frequently duplicated AAs other than R595 in 73 and 74% of patients respectively. Hence to check the effect of duplicated AAs E596 and AA E597, we generated deletion mutants FLT3-W51-ΔE603 and FLT3-W51-ΔY604 in FLT3-ITD-W51 (Figure 4.1.5.1). Over expression of mutants FLT3-W51-ΔE603 and FLT3-W51-ΔY604 showed factor independent growth, but there was not significant reduction in the transforming potential when compared to FLT3-ITD-W51 (Figure 4.1.5.6). FLT3-W51-ΔE603 and FLT3-W51-ΔY604 showed a transforming potential of 83% and 76% when compared to FLT3-ITD-W51.



4.1.5.6: Deletion of neighbouring AAs next to duplicated R595 in FLT3-ITD-W51 show no significant reduction in the transforming potential: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51-ΔE603, FLT3-W51-ΔY604 or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3. Viable cells were counted after 72 hours. The growth of cells in the presence of IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

4.1.5.7 Stimulation of R595 deletion/substitution mutants shows hyperproliferation upon stimulation with Flt3 ligand.

To simulate an in vivo situation, where both FLT3 and FLT3 ligand are coexpressed in leukemic blast cells, we stimulated Ba/F3 cells expressing all the mutants FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51ΔR602, FLT3-W51-ΔE603, FLT3-W51-ΔY604 and FLT3-NPOS-ΔR623 along with the controls FLT3-WT, FLT3-ITD-W51 and FLT3-ITD-NPOS FL (60 ng/ml). All the mutants showed hyperproliferation of the cells upon stimulation with FL (Figure 4.1.5.7). In detail FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51ΔR602, FLT3-W51-ΔE603, FLT3-W51-ΔY604 and FLT3-NPOS-ΔR623 showed a hyperproliferation of 72%, 62%, 97%, 42%, 96%, 84% and 42% respectively (Figure 4.1.5.7).



4.1.5.7: Deletion/susbstitution mutants of duplicated R595 show hyperproliferation upon stimulation with FL: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51ΔR602,FLT3-W51-ΔE603, FLT3-W51-ΔY604 or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of FL (60ng/ml). Viable cells were counted after 72 hours. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

4.1.5.8 FLT3-ITDs duplicated R595 substitution/deletion mutants show a reduced capacity to activate STAT5 compared to FLT3-ITDs

To investigate the activation of the STAT5, AKT and MAPK signalling pathways we prepared crude cell lysates of serum starved Ba/F3 cells, transduced with either vector control (MIY), FLT3-WT, FLT3-ITD-W51, FLT3-ITD-NPOS, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51-ΔR602, FLT3-W51-ΔE603, and FLT3-NPOS-ΔR623. All the mutants FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-ΔR602, and FLT3-NPOSΔ623R in Ba/F3 cells showed reduced phosphorylation of the STAT5 compared to non-manipulated FLT3-ITD constructs, (Figure 4.1.5.8a,b). STAT5, AKT and MAPK activation of FLT3-W51-R602K was similar to the FLT3-ITDs (Figure 4.1.5.8a, b). Thus, the activation of the most important signalling pathways downstream of FLT3, STAT5, AKT and MAPK are downregulated in FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51Δ602R, and FLT3-NPOSΔ623R, but not in FLT3-W51-R602K expressing cells and correlates with IL-3 independent growth.

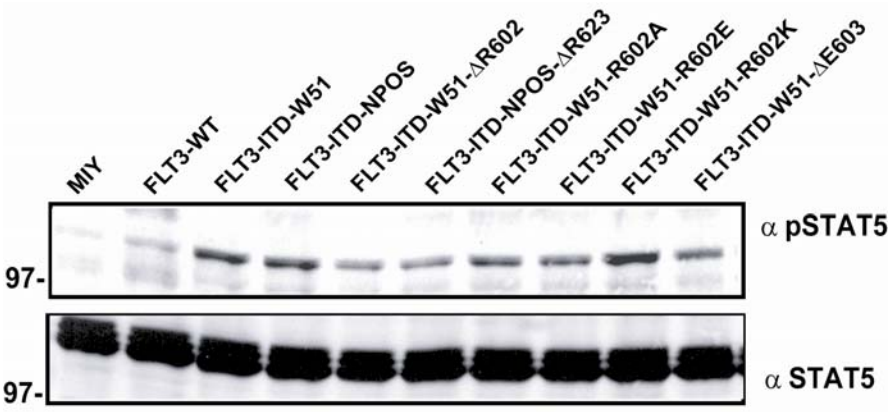


Figure 4.15.8a: The constitutive STAT5 activation is reduced in substitution/deletion mutants of duplicated R595: FLT3-WT, FLT3-ITD-W51, FLT3-ITD-NPOS, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51-ΔR602, FLT3-W51-ΔE603 and FLT3-NPOS-ΔR623 expressing cells were starved for 24 hours in the presence of of 0.3% FCS. Blots were incubated with anti-phospho-STAT5 antibody, stripped and reblotted with anti-STAT5 antibody.

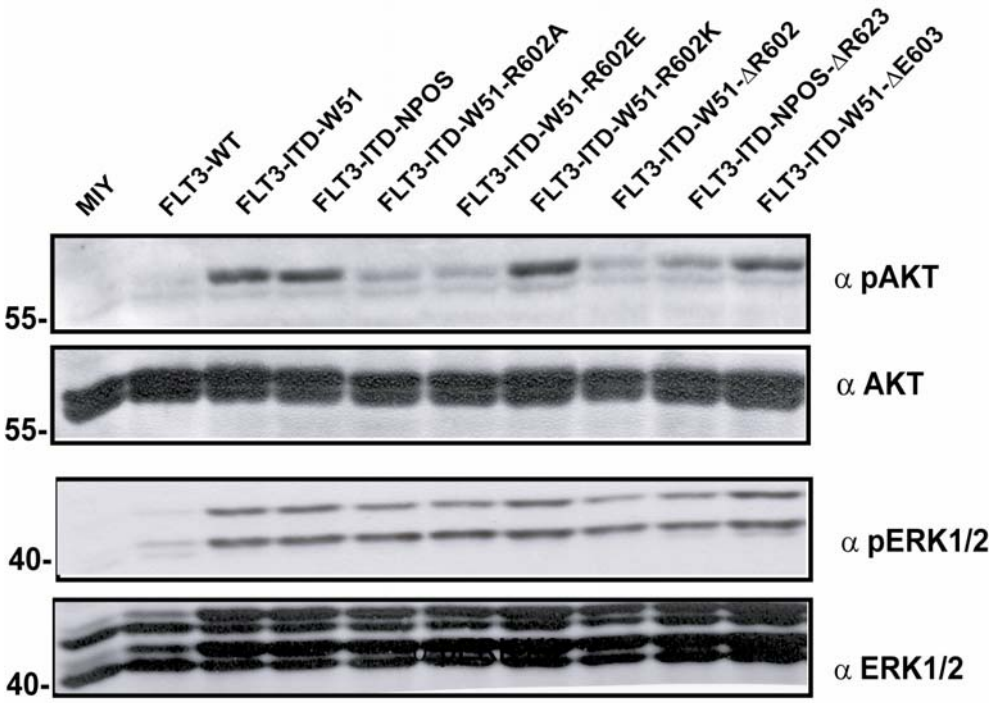


Figure 4.1.5.8b: The constitutive AKT and MAPK activation is reduced in substitution/deletion mutants of duplicated R595: FLT3-WT, FLT3-ITD-W51, FLT3-ITD-NPOS, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51-ΔR602, FLT3-W51-ΔE603 and FLT3-NPOS-ΔR623 expressing cells were starved for 24 hours in the presence of of 0.3% FCS. Blots were incubated with anti-phospho-AKT and anti-phospho-MAPK antibodies, stripped and reblotted with anti-AKT and anti-MAPK antibodies.

4.1.5.9 The substitution/deletion arginine mutants of FLT3-ITDs are sensitive to the FLT3 PTK inhibitor PKC412

PKC412 (Novartis), an inhibitor initially discovered as an inhibitor of protein kinase C, was found to block the phosphorylation and activity of FLT3-WT and mutant FLT3-receptors (Stone et al., 2005; Weisberg, 2002). The inhibitory activity of PKC412 against the arginine mutants of FLT3-ITDs was analyzed by treating the FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51Δ602R, and FLT3-NPOSΔ623R expressing cells with different concentrations of PKC412 ranging from 1 to 100 nM. PKC412 showed a strong growth inhibitory effect on FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51Δ602R, and FLT3-NPOSΔ623R receptors expressing cells in the absence but not in the presence of IL-3. The IC_{50} of PKC412 was significantly lower in arginine substitution/deletion mutants (0.6-1 nM) compared to FLT3-ITD mutants (4 nM) (Table 4.1.5.9).

FLT3-ITD(W51/NPOS)	4.0 nM
FLT3-W51-R602A	0.9 nM
FLT3-W51-R602E	0.8 nM
FLT3-W51-R602K	4.0 nM
FLT3-W51-ΔR602	0.6 nM
FLT3-NPOS-ΔR623	0.7 nM

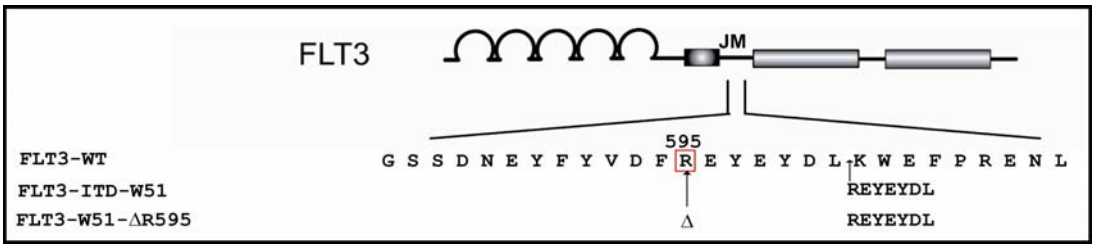
Table 4.1.5.9: IC_{50} for PKC412 values in different FLT3-ITD arginine mutants: Ba/F3 cells expressing FLT3-WT, FLT3-ITD-W51, FLT3-ITD-NPOS, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51-ΔR602 and FLT3-NPOS-ΔR623 seeded at a density of 4 $X10^4$ cells/mL in the absence or presence of different concentrations of PKC412 (0 to 100 nM) and counted after 72 hours. All cell lines were also cultured in the presence of IL-3 and 100 nM PKC412 to confirm nontoxicity of PKC412 to Ba/F3 cells. The IC_{50} was calculated from three independent experiments (IC_{50} is defined as the concentration of inhibitor required to induce a growth reduction of 50% compared to the cells grown in the absence of inhibitor).

4.1.6 Deletion of wild type R595 reduces the transforming potential and activation of STAT5 in FLT3-ITD mutant

Since the duplicated arginine plays an important role for the transforming potential the FLT3-ITDs, we asked whether wild type R595 also has a critical role in the transforming capacity of FLT3-ITDs. We therefore created a deletion mutant of R595 in FLT3-ITD-W51 (FLT3-W51-ΔR595) (Figure 4.1.6.1A).

Overexpression of the wild type arginine deletion mutant FLT3-W51-ΔR595 reduced the IL-3 independent growth by 64% when compared to FLT3-ITD-W51 (Figure 4.1.6.1B).

A



B

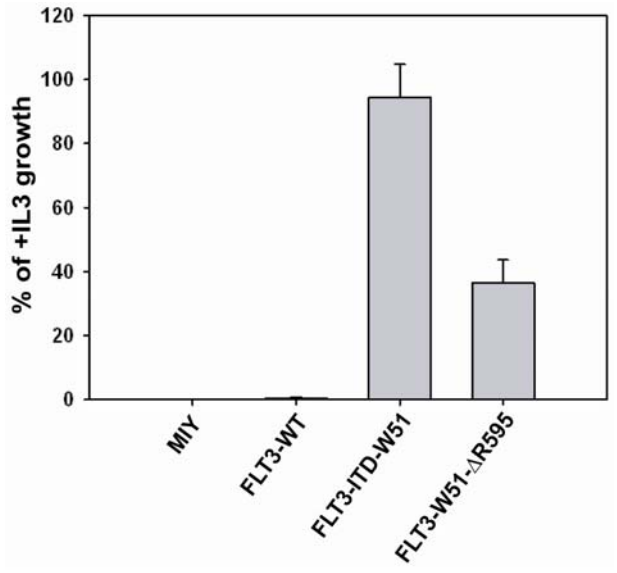


Figure 4.1.6.1: Deletion of wild type R595 in FLT3-ITD-W51 results in reduced transforming potential in Ba/F3 cells: (A) Schematic representation of wild type R595 (FLT3-W51-ΔR595) deletion mutant in the FLT3-ITD-W51 construct. (B) Ba/F3 cells expressing the FLT3-WT**,** FLT3-W51-ΔR595, FLT3-ITD-W51 and mock transduced cells were seeded at a density of 4 x10^4 cells/ml in the absence or presence of IL-3. Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). The standard error of the mean calculated from three independent experiments is indicated.

These proliferation data were supported by the measurement of STAT5 activation (Figure 4.6.2). STAT5 activation was significantly lower in the cells expressing FLT3-W51-ΔR595, when compared to FLT3-ITD-W51. These data suggest that not only the duplicated R595 but also the wild type R595 plays an important role in the transforming potential and STAT5 activation of FLT3-ITD-W51.

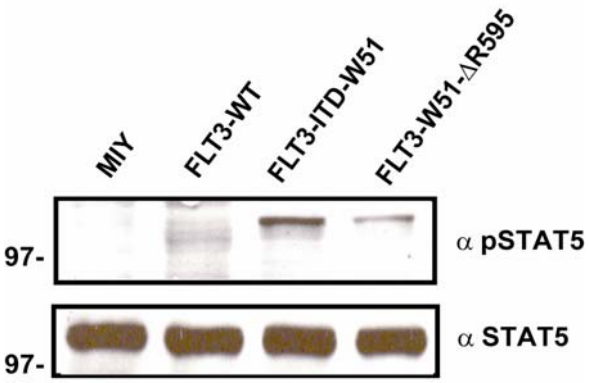


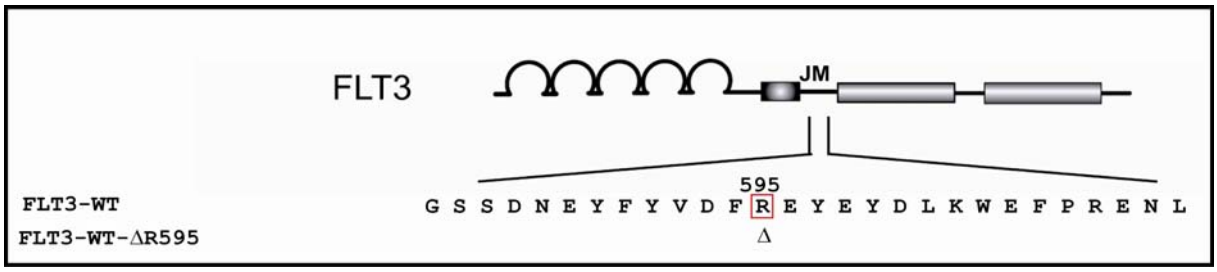
Figure 4.1.6.2: Deletion of wild type R595 in FLT3-ITD-W51 results in reduced phosphoSTAT5 activation in Ba/F3 cells: Western blot image showing the activation of STAT5 in cells expressing FLT3-W51-ΔR595 and FLT3-ITD-W51, when compared to FLT3-WTor mock transduced cells. Cells were starved in the presence of 0.3% FBS for 24 hours. Blots were incubated with anti-phospho-STAT5 antibody, stripped and reblotted with anti-STAT5 antibody.

4.1.7 Deletion of R595 in FLT3-WT abrogates the growth of cells upon FL stimulation

Since duplicated arginine plays an important role for the transforming potential of FLT3-ITDs, we wanted analyse the role of the wild type arginine 595 in the signalling properties of the FLT3-WT. Hence we created a deletion mutant of arginine 595 in FLT3-WT (FLT3-WT-ΔR595) (Figure 4.1.7.1A). We stably transduced the pro-B cell line Ba/F3 with FLT3-WT-ΔR595, using FLT3-WT and FLT3-ITD (W51) as a control. Identical surface expression levels of FLT3 were confirmed by CD135 antibody staining and FACS analysis (data not shown).

Overexpression of the arginine deletion mutant FLT3-WT-ΔR595, did not show any –IL3 independednt growth, but to the surprise it also did not show any growth of the cells on stimulation with FL, when compared to FLT3-WT (Figure 4.1.7.1B).

A



B

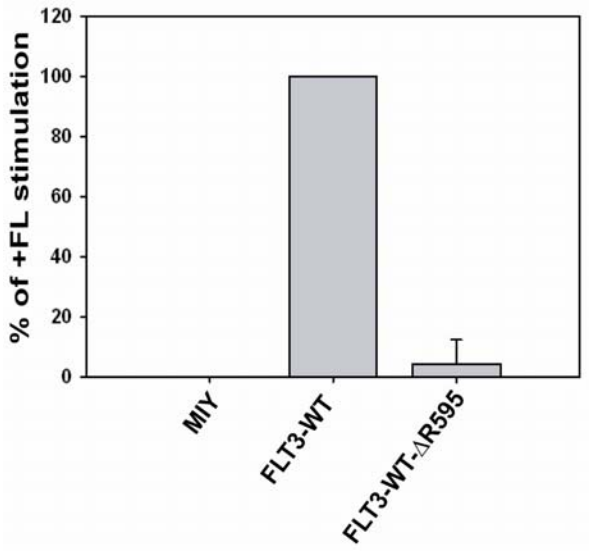


Figure 4.1.7.1: Wild type R595 deletion in FLT3 results loss FL induced growth of cells: (A) Schematic representation of wild type R595 (FLT3-WT-ΔR595) deletion mutant in the FLT3-WT construct. (B) Ba/F3 cells expressing the FLT3-WT**,** FLT3-WT-ΔR595, FLT3-WT and mock transduced cells were seeded at a density of 4 x10^4 cells/ml in the absence or presence of IL-3 and FL (60ng/ml). Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). The standard error of the mean calculated from three independent experiments is indicated.

Since it has been shown that STAT5 plays an important role in signal transduction of FLT3, we prepared crude cell lysates of serum starved Ba/F3 cells transduced with MIG, FLT3-WT, and FLT3-WT-ΔR595 and Flt3-ITD-W51 acting as positive control. Lysates were analysed by immunoblotting with a specific antibody against phospho-STAT5. As expected we could not detect any STAT5 actiovation in the FLT3-WT-ΔR595, when compared to FLT3-WT expressing cells, suggesting that arginine 595 plays important role in the STAT5 activation of FLT3-WT stimulated with FL (Figure 4.1.7.2).

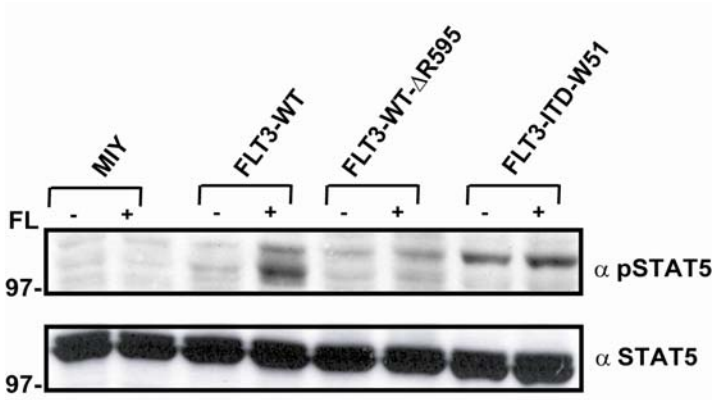


Figure 4.1.7.2: Deletion of wild type R595 in FLT3-WT results in reduced phosphoSTAT5 activation in Ba/F3 cells: Western blot image showing the activation of STAT5 in cells expressing FLT3-WT-ΔR595 and FLT3-WT, when compared to FLT3-WT or mock transduced cells in the absence and presence of FL (60ng/ml). Cells were starved in the presence of 0.3% FBS for 24 hours. Blots were incubated with anti-phospho-STAT5 antibody, stripped and reblotted with anti-STAT5 antibody.

4.2 The role of tyrosine residues in the juxtamembrane domain of FLT3 in the activation of FLT3-ITDs

4.2.1 Generation of substitution mutants of juxtamembrane domain tyrosine residues

Tyrosine residues of the juxtamembrane domain have been shown to be playing important role in maintaining the unphosphorylated and phosphorylated states of FLT3. But no study has been carried out in detail about the role of tyrosoine residues 589, 591, 597 and 599 in the FLT3-ITDs and theire role in transforming potential of FLT3-ITDs. For this purpose, we substituted the tyrosine residues 589, 591, 597 and 599 with phenyalanine as single and double mutants in different combinations (Figure 4.2.1).

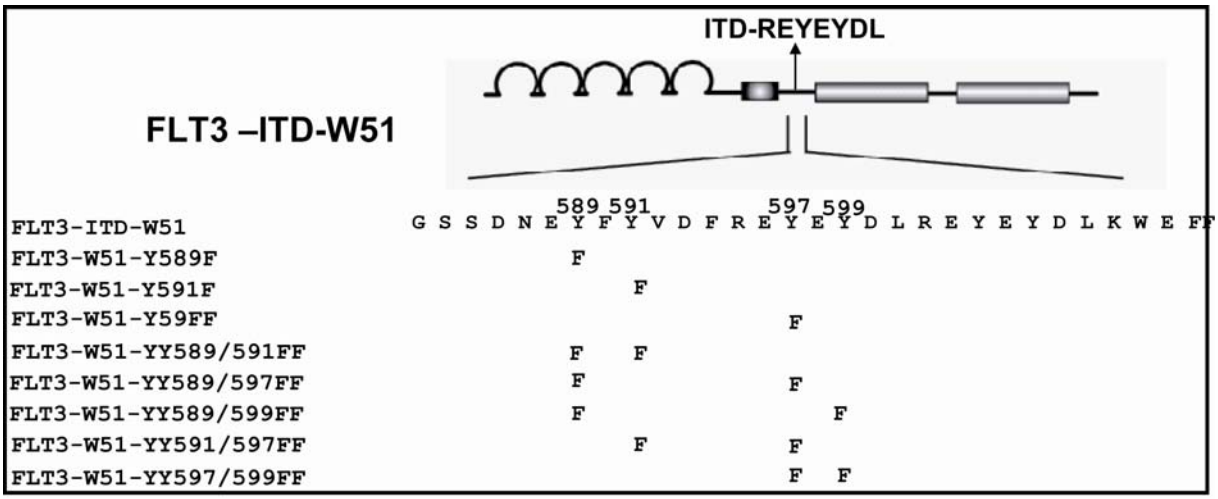


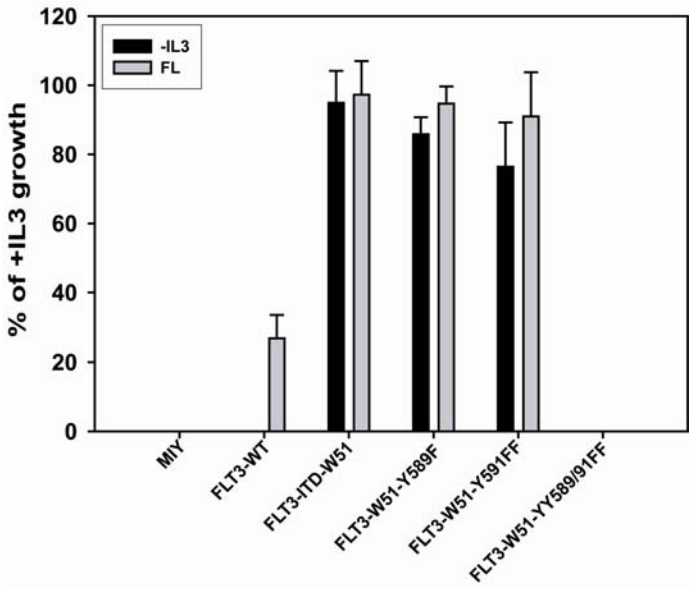
Figure 4.2.1: Map of sustitution mutants of tyrosine residues in the juxtamembrane of FLT3-ITD-W51duplicated R595: Panel shows three different single and double combinatorial substitution mutants of tyrosine residues 589, 591, 597 and 599 of the juxtamebrane domain with phenylalanine made in the background of FLT3-ITD-W51.

4.2.2 Generation of duplicated R595 substitution/deletion mutant cell lines

To check the overexpression of the tyrosine substitution, Ba/F3 cells were stably transduced with FLT3-W51-Y589F, FLT3-W51-Y591F, FLT3-W51-Y597F and double substitutions FLT3-W51-YY589/591FF, FLT3-W51-YY589/597FF, FLT3-W51-YY589/599FF, FLT3-W51-YY91/597FF, FLT3-W51-YY597/599FF and with FLT3-WT, FLT3-ITD-W51 as controls. Transduced EYFP-positive cells were sorted by FACS on the basis of EYFP fluorescence and were expanded in the presence of IL-3. After expansion the positive cells were checked for FLT3 expression using PE-conjugated CD135, a FLT3 specific surface antibody in FACS caliber (data not shown).

4.2.3 Substitution of tyrosine residues 589 and 591 in FLT3-ITD-W51 abolishes the transforming potential of FLT3-ITD

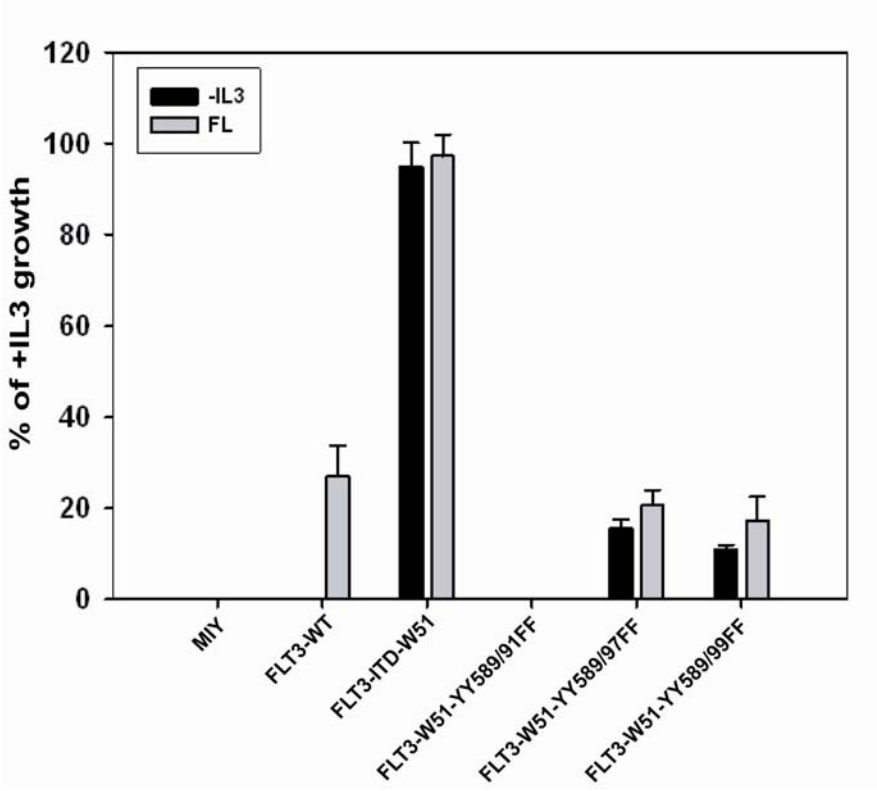
Patient samples analysis, showed that in the majority of the AML patients harbouring FLT3-ITDs, the amino acids 589 and 591 of FLT3 are not duplicated. Hence, we hypothesised that these residues play an important role in the transforming potential of FLT3-ITDs. Therefore, we generated single substitutions of 589 (FLT3-W51-Y589F), 591 (FLT3-W51-Y591F) and double substitutions (FLT3-W51-YY589/91FF) with phenyalanine in the background of FLT3-ITD-W51 (Figure 4.2.1). Overexpression of these mutants in Ba/F3 cells showed that mutants FLT3-W51-Y589F and FLT3-W51-Y591F did not show any significant decrease in the transforming potential of FLT3-ITDs (Figure 4.2.3). In contrast, the mutant FLT3-W51-YY589/91FF completely lost the transforming potential when compared to unmanipulated FLT3-ITD-W51 (Figure 4.2.3). Quite interestingly, cells expressing the double substitution mutant FLT3-W51-YY589/91FF also lost the ability to grow upon stimulation with FL, signifying the role of these residues in transforming potential of FLT3-ITDs (Figure 4.2.3).



4.2.3: Substitution of tyrosines 589 and 591 abolishes the transforming potential of FLT3-ITD-W51: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51,FLT3-W51-Y589F, FLT3-Y591F, FLT3-W51-YY589/591FF, or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3 and FL (60ng/ml). Viable cells were counted after 72 hours. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

4.2.4 Substitution of residues 591 and 599 in combination with 589 reduces the transforming potential of FLT3-ITDs

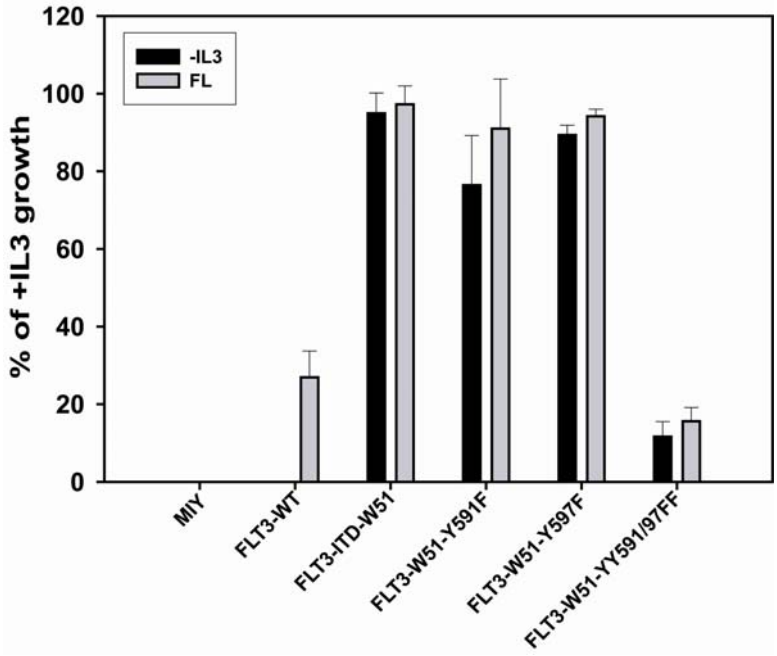
A recent study in has shown that tyrosines 589 and 599 is the phosphoryated upon stimulation with FL (Heiss et al., 2006). But in our hands, single substitution of tyrosine 589 did not have any effect on transforming potential of FLT3-ITDs where as the double mutant FLT3-W51-YY589/91FF showed no transforming potential (Figure 4.2.1). Hence, for this purpose we made different double substation mutants of 589 in combination with other tyrosine residues 597 (FLT3-W51-YY589/97FF) and 599 (FLT3-W51-YY589/99FF). Overexpression of these mutants FLT3-W51-YY589/97FF and FLT3-W51-YY589/99FF in the IL-3 dependent Ba/F3 cells showed that these mutants gave factor independent growth to the cells, but the transforming potential was very weak when compared to the unmanipulated FLT3-ITD-W51 (Figure 4.2.4)..



4.2.4: Substitution of tyrosine 589 along with tyrosines 597 and 599 reduces the transforming potential of FLT3-ITD expressing cells: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51, FLT3-W51-YY589/591FF, FLT3-W51-YY589/597FF, FLT3-W51-YY589/599FF or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3 and FL (60ng/ml). Viable cells were counted after 72 hours. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

4.2.5 Substitution of residues 597 in combination with 591 reduces the transforming potential of FlT3-ITDs

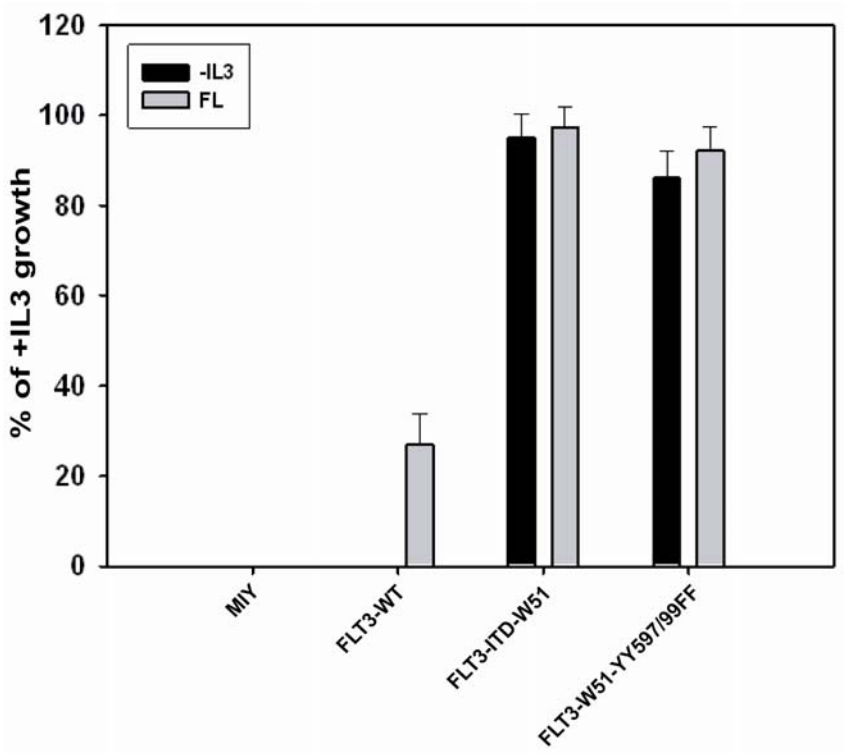
Having shown that when tyrosine residues 589 mutated along with 597 or 599 residue decreases the transforming potential of FLT3-ITD-W51, we now checked, the role of tyrosine residue 591, when it is mutated in combination with either 597/599. Hence, for this purpose we generated double substation mutants of 591 in combination with tyrosine residues 597 (FLT3-W51-YY591/97FF) (Figure 4.2.1). Over expression of these mutants FLT3-W51-YY591/97FF in the IL-3 dependent Ba/F3 cells showed that these mutants conferred factor independent growth to the cells, but the transforming potential was very weak when compared to the unmanipulated FLT3-ITD-W51 (Figure 4.2.5).



4.2.5: Substitution of tyrosine 591 and 597 together reduces the transforming potential of FLT3-ITD: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51, FLT3-W51-Y591F, FLT3-Y597F, FLT3-W51-YY591/597FF or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3 and FL(60ng/ml). Viable cells were counted after 72 hours. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

4.2.6 Substitution of residues 597 together with 599 do not affect the transforming potential of FlT3-ITD

Having shown that when tyrosine residues 589/591 mutated along with 597 or 599 residue decreases the transforming potential of FLT3-ITD-W51, we wanted to check if the same effect could be seen when we mutate the tyrosine residues 597/599.. Hence, for this purpose we generated double substitution mutant of 597 in combination with tyrosine residues 599 (FLT3-W51-YY591/99FF) (Figure 4.2.1). Over-expression of these mutants FLT3-W51-YY591/99FF in the IL-3 dependent Ba/F3 cells showed that these mutants conferred factor independent growth to the cells, and there was no significant decrease in the transforming potential when compared to the unmanipulated FLT3-ITD-W51 (Figure 4.2.6).



4.2.6: Substitution of tyrosine 591 and 597 together reduces the transforming potential of FLT3-ITD: Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51-YY597/599FF or mock transduced cells were seeded at a density of 4 x 10^4 cells/ml in the absence or presence of IL-3 and FL(60ng/ml). Viable cells were counted after 72 hours. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from three independent experiments is indicated.

5 DISCUSSION

5.1 Arginine 595 is duplicated in patients with acute leukemias carrying internal tandem duplications of FLT3 and is critical for its transforming potential

FLT3-ITDs are found in 20-25% of AML patients and are associated with an adverse prognosis. Recently, three different studies have reported conflicting results, stating the importance of the length of the duplication for the prognostic significance of the mutation. One study showed that longer duplications are associated with an unfavorable prognosis in patients with AML, whereas another study found the opposite (Kusec et al., 2006; Stirewalt et al., 2006) but a more recent study showed that the length of duplication in FLT3 has no role at all in the prognosis of AML patients (Ponziani et al., 2006). Given the substantial structural heterogeneity of FLT3-ITD mutations, no common motifs or amino acids have been identified so far that is duplicated in all patients and which might play a important role in the prognosis of patients. Hence, we sequenced the JM region of FLT3 in 284 patients with acute leukemias carrying internal tandem duplications in order to identify a common signature in these ITDs.

Analysis of the juxtamembrane region of FLT3-ITDs, confirms previous data showing that internal tandem duplications are heterogenous with respect to length. The length of the duplication varied from 2-68 amino acids with a median of 17 AA. In 111 patients there were pure tandem duplications whereas in 163 patients there were additional insertions at the start of duplication were found that maintained a reading frame. The analysis of single residues within the duplicated region showed that R595 was the single most frequently duplicated AA in 77% of the patients, followed by Y597 in 73%, F594 and E596 in 71% of all patients. In 95% of the patients at least one amino acid of the stretch between amino acids 591-599 was duplicated. A detailed statistical analysis of AA combinations from 1 to 30 AA in the duplicated regions showed that, although there is no central motif common in all patients, the site of insertion (mutational hotspot) is frequently located in or around the Y-rich strech region from AA 591 to 599 (YVDFREYEY) of FLT3. R595 is duplicated in 77% of the patients followed by a combination of EY (E596 and Y597) in 70% of patients. *In vitro* studies showed that duplication of R595 in FLT3 is able to confer factor independent growth to Ba/F3 cells and led to activation of STAT5. Substitution of the duplicated R595 with alanine (R602A) or glutamic acid (R602E) in a representative FLT3-ITD-W51 construct showed a significant reduction in the transforming potential of FLT3-ITD-W51. In contrast,

substitution of the duplicated R595 with the positively charged AA (R602K) did not alter the transforming potential of cells compared to FLT3-ITD-W51 construct.

Statistical analysis of our data showed that R595 is the most frequently duplicated single AA, Moreover, the analysis of patients with the shortest duplications showed that R595 was duplicated in 84% of these patients. Interestingly, the shortest duplication found in our cohort was R595/E596 duplication. This prompted us to check if a single R595 duplication was sufficient to confer factor independent growth to Ba/F3 cells. *In vitro*, duplication of a single AA, R595 (FLT3-ins595R) was sufficient to activate the transforming potential of FLT3. Previous studies have shown that factor-independent growth *in vitro* and myeloproliferative disease can be caused by FLT3-ITD in transgenic mice and murine bone marrow transplantation model (Ihle et al., 1995; Kelly et al., 2002a). The weak transforming phenotype of FLT3-ins595R could be attributed to a slight disturbance in the autoinhibitory conformation of the JM region caused by a single amino acid insertion. Similar results were reported by our group on point mutations in the JM region found in AML patients that have a weak transforming potential, when compared to FLT3-ITDs (Reindl et al., 2006). Addition of amino acids next to arginine, (FLT3-ins596RE, FLT3-ins597REY) increased the transforming potential when compared to FLT3-ins595R. Moreover, FLT3-ITD mutant lacking the R595 duplication, i.e. FLT3-ins595EY showed a weak transforming potential when compared to FLT3-ins595R, FLT3-ins596RE, FLT3-ins597REY and FLT3-ITD-W51. These data clearly show that the duplicated R595 plays a critical role for the transforming potential of FLT3-ITDs. Activation of FLT3 by FLT3 ligand (FL), leads to receptor dimerization and transphosphorylation of specific tyrosine residues, (Turner et al., 1996) which further activates the downstream signalling pathways including STAT5, Ras/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (Srinivasa and Doshi, 2002; Zhang and Broxmeyer, 1999; Zhang and Broxmeyer, 2000; Zhang et al., 2000; Zhang et al., 1999). Ba/F3 cells expressing FLT3-ins595R, FLT3-ins597EY, FLT3-ins596RE, FLT3-ins597REY showed hyperresponsiveness to FLT3 ligand, with a 1.5 to 3 times higher proliferation rate compared to FLT3-WT cells. This experimental setting probably reflects the *in vivo* situation, as it has been found that FL is coexpressed in AML blasts (Zheng et al., 2004).

FLT3-ITDs mutants are strong activators of STAT5 (Rocnik et al., 2006; Spiekermann et al., 2003), MAPK/ERK1/2 (Hayakawa et al., 2000) and AKT (Brandts et al., 2005; Mizuki et al.,

2000; Scheijen et al., 2004). STAT5 has been shown to be activated in blasts of 20-80% of patients with AML (Benekli et al., 2003; Gouilleux-Gruart et al., 1996; Hayakawa et al., 2000). Previous studies have also shown that these pathways regulate the expression of early cytokine genes like PIM-1 (Kim et al., 2005; Lilly and Kraft, 1997), FOXO proteins (Scheijen et al., 2004) and Bcl-xl (Vander Heiden et al., 1997), which are responsible for cell survival and growth, and leads to tumour development and progression. In accordance with the proliferation data, all the mutants FLT3-ins595R, FLT3-ins596RE, FLT3-ins597REY and FLT3-ins597EY showed activation of STAT5, but to a lesser degree compared to the FLT3-ITD-W51 mutant. All the mutants showed activation of MAPK and AKT, but there was no notable difference in the activation level in all the mutants.

Determination of the crystal structure of FLT3-WT has shown that the JM can be divided into three parts (Griffith et al., 2004). The JM binding motif (JM-B) acts as an autoinhibitory domain, by preventing the rotation of the N lobe towards the C lobe of the tyrosine kinase domain (TKD) to generate the activated kinase fold, whereas the JM switch motif (JM-S) that lies next to JM-B provides a rigid and properly oriented scaffold for the interposition of tyrosines 589 and 591 between the JM-S and the C lobe of the kinase. FLT3-ITDs are frequently found in the JM zipper region (JM-Z) that aligns and maintains the JM-S in the proper orientation during and after the transition between activated and inactive states of FLT3. It is hard to predict the mechanisms by which these FLT3-ITDs change the conformation of the FLT3 protein, but it is hypothesized that the duplications offset the position of the JM-S in the FLT3 structure (Griffith et al, 2004). This results in disturbing or preventing the optimal orientation of JM-S as it tries to position the JM-B in its binding site. Since FLT3-ins597EY showed a transforming potential 2-4 times lower than FLT3-ins595R, FLT3-ins596RE and FLT3-ins597REY, we hypothesized that the positive charge of R595 might be involved in forming crucial interactions with other AAs. These new interactions might not only disrupt the autoinhibitory loop formed by JM-B, but also promote higher tyrosine phosphorylation by rendering the JM domain more accessible for autophosphorylation.

To analyze whether the positive charge of the duplicated R595 has any role for the oncogenic potential of FLT3-ITDs, we generated substitution mutants of the duplicated R595 with an aliphatic AA, alanine (FLT3-W51-R602A), and a negatively charged AA, glutamic acid (FLT3-W51-R602E) as well as a deletion mutant of the duplicated R595 (FLT3-W51-ΔR602)

in the strongly transforming FLT3-ITD-W51. All mutants showed factor independent growth in Ba/F3 cells, but the transforming potential of these mutants was reduced by 55%-70% compared to FLT3-ITD-W51. The same degree of reduction was seen by deleting the duplicated R595 (FLT3-NPOS-ΔR623) in another FLT3-ITD (FLT3-ITD-NPOS) construct. Interestingly, there was no effect on the transforming potential when duplicated R595 was replaced by another positively charged AA, e.g. lysine (FLT3-W51-R602K). Deletion of the AA adjacent to arginine in FLT3-ITD-W51 (FLT3-W51ΔE603) did not significantly reduce the transforming potential of FLT3-ITD-W51, confirming that the positive charge of the duplicated R595 plays an important role for the transforming potential of FLT3-ITDs.
All deletion and substitution mutants of wild type and duplicated R595 in FLT3-ITDs (W51 and NPOS) showed a reduction in the activation of STAT5, MAPK and AKT pathways except for the FLT3-W51-R602K suggesting that the positive charge of duplicated R595 has an essential role in the activation of these pathways.

The hypothesis that the positive charge of arginine plays an important role in the transforming potential of FLT3-ITD was further confirmed by the deletion of the wild type R595 in the FLT3-ITD-W51 ((Flt3-W51-ΔR595). Deletion of wild type R595 decreased the transforming potential of FLT3-ITD by 64% which was in consonance to STAT5 reduction in this cell line.

Since the positive charge of both wild type and duplicated R595 had a strong influence on the transforming potential of FLT3-ITD, we hypothesised that R595 must also have an important role in the activation of FLT3-WT by FL. Deletion of R595 in FLT3-WT (FLT3-WT-ΔR595) and expression in Ba/F3 cells nearly abolished the growth of cells (15%) and STAT5 activation upon stimulation with FL, when compared to FLT3-WT(100%). This suggests that positively charged R595 has important role in the activation of STAT5 signalling which leads to growth and proliferation of cells.

Our results clearly show that R595 is duplicated in 77% of the patients with FLT3-ITDs and plays a critical role for the transforming potential of FLT3-ITD mutants. For the first time, we have shown at the molecular level *in vitro,* that not only the length, but duplication of R595 is neccessary for the transformation of hematopoietic cells by FLT3-ITD mutants.

5.2 The role of tyrosine residues in the juxtamembrane domain of FLT3 in the activation of FLT3-ITDs

Resolving the crystal structure of FLT3-WT (Griffith et al., 2004), has shown that the tyrosine rich juxtamembrane region of FLT3 acts as a autoinhibitory loop in the inactivated state. Previous studies of c-KIT JM domain in controlling receptor phosphorylation was well examined by generating a series of mutant c-KIT constructs with single alanine substitutions for residues Methionine-552 through Isoleucine-563 (Ma et al., 1999). This region included a putative-helix and most of the mutated residues found in gastrointestinal stromal tumors and in mastocytomas. This work demonstrated that the Y553F mutant showed receptor autophosphorylation but that the E561A mutant impaired ligand-induced phosphorylation. The tyrosine-rich stretch of the FLT3-JM domain is predicted as a putative-helix, though it lies downstream of the region analysed in c-KIT. It is, therefore, conceivable that the tyrosine-rich stretch exerts inhibitory effects on spontaneous phosphorylation. In addition, since several SH2-containing molecules are reportedly recruited to tyrosine residues of the JM domain in other Class III RTKs, we examined the role of tyrosine residues (Y589, Y591, Y597 and Y599) in the JM domain in the activation of FLT3-ITDs. In FLT3-WT, these tyrosine residues have been shown to be important for the FL-dependent activation, because 4F-FLT3-expressing (four tyrosines 589, 591,597,599 substituted with phenyalanine) 32D cells could not proliferate with FL alone and FLT3 was not tyrosine-phosphorylated even after FL-stimulation (Kiyoi et al., 2002).

Analysis of the FLT3 juxtamembrane region in 284 patients carrying FLT3-ITDs showed that in the majority of the patients the tyrosines residues 589 and 591 were not duplicated. Hence we hypothesised that these residues might have important role for the transforming potential of FLT3-ITDs. Therefore we substituted the Y589 (FLT3-W51-Y589F) and Y591 (FLT3-W51-Y591F) with phenylalanine in the background of FLT3-ITD-W51. Expression of the substitution mutants in Ba/F3 cells did not show any effect in the transforming potential of FLT3-ITDs. But, simultaneous substitution of Y589 and 591 (FLT3-W51-YY589/591FF) in FLT3-ITD-W51, totally abrogated the transforming potential of FLT3-ITD when expressed in Ba/F3 cells. The mutant FLT3-W51-YY589/591FF also did not show any growth of cells when stimulated with FL. These results suggest that these residues might be acting as phosphorylation sites and enable the binding of downstream signaling molecules. The juxtamembrane tyrosine residues analogous to 589 and 591 in other members of related receptor tyrosine kinases have also been examined for their role in kinase activation. In

PDGFRb the tyrosine to phenylalanine mutations of Y579 and Y581 resulted in a significant decrease in kinase activity to varying degrees dependent on cell type (Baxter et al., 1998; Drummond-Barbosa et al., 1995; Mori et al., 1993; Vaillancourt et al., 1995). In addition, phenylalanine substitutions of the corresponding juxtamembrane tyrosines in c-kit (Kimura et al., 2004) and CSF-1R (Rohde et al., 2004) also impaired kinase activity.

Y589 and Y591 were also attractive candidate residues that might account for the signal transduction differences between FLT3-ITD and FLT3-WT in that they are localized to the juxtamembrane domain that harbors the ITD mutation. Determination of the crystal structure of FLT3-WT has indicated that the function of the JM switch motif (JM-S) is to provide a rigid and properly oriented framework for the interposition of tyrosines 589 and 591 between the JM-S and the C lobe of the kinase (Griffith et al., 2004). These finding suggest that it may be possible that ITDs within the JM domain of FLT3 result in the loss of structure leading to constitutive activation of the kinase and exposure of Y589 and Y591 via release from the JM-S interface with the C loop might subsequently enable phosphorylation and engagement of STAT5.

To check if other tyrosine residues 597 and 599 have any role in the transforming potential FLT3 we generated different combination mutants of 597 and 599 with 589 and 591. Substitution mutants of Y589 in combination with Y597 (FLT3-W51-YY589/597FF) and Y599 (FLT3-W51-YY589/599FF) when expressed in Ba/F3 cells showed a factor independent growth, but the transforming potential was reduced by nearly 70-80% and were able to show hyperproliferation upon stimulation with FL. Similar result was found with substitution mutant of Y591 in combination with Y597 (FLT3-ITD-YY591/597FF). These results suggest that the combination of tyrosine residues 589 and 591 is critical for the transforming potential of FLT3-ITDs.

In conclusion, we have identified specific tyrosine residues that are required for the transforming potential of FLT3-ITDs. Our data provide important insights into the molecular mechanisms of FLT3 activation by oncogenic mutation in AML.

6 SUMMARY

Activating mutations in the juxtamembrane domain of FLT3 (FLT3-internal tandem duplications, FLT3-ITDs) represent the most frequent genetic alterations in acute myeloid leukemia (AML). FLT3-internal tandem duplications (FLT3-ITDs) are a heterogenous group of mutations in patients with acute leukemias that are prognostically important. To characterize the mechanism of transformation by FLT3-ITDs, we sequenced the juxtamembrane region (JM) of FLT3 from 284 patients with acute leukemias. The length of FLT3-ITDs varied from 2 to 42 amino acids (AA) with a median of 17 AA. The analysis of duplicated AAs showed that in the majority of patients, the duplications localize between AA 591 to 599 (YVDFREYEY). Arginine 595 (R595) within this region is duplicated in 77% of patients. Single duplication of R595 in FLT3 conferred factor-independent growth to Ba/F3 cells and activated STAT5. Moreover, deletion or substitution of the duplicated R595 in two FLT3-ITD constructs as well as the deletion of wildtype-R595 in FLT3-ITD substantially reduced the transforming potential, pointing to a critical role of the positive charge of R595 in stabilizing the active confirmation of FLT3-ITDs. Deletion of R595 in the FLT3-WT inhibited the growth of cells upon FL stimulation and the STAT5 activation.

In this study we could also show that the tyrosine residues 589 and 591 of the FLT3-ITDs could be important phosphorylation sites and are very crucial for the activation of FLT3-ITDs. Simultaneous substitution of these two tyrosine residues with phenyalanine showed complete inhibition of the transforming potential of FLT3-ITDs and STAT5 activation. The substitution of tyrosine residues 597 and 599 did not show any effect on the transforming potential of FLT3-ITDs, supporting the previous hypothesis that these tyrosines may be only important to maintain the integrity of FLT3-WT in its inactive state.

Our data provide important insights into the role of the juxtamembrane domain in the mechanism of transformation by FLT3-ITDs.

7 Zusammenfassung

Aktivierende Mutationen in der juxtamembranösen Domäne von FLT3 (FLT3-Längenmutationen, FLT3-LM) sind die häufigsten genetischen Alterationen in der akuten myeloischen Leukämie (AML). FLT3-interne Tandemduplikationen sind eine heterogene Gruppe von Mutationen in Patienten mit akuten Leukämien, die von prognostischer Bedeutung sind. Um den Mechanismus der Transformation durch FLT3-ITDs zu charakterisieren, haben wir die juxtamembranöse Domäne von FLT3 von 284 Patienten mit akuten Leukämien sequenziert. Die Länge der FLT3-ITDs variierte von 2 bis 42 Aminosäuren (AA) mit einer medianen Länge von 17 AA. Die Analyse der duplizierten AAs zeigte, dass in der Mehrheit der Patienten die Duplikationen zwischen AA 591 und 599 (YVDFREYEY) lokalisiert sind. Arginin 595 (R595) innerhalb dieser Region ist in 77% der Patienten dupliziert. Die einzelne Duplikation des R595 in FLT3 führte zu wachstumsfaktor-unabhängigem Wachstum von Ba/F3-Zellen und aktivierte STAT5. Darüber hinaus führte die Deletion oder Substitution des duplizierten R595 in zwei FLT3-ITD Konstrukten sowie die Deletion des wildtyp-R595 in FLT3-ITD zu einer signifikanten Reduktion des transformierenden Potentials, was auf eine kritische Rolle der positiven Ladung des R595 in der Stabilisierung der aktiven Konformation der FLT3-ITDs hinweist. Die Deletion des R595 in FLT3-WT inhibierte das Wachstum der Zellen nach FL-Stimulierung und die STAT5 Aktivierung der Zellen.

In dieser Studie konnten wir weiterhin zeigen, dass die Tyrosinreste 589 und 591 der FLT3-ITDs die Phosphorylierungsstellen sein könnten und entscheidend für die Aktivierung der FLT3-ITDs sind. Die gleichzeitige Substitution dieser beiden Tyrosinreste durch Phenylalanin führte zur vollständigen Inhibition des transformierenden Potentials der FLT3-ITDs und STAT5 Aktivierung. Die Substitution der Tyrosinreste 597 und 599 hatte keine Auswirkung auf das transformierende Potential der FLT3-ITDs und unterstützt damit die Hypothese, dass diese Tyrosinreste möglicherweise nur für die Integrität des FLT3-WT Proteins in der inaktiven Konformation wichtig sind.

Unsere Daten erlauben wichtige Einblicke in die Funktion der juxtamembranösen Domäne im Mechanismus der Transformation durch FLT3-ITDs.

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9 Publications

Sridhar Vempati, Carola Reindl, Seshu Kumar Kaza, Ruth Kern, Theodora Malamoussi, Martin Dugas, Gudrun Mellert, Susanne Schnittger, Wolfgang Hiddemann and Karsten Spiekermann. Arginine 595 duplicated in the majority of patients with AML is critical for the transforming potential of FLT3-length mutations. **Blood. 2007 [Epub ahead of print]**

Sridhar Vempati, Ruth Kern , Ulla Wolf , Konstantin Petropoulos , Naidu Vegi , Christian Buske , Wolfgang Hiddemann , Tobias Kohl and Karsten Spiekermann. Transformation by oncogenic mutants and ligand dependent activation of FLT3-WT requires the tyrosine residues 589 and 591. (Submitted)

Carola Reindl, Sridhar Vempati, H. Quentenemeir, Gudrun Mellert, Hiddemann W and KarstenSpiekermann K. Oncogenic CBL mutants confer a transforming potential to hematopoietic cells expressing the FLT3 tyrosine kinase. (In preparation)

Carola Reindl, Ksenia Bagrintseva, Sridhar Vempati, Susanne Schnittger, Joachim W. Ellwart, Katja Wenig, Karl-Peter Hopfner, Wolfgang Hiddemann, and Karsten Spiekermann. Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML. Blood(2006)107:3700-3707

Kumarasamy Thangaraj, Vempati Sridhar, Toomas Kivisild, Alla G. Reddy, Gyaneshwer Chaubey, Vijay K. Singh, Suminder Kaur, Pooja Agarawal, Amit Rai, Jalaj Gupta, Chandana B.Mallick, Niraj Kumar, Thrimulaisamy P. Velavan, Rajanbabu Suganthan, Divi Udaykumar, Rashmi Kumar, Rachana Mishra, Arif Khan, Chitikineni Annapurna and Lalji Singh: Different population histories of the Mundari- and Mon-Khmer-speaking Austro-Asiatic tribes inferred from the mtDNA 9-bp deletion/insertion polymorphism in Indian populations. Human genetics(2005)116:507-517

Rao Raghavendra V, Thangaraj Kumarasamy, Reddy Alla G, Sridhar V, Singh Lalji: Pairwise MtDNA-HVRII sequence differences and geographic maternal distances among Korku, an Austro-Asiatic tribe in Central India: Indian journal of human genetics(2003) 9:25-28

10 CURRICULUM VITAE

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Minor: Chemistry

PUBLICATIONS

Sridhar Vempati, Carola Reindl, Seshu Kumar Kaza, Ruth Kern, Theodora Malamoussi, Martin Dugas, Gudrun Mellert, Susanne Schnittger, Wolfgang Hiddemann and Karsten Spiekermann. Arginine 595 duplicated in the majority of patients with AML is critical for the transforming potential of FLT3-length mutations. Blood. 2007 [Epub ahead of print]

Sridhar Vempati, Ruth Kern , Ulla Wolf , Konstantin Petropoulos , Naidu Vegi , Christian Buske , Wolfgang Hiddemann , Tobias Kohl and Karsten Spiekermann. Transformation by oncogenic mutants and ligand dependent activation of FLT3-WT requires the tyrosine residues 589 and 591. (Submitted)

Carola Reindl, Sridhar Vempati, H. Quentenemeir, Gudrun Mellert, Hiddemann W and KarstenSpiekermann K. Oncogenic CBL mutants confer a transforming potential to hematopoietic cells expressing the FLT3 tyrosine kinase. (In preparation)

Carola Reindl, Ksenia Bagrintseva, Sridhar Vempati, Susanne Schnittger, Joachim W. Ellwart, Katja Wenig, Karl-Peter Hopfner, Wolfgang Hiddemann, and Karsten Spiekermann. Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML. Blood(2006)107:3700-3707

Kumarasamy Thangaraj, Vempati Sridhar, Toomas Kivisild, Alla G. Reddy, Gyaneshwer Chaubey, Vijay K. Singh, Suminder Kaur, Pooja Agarawal, Amit Rai, Jalaj Gupta, Chandana B.Mallick, Niraj Kumar, Thrimulaisamy P. Velavan, Rajanbabu Suganthan, Divi Udaykumar, Rashmi Kumar, Rachana Mishra, Arif Khan, Chitikineni Annapurna and Lalji Singh: Different population histories of the Mundari- and Mon-Khmer-speaking Austro-Asiatic tribes inferred from the mtDNA 9-bp deletion/insertion polymorphism in Indian populations. Human genetics(2005)116:507-517

Rao Raghavendra V, Thangaraj Kumarasamy, Reddy Alla G, Sridhar V, Singh Lalji: Pairwise MtDNA-HVRII sequence differences and geographic maternal distances among Korku, an Austro-Asiatic tribe in Central India: Indian journal of human genetics(2003) 9:25-28

RESEARCH EXPERIENCE

University Hospital of the Ludwigs Maximillilans University at Grosshadern and the GSF Hematology

Doctoral student: "Role of Juxtamembrane of FLT3 in leukemogenesis'' June 2004-present

In my doctoral thesis I have worked on identifying key residues and interacting proteins of FLT3 in juxtamembrane which are responsible for the transforming potential of FLT3-ITDs. FLT3 has been shown to be the most frequent gentic lesions found in AML. FLT3-ITDs have been shown to give factor independent growth to cell invitro and myeloproliferation invivo. In my work I have been able to find a common motif which is duplicated in majority of AML patients. I have identified a key amino acid which is responsible for increasing the transforming potential of these mutations. Also I have identified some key tyrosine residues which are required for the activity of these mutations. I am in the process of identifying the role of these residues in mice models. In my work I have also used yeast two hybrid system and proteomics to identify the interacting proteins of FLT3 and FLT3-ITD.

Centre for Cellular and Molecular Biology, Hyderabad, India

Research Assistant – "Human Genome Diversity in Indian Populations" November 2001-May 2004

Guru Nanak Dev University, Amritsar, India

Junior research Student: "Production of monoclonal antibody against 24KDa Mycobacterial antigen'' August 2000- May 2001

FELLOWSHIPS AND AWARDS

- DBT Scholarship for M. Sc 1998-2000
- CSIR-UGC Net Scholarship, India 2001
- ICMR Fellowship, India 2002

PRESENTATIONS

DGHO, Leipzig, Germany

Oral presentation – Annual Meeting of the German, Austrian and Swiss Societies for Hematology and Oncology, (DGHO) 2006

Sridhar Vempati, Carola Reindl, Seshu Kumar Kaza, Ruth Kern, Theodora Malamoussi, Martin Dugas, Gudrun Mellert, Susanne Schnittger, Wolfgang Hiddemann and Karsten Spiekermann. Arginine 595 duplicated in the majority of patients with AML is critical for the transforming potential of FLT3-length mutations

Acute Leukemias , Munich, Germany

Poster presentation – Leukemias XI, Leukemia and Lymphoma 2006

Sridhar Vempati, Carola Reindl, Ruth Kern, Theodora Malamoussi, Martin Dugas, Gudrun Mellert, Susanne Schnittger, Wolfgang Hiddemann and Karsten Spiekermann. Duplicated Arginine 595 is critical for the transforming potential of FLT3-length mutations

ASH, San Diego, USA

Poster presentation – 47th Annual Meeting of the American Society of Hematology (ASH) 2005

Sridhar Vempati, Ruth Kern, Theodora Malamoussi, Martin Dugas, Gudrun Mellert, Susanne Schnittger, Wolfgang Hiddemann and Karsten Spiekermann. Duplicated Arginine 595 is critical for the transforming potential of FLT3-length mutations

Functinal Genomics, Hyderabad, India

Poster presentation: Functinal genomics symposium 2002

Thangaraj K, Sridhar V, Singh BJ, Udayakumar, Patel P, Patel M, Bhurani I, Suhasini S, Singh AK, Merline R, Neelima G, Kaur S, Singhal R, Chandra Rao J, Reddy AG, Singh L. "Y-STR analysis of Indian tribes reveals that the expansion of paternal lineage is from South India.

ISHG, Trivandrum, India

Poster presentation: XXVII Annual conference of the Indian society of human genetics 2002

Rao VR, Thangaraj K, Reddy AG, Sridhar V, Singh L. "DNA polymorphisms in Korku and Nihali: a southernmost Austro-Asiatic group in central India.

LANGUAGES

- English – speak fluently and read/write with high proficiency
- Hindi – speak fluently and read/write with high proficiency
- Telugui – speak fluently and read/write with high proficiency
- Bengali -Speak fluently
- Punjabi -working knowledge of the language
- German– working knowledge of the language

Curriculum Vitae

PERSONAL DETAILS

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REFERENCES

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NEOPLASIA

Arginine 595 is duplicated in patients with acute leukemias carrying internal tandem duplications of FLT3 and modulates its transforming potential

Sridhar Vempati,[1,2] Carola Reindl,[1,2] Seshu Kumar Kaza,[1] Ruth Kern,[1,2] Theodora Malamoussi,[1,2] Martin Dugas,[3] Gudrun Mellert,[4] Susanne Schnittger,[5] Wolfgang Hiddemann,[1,2] and Karsten Spiekermann[1,2,4]

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FLT3–internal tandem duplications (FLT3-ITDs) comprise a heterogeneous group of mutations in patients with acute leukemias that are prognostically important. To characterize the mechanism of transformation by FLT3-ITDs, we sequenced the juxtamembrane region (JM) of FLT3 from 284 patients with acute leukemias. The length of FLT3-ITDs varied from 2 to 42 amino acids (AAs) with a median of 17 AAs. The analysis of duplicated AAs showed that in the majority of patients, the duplications localize between AAs 591 to 599 (YVDFREYEY). Arginine 595 (R595) within this region is duplicated in 77% of patients. Single duplication of R595 in FLT3 conferred factor-independent growth to Ba/F3 cells and activated STAT5. Moreover, deletion or substitution of the duplicated R595 in 2 FLT3-ITD constructs as well as the deletion of wild-type R595 in FLT3-ITD substantially reduced the transforming potential and STAT5 activation, pointing to a critical role of the positive charge of R595 in stabilizing the active confirmation of FLT3-ITDs. Deletion of R595 in FLT3-WT nearly abrogated the ligand-dependent activation of FLT3-WT. Our data provide important insights into the molecular mechanism of transformation by FLT3-ITDs and show that duplication of R595 is important for the leukemic potential of FLT3-ITDs. (Blood. 2007;0:000-000)

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Introduction

Mutations in the FMS-like tyrosine-kinase 3 (FLT3) are one of the most frequently found genetic alterations in patients with acute myeloid leukemia (AML),[1-13] myelodysplastic syndromes (MDSs; 10%-15%)[14,15] and acute lymphoblastic leukemia (ALL; 1%-3%).[9,16,17] FLT3 belongs to the class III of receptor tyrosine kinases, which are characterized by the presence of an extracellular immunoglobulin-like domain, a transmembrane and the cytoplasmic juxtamembrane (JM) domain, and the tyrosine kinase domain (TKD).[18] The class III receptors also include KIT, FMS, PDGFRA, and PDGFRB.[19,20]

Activation of FLT3 by FLT3 ligand (FL) leads to receptor oligomerization and transphosphorylation of specific tyrosine residues,[21] which activates the downstream signaling pathways including STAT5, Ras/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/AKT.[22-25] FLT3 is highly expressed in $CD34^+$ hematopoietic progenitor cells and plays an important role in normal hematopoiesis.[26-29] Three distinct activating mutations of FLT3 in hematologic malignancies have been reported: point mutations (FLT3-JM-PM)[30,31] and internal tandem duplications (FLT3-ITD) in the JM domain and mutations in the tyrosine-kinase domain (FLT3-TKD).[1,8,9,12,16,17,32]

The crystal structure of FLT3 has shown that the JM domain acts as an autoinhibitory domain in the inactive state.[33] The JM domain is highly conserved across all members of class III RTKs. Hence many tumors in humans show activating mutations of JM in class III RTKs.[34-37] FLT3-ITDs, found in a majority of acute leukemia patients, are in-frame duplications of a fragment of the JM domain. FLT3-ITDs are highly heterogeneous and vary in length from 2 to 68 AAs. These duplications are thought to disrupt the autoinhibitory mechanism of FLT3 and result in constitutive activation of the catalytic domain of FLT3. Activated FLT3 mutants promote cell proliferation and inhibit apoptosis, leading to factor-independent growth of murine hematopoietic cells in vitro and a myeloproliferative phenotype in vivo.[38]

FLT3-ITDs are present in the leukemic blasts of 20% to 30% of all AML patients. Recent studies have also shown that FLT3-ITDs are found in the leukemic stem cells.[39] Furthermore, the presence of a FLT3-ITD has been recognized as an independent poor prognostic factor in AML and is associated with a decreased survival due to an increased relapse rate.[8,9,11,12,40-43] Several factors influence the poor prognosis seen in AML patients harboring FLT3-ITDs (eg, a high FLT3-ITD/wild-type ratio).[9,44] A recent study has reported that the detection of FLT3-ITD mutation in less mature progenitor populations (eg, $CD34^+/CD33^-$) might be associated with drug resistance.[43]

In the present study, we asked whether any common duplicated motif exists in AML patients carrying FLT3-ITDs, which might be responsible for the transforming potential. To address this question, we sequenced and analyzed the JM region of FLT3 from 284 patient samples with acute leukemias carrying FLT3-ITDs. We found that the length of FLT3-ITDs varied from 2 to 42 amino acids (AAs) with a median of 17 AAs. Duplications were localized in the AA stretch from 591 to 599 (YVDFREYEY) of FLT3. Arginine 595 (R595), within this region, is duplicated in 77% of patients. In vitro studies showed that both wild-type and duplicated R595 are critical for the transforming potential of the FLT3-ITDs. These data

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provide important insight into the molecular mechanisms of malignant transformation by FLT3-ITDs.

Patients, materials, and methods

Patients and samples. Two hundred and eighty four patients diagnosed with acute leukemias and carrying FLT3-ITD listed in the patient data bank of the Laboratory for Leukemia Diagnostics, University Clinic of Grosshadern, Munich were used for this study. All patients gave informed consent before entering the study. The study design adhered to the principles of the Declaration of Helsinki and was approved by the ethics committees of the participating institutions. Clinical and laboratory data of patients analyzed in this study are given in Table 1. Ten patients included in this study had no clinical data.

Reagents and cell lines. Low passage murine Ba/F3 cells were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 10% WEHI conditioned medium as a source of murine IL-3 when indicated. PKC412 was kindly provided by Novartis Pharma (Basel, Switzerland).

Cell proliferation of Ba/F3 cells. Cells were seeded at a density of 4×10^4/mL for short-term proliferation and in the presence or absence of IL-3 and inhibitor as indicated. Viable cells were counted at the indicated time points in a standard hemacytometer after staining with trypan blue. Figures show mean values and standard deviations from 3 independent experiments unless otherwise indicated.

Antibodies. The following antibodies were used: anti-FLT3 antibody (Sc-480; Santa Cruz, Heidelberg, Germany), anti–phospho-STAT5-Tyr694 (New England Biolabs, Frankfurt, Germany), and anti-STAT5 (sc-835; Santa Cruz).

DNA constructs and vectors. The FLT3-ITD-W51 construct contains a 7–amino acid– duplicated sequence (REYEYDL) inserted between AAs 601/602 of human FLT3-WT, and the FLT3-ITD-NPOS contains a 28-AA–duplicated sequence (CSSDNEYFYDFREYEYDLKWEFPRENL) inserted between AAs 611/612 of FLT3-WT. All FLT3 constructs were subcloned in the MSCV-IRES-EYFP/EYFP retroviral expression vector (kindly provided by R. K. Humphries, The Terry Fox Laboratory, Vancouver, University of British Columbia).

In vitro mutagenesis. The mutants FLT3-WT-ins595R, FLT3-WT-ins597EY, FLT3-WT-ins596RE, FLT3-WT-ins597REY, FLT3-WT-ins591YFY, FLT3-WT-ins602KWE, and FLT3-WT-Δ595 in the FLT3-WT; FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51-ΔR602, FLT3-W51-ΔE603, and FLT3-W51-ΔR595 in the FLT3-ITD-W51; and FLT3-NPOS-ΔR623 in the FLT3-ITD-NPOS were created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The correct sequence of all constructs was confirmed by nucleotide sequencing.

Transient transfection of 293 cells and stable transduction of Ba/F3 cells were performed as previously described.[45]

Western blot analysis was performed as previously described.[45]

Statistical analysis. Analysis of duplicated amino acids was performed by PERL-programs (Version 5; http://www.perl.com). Sequences were parsed with regular expressions. Sequence patterns were analyzed as frequency tables of individual amino acids by position as well as frequency tables of subsequences by position. All subsequences of length 1 to 30 were extracted from the data set and sorted by frequency.

Table 1. Clinical and laboratory data of the patients analyzed in this study

	N
No. of patients	284
Age	
Range	18-89
Median	60.5
Sex	
Female	163
Male	121
FAB class	
AUL	2
Biphenotypic	3
ALL	4
AML M0	6
AML M1	63
AML M2	49
AML M3	28
AML M4	39
AML M5	21
AML M6	4
MDS	3
AML with unknown FAB	52
Patients without clinical data	10
WBC count	
Median	50000
Range	12000-67500
Cytogenetic abnormality	
Favorable	29
Intermediate	185
Adverse	11
Unknown	49

AUL indicates acute undifferentiated leukemia, AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; and WBC, white blood cell.

Favorable, intermediate, and unfavorable cytogenetic abnormality has been defined according to published data.[65]

Results

Internal tandem duplications are located in the common motif YVDFREYEY and include R595 in 77% of patients

The most common form of activating FLT3 mutation is an internal tandem duplication, which occurs in 20% to 25% of patients with AML[1,3,4] and 5% to 10% of patients with myelodysplastic syndromes (MDSs).[1,3,4] Since these duplications are of variable length and location, we aimed to identify a common duplicated motif in the FLT3-JM region.

The cDNA of 284 unselected patient samples carrying FLT3-ITDs was analyzed. Nucleotide sequencing of the JM region showed that 118 patients expressed pure tandem duplications, whereas 166 patients carried additional insertions always maintaining the reading frame (data not shown). The length of duplications varied from 2 AAs to 42 AAs, with the median length being 17 AAs. In 95% of the patients, at least one AA within the stretch Y591 to Y599 (YVDFREYEY) was duplicated (data not shown). Analysis of the frequency of single AAs in the duplicated region revealed that arginine 595 was the most frequently duplicated single AA in 77%, followed by Y597 in 74%, and F594 and E596 in 73% of all patients (Figure 1).

Next, we analyzed the frequency of AA combinations, ranging in length from 1 to 30 AAs within the duplicated region. As shown in Figure 2A, the single R595 is subsequently followed by the combination of amino acids EY (AA596-597) and REY (AA595-597) in 70% of all patients.

These findings point to a commonly duplicated motif that centers around R595 within the Y-rich stretch from AAs 591 to 599 (YVDFREYEY). We hypothesized that this region might play an important role for the transforming activity of FLT3-ITDs. To confirm this hypothesis, we analyzed the patients that carried the shortest ITDs. Figure 2B shows the duplicated sequences of 25

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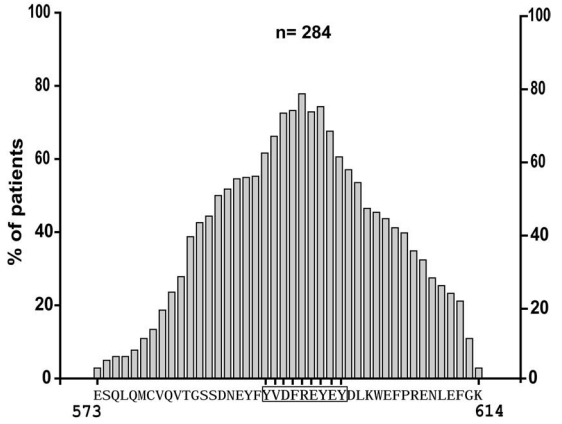

Figure 1. Analysis of the AA composition in the duplicated region in FLT3-ITDs. Frequency of single AAs by position in the duplicated region is provided. For each position, the most frequent single AA was selected.

patients ranging in length from 2 to 8 AAs. The patient with the shortest duplication (2 AAs) showed insertion of R595 and E596. All but 4 patients (21/25 = 84%) had duplications of R595. Moreover, all patients had duplications of at least one amino acid of the protein stretch REYEY (AAs 595 to 599).

Taken together, our data show that a common duplicated AA stretch can be defined in patients with FLT3-ITDs. This region includes R595, which is duplicated in 77% of all patients and supports the hypothesis that this residue might play an important role for the transforming potential of FLT3-ITDs.

Insertion of a single arginine between AAs 595 and 596 in FLT3-WT confers IL-3–independent growth to Ba/F3 cells along with activation of STAT5

The acquisition of FLT3-ITD mutations in the FLT3 gene was shown to induce constitutive activation of the receptor and ligand-independent cell growth in different cell lines.[5,16,32,46] To validate our hypothesis that duplication of R595 plays an important role for the transforming activity of the receptor, we introduced an arginine in the FLT3-WT cDNA between positions 595 and 596 (FLT3-ins595R). The second and third most frequently duplicated AA combinations E596/Y597 (FLT3-ins597EY) and R595/E596/Y597 (FLT3-ins597REY), and also the shortest duplication found in patients (ie, AA combination R595/E596 [FLT3-ins597RE]) were generated by in vitro mutagenesis (Figure 3A). Furthermore, we analyzed if tandem duplication of AAs outside the region of AA592-599 can confer factor-independent growth to cells. Hence, we generated 2 different hypothetical ITDs that have duplications outside the AAs 592-595 stretch (FLT3-WT-ins591YFY and FLT3-WT-ins602KWE in FLT3-WT) using in vitro mutagenesis (Figure 3A). F3 AQ: 14

We stably transduced the pro-B-cell line Ba/F3 with the FLT3-WT-ins595R/ins596RE/ins597EY/ins597REY/ins591YFY/ins602KWE constructs. In addition, Ba/F3 cells expressing FLT3-WT and FLT3-ITD-W51 were generated. Expression of all FLT3 constructs was confirmed by CD135 antibody staining and fluorescence-activated cell sorting (FACS) analysis as well as Western blotting (data not shown). AQ: 15

Overexpression of the mutant FLT3-ins595R, but not FLT3-WT, induced IL-3–independent growth in Ba/F3 cells (Figure 3B). In detail, the growth rate of FLT3-ins595R was 40% compared to cells expressing the FLT3-ITD-W51 construct, which served as a positive control. Similar results were obtained for cells expressing FLT3-ins597RE (50.7%) and FLT3-ins597REY (58.9%), but with slightly increased growth rate. When expressed in Ba/F3 cells, the FLT3-insEY construct induced a 15% growth rate compared to FLT3-ITD-W51 (Figure 3B). The cells expressing the constructs FLT3-WT-ins591YFY and FLT3-WT-ins602KWE also showed factor-independent growth of 28% and 24%, respectively, when compared to FLT3-ITD-W51 (Figure 3B), but again the transforming potential was weaker when compared to the cells expressing FLT3-ins595R, FLT3-ins596RE, and FLT3-ins597REY. To analyze whether the autonomous growth of these mutants might be further stimulated by exogenous ligand, all these mutant cell lines were grown in the presence of 60 ng FLT3 ligand (FL)/mL, and viable cells were counted after 72 hours. All mutants showed a growth rate which was 1.3 to 3 times higher, than that of FLT3-WT cells grown under identical conditions (Figure 3B). These data clearly indicate that the duplication of R595 is sufficient to activate the transforming potential of FLT3. Introduction of an ITD outside the AA stretch 591 to 595 resulted in weaker transforming potential compared to constructs carrying R595 duplication AQ: 26

STAT5 is the downstream target of the constitutively activated FLT3 receptor probably responsible for most of the transforming potential of the FLT3 receptor in vitro and in vivo.[46-48] To investigate the activation of the STAT5 signaling pathway, we prepared crude cell lysates of serum-starved Ba/F3 cells transduced with either vector control (MIY) or FLT3WT, FLT3-ITD-W51, AQ: 16

A

No: of AA	570 580 590 600 610	% of patients
	FRYESQLQMV QVTGSSDNEY FYVDFREYEY DLKWEFPREN LEFGKVLGSG	
1	R	77
2	EY	70
3	REY	70
4	FREY	63
5	DFREY	60
6	VDFREY	52
7	EY FYVDF	48
8	EY FYVDFR	46
9	DNEY FYVDF	44
10	DNEY FYVDFR	42
11	SDNEY FYVDFR	38
12	SSDNEY FYVDFR	36
13	SSDNEY FYVDFRE	33
14	SSDNEY FYVDFREY	32
15	GSSDNEY FYVDFREY	28
16	GSSDNEY FYVDFREYE	25
17	SSDNEY FYVDFREYEY D	19
18	NEY FYVDFREYEY DLKWE	16
19	EY FYVDFREYEY DLKWEFP	16
20	NEY FYVDFREYEY DLKWEFP	15
21	DNEY FYVDFREYEY DLKWEFP	14
22	SDNEY FYVDFREYEY DLKWEFP	13
23	SSDNEY FYVDFREYEY DLKWEFP	11
24	SDNEY FYVDFREYEY DLKWEFPRE	10
25	SSDNEY FYVDFREYEY DLKWEFPRE	9
26	GSSDNEY FYVDFREYEY DLKWEFPRE	8
27	SSDNEY FYVDFREYEY DLKWEFPREN L	6
28	GSSDNEY FYVDFREYEY DLKWEFPREN L	5
29	GSSDNEY FYVDFREYEY DLKWEFPREN LE	3
30	VTGSSDNEY FYVDFREYEY DLKWEFPREN L	3

B

580 590 600	no of AA
QVTGSSDNEY FYVDFREYEY DLKWEFPREN	
RE	2
FREYE	5
REYEY D	6
DYVDFR	6
GDFREY	7
HVDFREY	7
YEY DLKW	7
REYEY DL	7
DVDFREY	7
FDFREYE	7
VDFREYE	7
REYEY DL	7
REYEY DL	7
DVDFREY	7
REYEY DL	7
FREYEY D	7
YEY DLKW	7
Y DLKWEFP	8
FREYEY DL	8
EY DLKWEF	8
YVDFREYE	8
DYVDFREY	8
NREYEY DL	8
XYVDFREY	8
DYVDFREY	8

Figure 2. Duplications locate in the motif YVDFREYEY and include R595 in 77% of patients. (A) Most frequent AA combinations within the duplicated region, sorted by length from 1 to 30 AAs. (B) Panel showing the duplicated sequences of 25 patients ranging from 2 to 8 AAs.

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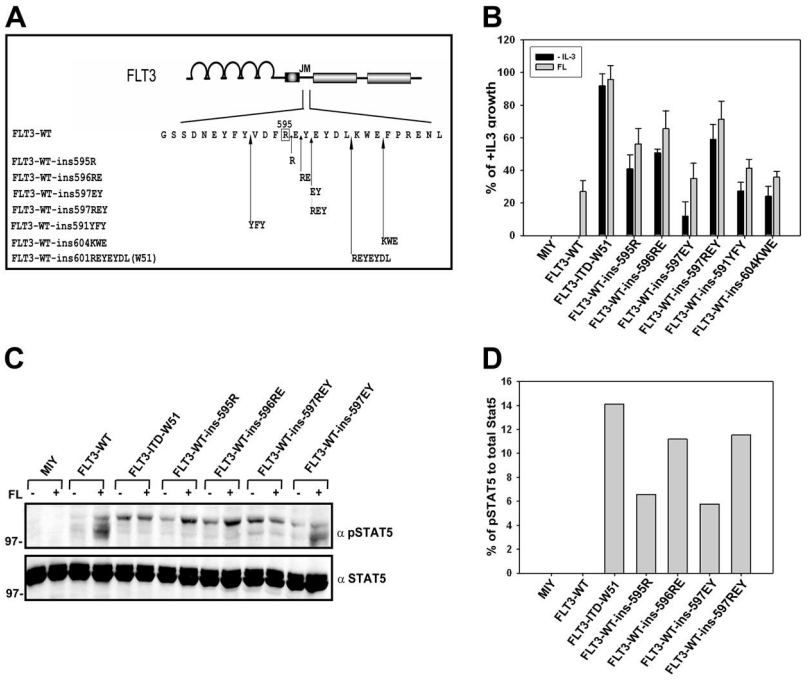

Figure 3. Duplication of single arginine 595 in FLT3 induces IL-3–independent growth in Ba/F3 cells. (A) Localization of 6 insertion mutants of FLT3 generated by duplication of 1 to 3 AAs of the stretch between 3 AA regions: 595 to 597, 589 to 591, and 602 to 604. (B) Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-ins595R, FLT3-ins596RE, FLT3-ins597EY, FLT3-ins597REY, FLT3-WT-ins591YFY, FLT3-WT-ins602KWE, or mock-transduced cells were seeded at a density of 4×10^4 cells/mL in the absence or presence of IL-3 or FL (60 ng/mL). Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from 3 independent experiments is shown. (C) Western blot showing the autoactivation of STAT5 in the mutants FLT3-ins595R, FLT3-ins597EY, FLT3-ins597REY, and FLT3-ITD-W51 when compared to FLT3-WT in the unstimulated cells. FLT3-WT-ins595R, FLT3-WT-ins597EY, FLT3-WT-ins597REY, FLT3-WT-ins596RE, FLT3-ITD-W51, FLT3-WT, or mock-transduced cell lines were starved for 24 hours in the presence of 0.3% FBS and stimulated with 60 ng FL/mL for 5 minutes. Crude cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on a nitrocellulose membrane. Blots were then incubated with anti–phospho-STAT5 antibody, stripped, and reblotted with anti-STAT5 antibody. (D) Densitometric analysis of the Western image in panel C was used to quantify the ratio of phospho-STAT5 to total STAT5.

AQ: 24

FLT3-ins595R, FLT3-ins597EY, FLT3-ins597RE, and FLT3-ins597REY. Lysates were analyzed by immunoblotting with a specific antibody against phospho-STAT5. We could clearly demonstrate that the expression of FLT3-ins595R, FLT3-ins597EY, FLT3-ins597RE, and FLT3-ins597REY led to increased phosphorylation of tyrosine at position 694 of STAT5 compared to FLT3-WT in accordance with the proliferation data; the FLT3-ins597EY mutant showed a significantly weaker STAT5 activation (Figure 3C-D)

AQ: 17

Deletions or substitutions of arginine 595 with alanine or glutamic acid in the duplicated region of FLT3-ITD reduce the proliferation rate of FLT3-ITD–transformed cells

Having shown that duplication of R595 can activate the transforming activity of FLT3, we asked whether R595 is necessary for the oncogenic potential of FLT3-ITD mutants.

For this purpose, we substituted the positively charged duplicated AA arginine (R602) with the neutral AA alanine (FLT3-W51-R602A) and also with the negatively charged AA glutamic acid (FLT3-W51-R602E) (Figure 4A). Furthermore, we generated a deletion mutant of duplicated R595 (FLT3-W51ΔR602) (Figure 4A) and the deletion of neighboring AA glutamic acid (FLT3-W51ΔE603) (Figure 4A). Ba/F3 cells were stably transduced with these different FLT3-ITD-W51 mutants and with FLT3-WT and FLT3-ITD-W51 as controls.

F4

In proliferation assays, FLT3-W51ΔR602–, FLT3-W51-R602E–, and FLT3-W51-R602A–expressing cells showed a growth reduction of approximately 55% to 70% when compared to FLT3-W51 cells (Figure 4B), with the deletion mutant showing the maximum growth reduction of 70%. Next, we generated a deletion mutant of duplicated R595 (FLT3-NPOS-Δ623R) in a structurally different FLT3-ITD construct, FLT3-ITD-NPOS, that contains a 28-AA–duplicated sequence (CSSDNEYFYDFREYEYDLKWEFPRENL) inserted between AAs 611/612 of FLT3-WT (Figure 4A). Expression of the deletion mutant (FLT3-NPOS-Δ623R) showed a similar phenotype compared to FLT3-W51ΔR602 with a reduction of approximately 70% of its proliferative capacity when compared to the FLT3-ITD-NPOS construct (Figure 4C). Deletion mutant of neighboring AA (FLT3-W51ΔE603) did not result in a significant growth reduction (Figure 4B).

AQ: 18

Substitution of duplicated R595 with lysine did not alter the proliferation rate of the FLT3-ITD mutants

Having shown that the positively charged arginine plays an important role in the transforming potential of FLT3-ITDs, we asked if the duplicated arginine can be replaced with another positively charged amino acid. Hence, we substituted the duplicated arginine with the positively charged AA lysine (FLT3-W51-R602K) (Figure 4A). Overexpression of FLT3-W51-R602K in Ba/F3 cells induced factor-independent growth and did not show any reduction of the transforming potential when compared to FLT3-ITD-W51 (Figure 4D). These data clearly show that the positive charge of the AA (arginine or lysine) plays a crucial role in the transforming potential of FLT3-ITDs.

FLT3-ITD–duplicated R595 substitution/deletion mutants show a reduced capacity to activate STAT5 compared to FLT3-ITDs

To investigate the activation of the STAT5 signaling pathway, we prepared crude cell lysates of serum-starved Ba/F3 cells, transduced with either vector control (MIY) or FLT3-WT, FLT3-ITD-W51, FLT3-ITD-NPOS, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51-ΔR602, FLT3-W51-ΔE603, and FLT3-NPOS-ΔR623. All the mutants FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-ΔR602, and FLT3-NPOSΔ623R in Ba/F3 cells reduced the STAT5 activation compared to nonmanipulated FLT3-ITD constructs (Figure 5). STAT5 activation of FLT3-W51-R602K was similar to the FLT3-ITDs. Thus, the activation of the most important signaling pathway downstream of FLT3, STAT5, showed reduced phosphorylation in FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51Δ602R, and FLT3-NPOSΔ623R, but not in FLT3-W51-R602K–expressing cells, which correlates with IL-3–independent growth.

F5

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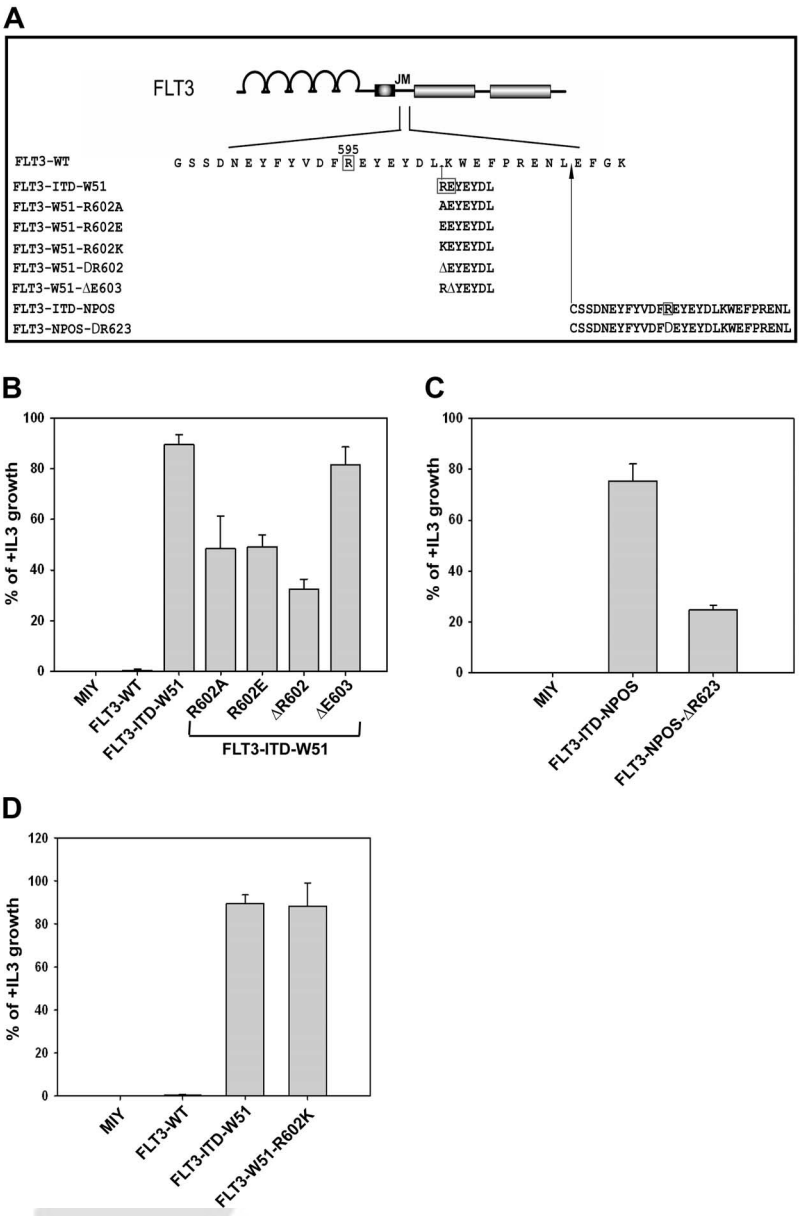

Figure 4. The positive charge of duplicated arginine 595 is critical for transforming potential of FLT3-ITDs. (A) Shows 3 different substitution and 2 deletion mutants of duplicated R595 generated in 2 different FLT3-ITDs (W51 and NPOS). The deletion mutant of duplicated E596 in FLT3-ITD-W51 is also shown. (B) Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD-W51, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-ΔR602, FLT3-W51-ΔE603, or mock-transduced cells were seeded at a density of 4×10^4 cells/mL in the absence or presence of IL-3. Viable cells were counted after 72 hours. The growth of cells with IL-3 was defined as 100% (control). Standard error of the mean calculated from 3 independent experiments is indicated. (C-D) The mutants FLT3-NPOSΔR623 and FLT3-W51-R602K were expressed in Ba/F3 cells and analyzed as described in panel B.

Deletion of wild-type R595 reduces the transforming potential and STAT5 activation of FLT3-ITD mutants

Since the duplicated arginine plays an important role in the transforming potential of the FLT3-ITDs, we asked whether wild-type R595 also has a critical role in the transforming capacity of FLT3-ITDs. We therefore created a deletion mutant of R595 in FLT3-ITD-W51 (FLT3-W51-ΔR595) (Figure 6A). F6

Overexpression of the wild-type arginine deletion mutant FLT3-W51-ΔR595 reduced the IL-3–independent growth by 64% when compared to FLT3-ITD-W51 (Figure 6B). These proliferation data were supported by the measurement of STAT5 activation (Figure 6C). STAT5 activation was significantly lower in the cells expressing FLT3-W51-ΔR595, when compared to FLT3-ITD-W51. These data suggest that not only the duplicated R595 but also the wild-type R595 plays an important role in the transforming potential and STAT5 activation of FLT3-ITD-W51.

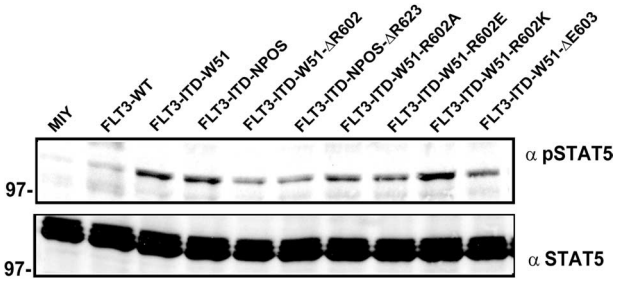

Figure 5. The constitutive STAT5 activation is reduced in substitution/deletion mutants of duplicated R595. FLT3-WT–, FLT3-ITD-W51–, FLT3-ITD-NPOS–, FLT3-W51-R602A–, FLT3-W51-R602E–, FLT3-W51-R602K–, FLT3-W51-ΔR602–, FLT3-W51-ΔE603–, and FLT3-NPOS-ΔR623–expressing cells were starved for 24 hours in the presence of 0.3% FBS. Blots were incubated with anti–phospho-STAT5 antibody, stripped, and reblotted with anti-STAT5 antibody.

Deletion of R595 in FLT3-WT nearly abrogates the growth of cells upon FL stimulation

As wild-type R595 had a significant effect on the transforming properties of FLT3-ITDs, we analyzed the role of the wild-type arginine 595 in the signaling properties of the FLT3-WT receptor.

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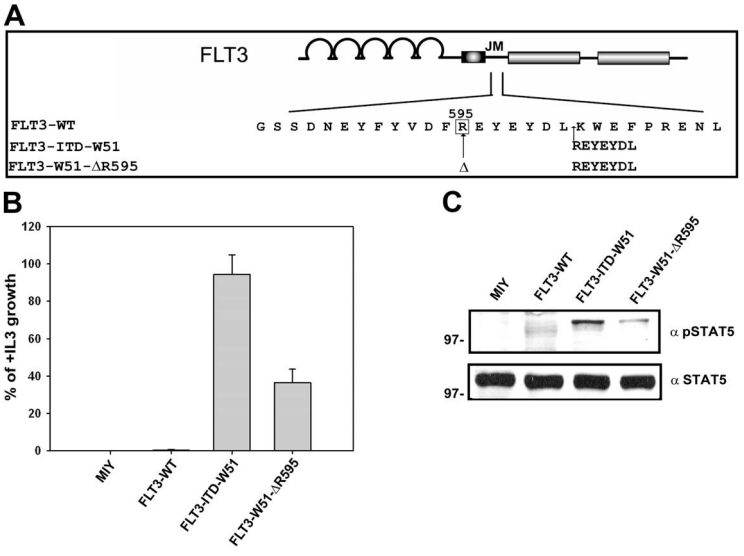

Figure 6. Deletion of wild-type R595 in FLT3-ITD-W51 results in reduced transforming potential and STAT5 activation in Ba/F3 cells. (A) Schematic representation of wild-type R595 (FLT3-W51-ΔR595) deletion mutant in the FLT3-ITD-W51 construct. (B) Ba/F3 cells expressing the FLT3-WT, FLT3-W51-ΔR595, FLT3-ITD-W51, and mock-transduced cells were seeded at a density of 4×10^4 cells/mL in the absence or presence of IL-3. Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). The standard error of the mean calculated from 3 independent experiments is indicated. (C) Western blot image showing the activation of STAT5 in cells expressing FLT3-W51-ΔR595 and FLT3-ITD-W51, when compared to FLT3-WT or mock-transduced cells. Cells were starved in the presence of 0.3% FBS for 24 hours. Blots were incubated with anti–phospho-STAT5 antibody, stripped, and reblotted with anti-STAT5 antibody.

Hence, we created a deletion mutant of R595 in FLT3-WT (FLT3-WT-ΔR595) (Figure 7A) and expressed it in Ba/F3 cells. F7

Overexpression of FLT3-WT-ΔR595 did not show any IL-3–independent growth, but cell proliferation was almost abrogated in the presence of FL, when compared to FLT3-WT (Figure 7B). In detail there was a reduction of approximately 90% in the proliferation of cells expressing FLT3-WT-ΔR595 after stimulation with FL, when compared to cells expressing FLT3-WT (taken as 100%)

To check the signaling pathway of STAT5, we prepared crude cell lysates of serum-starved Ba/F3 cells transduced with MIG, FLT3-WT, and FLT3-WT-ΔR595 and FLT3-ITD-W51 acting as positive control. Lysates were analyzed by immunoblotting with a specific antibody against phospho-STAT5. We detected very slight STAT5 activation in the FLT3-WT-ΔR595 in the presence of FL, when compared to FLT3-WT–expressing cells (Figure 7C).

The arginine substitution/deletion mutants of FLT3-ITDs are sensitive to the FLT3 PTK inhibitor PKC12

AQ: 19 PKC412 (Novartis Pharma), an inhibitor initially discovered as an inhibitor of protein kinase C, was found to block the phosphorylation and activity of FLT3-WT and mutant FLT3 receptors.[49,50] The inhibitory activity of PKC412 against the arginine mutants of FLT3-ITDs was analyzed by treating the FLT3-W51-R602A–, FLT3-W51-R602E–, FLT3-W51Δ602R–, and FLT3-NPOSΔ623R–expressing cells with different concentrations of PKC412 ranging from 1 to 100 nM. PKC412 showed a strong growth inhibitory effect on FLT3-W51R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51Δ602R, and FLT3-NPOSΔ623R receptors expressing cells in the absence but not in the presence of IL-3. The IC_{50} of PKC412 was significantly lower in arginine substitution/deletion mutants (0.5-1 nM) compared to FLT3-ITD mutants (4 nM) (Table 2). T2

Discussion

FLT3-ITDs are found in 20% to 25% of AML patients and are associated with an adverse prognosis. Recently, 2 different studies have reported conflicting results, stating the importance of the length of the duplication for the prognostic significance of the

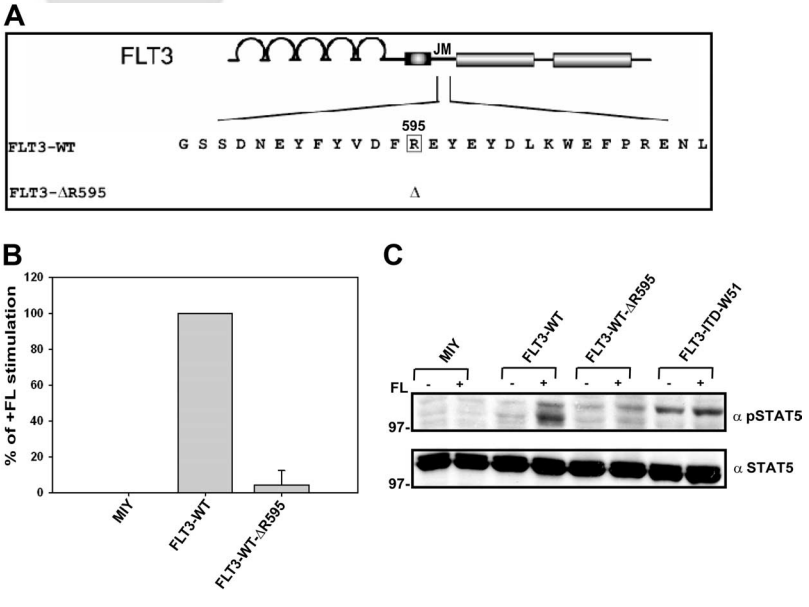

Figure 7. Deletion of wild-type R595 in FLT3-WT abrogates the ligand-dependent activation of FLT3-WT in Ba/F3 cells. (A) Schematic representation of wild-type R595 (FLT3-WT-ΔR595) deletion mutant in the FLT3-WT construct. (B) Ba/F3 cells expressing the FLT3-WT, FLT3-WT-ΔR595, FLT3-ITD-W51, and mock-transduced cells were seeded at a density of 4×10^4 cells/mL in the absence or presence of IL-3 and FL (60 ng/ml). Viable cells were counted after 72 hours by trypan blue exclusion. The growth of cells with IL-3 was defined as 100% (control). The standard error of the mean calculated from 3 independent experiments is indicated. (C) Western blot image showing the abrogation of activation of STAT5 in cells expressing FLT3-WT-ΔR595, when compared to FLT3-ITD-W51 and FLT3-WT, upon stimulation of FL (60 ng/mL). Cells were starved in the presence of 0.3% FBS for 24 hours. Blots were incubated with anti–phospho-STAT5 antibody, stripped, and reblotted with anti-STAT5 antibody. AQ: 25

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Table 2. IC_{50} of PKC412 values in different FLT3-ITD arginine mutants

Mutant	IC_{50}, nM
FLT3-ITD(W51/NPOS)	4.0
FLT3-W51-R602A	0.9
FLT3-W51-R602E	0.8
FLT3-W51-R602K	4.0
FLT3-W51-ΔR602	0.6
FLT3-NPOS-ΔR623	0.7

Ba/F3 cells expressing FLT3-WT, FLT3-ITD-W51, FLT3-ITD-NPOS, FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-R602K, FLT3-W51-ΔR602, and FLT3-NPOS-ΔR623 seeded at a density of 4×10^4 cells/mL in the absence or presence of different concentrations of PKC412 (0 to 100 nM) and counted after 72 hours. All cell lines were also cultured in the presence of IL-3 and 100 nM PKC412 to confirm nontoxicity of PKC412 to Ba/F3 cells. The IC_{50} was calculated from 3 independent experiments (IC_{50} is defined as the concentration of inhibitor required to induce a growth reduction of 50% compared to the cells grown in the absence of inhibitor).

mutation. One study showed that longer duplications are associated with an unfavorable prognosis in patients with AML, whereas another study found the opposite.[51,52] Given the substantial structural heterogeneity of FLT3-ITD mutations, no common motif has been identified so far that is duplicated in all patients. We sequenced the JM region of FLT3 in 284 patients with acute leukemias carrying internal tandem duplications in order to look for a common signature in these ITDs.

Our results confirm previous data showing that internal tandem duplications are heterogeneous with respect to length. The analysis of single residues within the duplicated region showed that R595 was the single most frequently duplicated AA in 77% of the patients, followed by Y597 in 73%, and F594 and E596 in 71% of all patients. A detailed statistical analysis of AA combinations from 1 to 30 AAs in the duplicated regions showed that, although there is no central motif common in all patients, the site of insertion (mutational hotspot) is frequently located in or around the Y-rich stretch region from AAs 591 to 599 (YVDFREYEY) of FLT3. R595 is duplicated in 77% of the patients followed by a combination of EY (E596 and Y597) in 70% of patients. In vitro studies showed that duplication of R595 in FLT3 is able to confer factor-independent growth to Ba/F3 cells and led to activation of STAT5. Substitution of the duplicated R595 with alanine (R602A) or glutamic acid (R602E) in a representative FLT3-ITD-W51 construct showed a significant reduction in the transforming potential of FLT3-ITD-W51. In contrast, substitution of the duplicated R595 with the positively charged AA (R602K) did not alter the transforming potential of cells compared to FLT3-ITD-W51 construct.

Statistical analysis of our data showed that R595 is the most frequently duplicated single AA. Moreover, the analysis of patients with the shortest duplications showed that R595 was duplicated in 84% of these patients. Of interest, the shortest duplication found in our cohort was R595/E596 duplication. This prompted us to check if a single R595 duplication was sufficient to confer factor-independent growth to Ba/F3 cells. In vitro, duplication of a single AA, R595 (FLT3-ins595R), was sufficient to activate the transforming potential of FLT3. The weak transforming phenotype of FLT3-ins595R could be attributed to a slight disturbance in the autoinhibitory conformation of the JM region caused by a single amino acid insertion. Similar results were reported by our group on point mutations in the JM region found in AML patients that have a weak transforming potential, when compared to FLT3-ITDs.[31] Addition of AAs next to arginine (FLT3-ins596RE, FLT3-ins597REY) increased the transforming potential but only slightly when compared to FLT3-ins595R. Moreover, FLT3-ITD mutant lacking the R595 duplication (ie, FLT3-ins595EY or FLT3-WT-ins591YFY/FLT3-WT-ins602KWE, which carry duplications outside AAs 592-599 stretch) showed a weaker transforming potential when compared to FLT3-ins595R (40.9%), FLT3-ins596RE (50.7%), FLT3-ins597REY (58.9%), and FLT3-ITD-W51 (92%). These data clearly show that the duplicated R595 plays an important role for the higher transforming potential of FLT3-ITDs. Ba/F3 cells expressing FLT3-ins595R, FLT3-ins597EY, FLT3-ins596RE, FLT3-ins597REY, FLT3-WT-ins591YFY, and FLT3-WT-ins602KWE showed hyperresponsiveness to FLT3 ligand, with a 1.3 to 3 times higher proliferation rate compared to FLT3-WT cells. This experimental setting probably reflects the in vivo situation, as it has been found that FL is coexpressed in AML blasts.[54]

FLT3-ITDs mutants are strong activators of STAT5,[47,55] and STAT5 has been shown to be activated in blasts of 20% to 80% of patients with AML.[56-59] Previous studies have also shown that STAT5 regulates the expression of early cytokine genes, such as PIM-1,[60] which are responsible for cell survival and growth, and leads to tumor development and progression.[61] In accordance with the proliferation data, all the mutants FLT3-ins595R, FLT3-ins596RE, FLT3-ins597REY, and FLT3-ins597EY showed activation of STAT5, but to a lesser degree compared to the FLT3-ITD-W51 mutant.

Determination of the crystal structure of FLT3-WT has indicated that the JM can be divided into 3 parts.[33] The JM binding motif (JM-B) acts as the autoinhibitory domain, by preventing the rotation of the N lobe toward the C lobe of the tyrosine kinase domain (TKD) to generate the activated kinase fold, whereas the JM switch motif (JM-S) that lies next to JM-B provides a rigid and properly oriented scaffold for the interposition of tyrosines 589 and 591 between the JM-S and the C lobe of the kinase. FLT3-ITDs are frequently found in the JM zipper region (JM-Z) that aligns and maintains the JM-S in the proper orientation during and after the transition between activated and inactive states of FLT3. It is hard to predict the mechanisms by which these FLT3-ITDs change the conformation of the FLT3 protein, but it is hypothesized that the duplications offset the position of the JM-S in the FLT3 structure. This results in disturbing or preventing the optimal orientation of JM-S as it tries to position the JM-B in its binding site. Since FLT3-ins597EY showed a transforming potential 2 to 4 times lower than FLT3-ins595R, FLT3-ins596RE, and FLT3-ins597REY, we hypothesized that the positive charge of R595 might be involved in forming crucial interactions with other AAs. These new interactions might not only disrupt the autoinhibitory loop formed by JM-B, but also promote higher tyrosine phosphorylation by rendering the JM domain more accessible for autophosphorylation.

To analyze whether the positive charge of the duplicated R595 has any role for the oncogenic potential of FLT3-ITDs, we generated substitution mutants of the duplicated R595 with an aliphatic AA, alanine (FLT3-W51-R602A), and a negatively charged AA, glutamic acid (FLT3-W51-R602E), as well as a deletion mutant of the duplicated R595 (FLT3-W51-ΔR602) in the strongly transforming FLT3-ITD-W51. All mutants showed factor-independent growth in Ba/F3 cells, but the transforming potential of these mutants was reduced by 55% to 70% compared to FLT3-ITD-W51. The same degree of reduction was seen after deletion of the duplicated R595 in a different FLT3-ITD (FLT3-NPOS-ΔR623) construct. Of interest, there was no effect on the transforming potential when the duplicated R595 was replaced by another positively charged AA (eg, lysine; FLT3-W51-R602K). Deletion of the AA adjacent to arginine in FLT3-ITD-W51 (FLT3-W51ΔE603)

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did not significantly reduce the transforming potential of FLT3-ITD-W51, confirming that the positive charge of the duplicated R595 plays an important role in transforming potential of FLT3-ITDs. This hypothesis is further supported by the finding that deletion of WT-R595 in FLT3-ITD-W51 (FLT3-W51ΔR595) reduces the growth rate of Ba/F3 cells by 64% compared to the FLT3-ITD-W51 construct. All deletion and substitution mutants of wild-type and duplicated R595 in FLT3-ITDs (W51 and NPOS) showed a reduction in the activation of STAT5 except for the FLT3-W51-R602K, suggesting that the positive charge of wild-type and duplicated R595 has an essential role in the activation of STAT5.

As the positive charge of both duplicated and wild-type R595 was critical on the transforming potential of FLT3-ITDs, we wanted to check for the effect of R595 in the ligand-dependent activation of FLT3-WT. Deletion of R595 in FLT3-WT (FLT3-WT-ΔR595) nearly abrogated the growth of cells expressing FLT3-WT-ΔR595 upon stimulation with FL when compared to cells expressing FLT3-WT. This was further confirmed by the STAT5 signaling where the cells expressing the FLT3-WT-ΔR595 showed very weak activation of STAT5 when compared to FLT3-WT. These data clearly show that R595 regulates the mitogenic activity of FLT3-WT and its constitutively activated receptor mutants.

The importance of FLT3 for the survival and proliferation of AML blasts, and its mutation and overexpression in a large cohort of AML patients, has led to development of selective FLT3 protein tyrosine kinase inhibitors such as PKC412 and CEP-701. These inhibitors block the FLT3 kinase activity, thereby inducing apoptosis in FLT3-expressing cell lines, and cause cytotoxicity in primary ALL and AML blasts.[49,62-64] PKC412, which was originally developed as an inhibitor of PKC, has also been found to inhibit FLT3 phosphorylation in vitro and in vivo.[49,50,62] We tested the effect of PKC412 on the described substitution and deletion mutants of duplicated R595 in FLT3-ITD compared to nonmanipulated FLT3-ITD constructs as a control. The IC_{50} of PKC412 in FLT3-W51-R602A–, FLT3-W51-R602E–, FLT3-W51-ΔR602–, and FLT3-NPOS-ΔR623–expressing Ba/F3 cells was approximately 5 times lower in Ba/F3 cells expressing nonmanipulated FLT3-ITDs. In contrast, there was no difference in the IC_{50} of PKC412 for FLT3-W51-R602K–expressing Ba/F3 cells compared to FLT3-ITD-W51 cells. The high sensitivity of FLT3-W51-R602A, FLT3-W51-R602E, FLT3-W51-ΔR602, and FLT3-NPOS-ΔR623 toward PKC412 might be due to their weak transforming potential as seen in our study for point mutants in the JM region of FLT3.[31]

Our results clearly show that R595 is duplicated in 77% of the patients with FLT3-ITDs and plays a critical role for the transforming potential of FLT3-ITD mutants and ligand-dependent activation of FLT3-WT.

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Authorship

Contribution: S.V. designed research, performed research, and wrote the paper; C.R. performed research and edited the paper; S.K.K., R.K., and G.M. performed research; T.M. and M.D. performed statistical analysis; S.S. performed research; W.H. designed research; K.S. designed research and wrote the paper.

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Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML

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In acute myeloid leukemia (AML), two clusters of activating mutations are known in the FMS-like tyrosine kinase-3 *(FLT3)* gene: FLT3-internal tandem duplications (FLT3-ITDs) in the juxtamembrane (JM) domain in 20% to 25% of patients, and FLT3 point mutations in the tyrosine-kinase domain (FLT3-TKD) in 7% to 10% of patients, respectively. Here, we have characterized a new class of activating point mutations (PMs) that cluster in a 16–amino acid stretch of the juxtamembrane domain of FLT3 (FLT3-JM-PMs). Expression of 4 FLT3-JM-PMs in interleukin-3 (IL-3)–dependent Ba/F3 cells led to factor-independent growth, hyperresponsiveness to FLT3 ligand, and resistance to apoptotic cell death. FLT3-JM-PM receptors were autophosphorylated and showed a higher constitutive dimerization rate compared with the FLT3–wild-type (WT) receptor. As a molecular mechanism, we could show activation of STAT5 and up-regulation of Bcl-x(L) by all FLT3-JM-PMs. The FLT3 inhibitor PKC412 abrogated the factor-independent growth of FLT3-JM-PM–expressing cells. Compared with FLT3-ITD and FLT3-TKD mutants, the FLT3-JM-PMs showed a weaker transforming potential related to lower autophosphorylation of the receptor and its downstream target STAT5.

Mapping of the FLT3-JM-PMs on the crystal structure of FLT3 showed that these mutations reduce the stability of the autoinhibitory JM domain, and provides a structural basis for the transforming capacity of this new class of gain-of-function mutations of FLT3. (Blood. 2006;107:3700-3707)

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Introduction

FMS-like tyrosine kinase-3 (FLT3) has been shown to be mutated in about one-third of patients with acute myeloid leukemia (AML), representing one of the most frequently occurring mutations in this disease.[1,2] Until now, two distinct clusters of activating mutations are known: FLT3-internal tandem duplications (FLT3-ITDs) in the juxtamembrane (JM) domain in 20% to 25% of patients, and point mutations (PMs) in the tyrosine-kinase domain (FLT3-TKD) in 7% to 10% of patients.[3-9]

Recently, the crystal structure of the autoinhibited form of FLT3 was resolved.[10] The structure conforms to the prototypical conformation common to other inactive kinases that have a "closed" activation loop, but the remarkable feature is the complete JM domain serving as a critical autoinhibitory loop and interacting with all key features of FLT3. This domain can be divided into three distinct parts: the JM binding motif (JM-B), JM switch motif (JM-S), and the zipper or linker peptide segment (JM-Z). According to that model, the JM-B region is nearly buried in the FLT3 structure. It serves as an autoinhibitory domain, which in an inactive state prevents the N lobe from rotating toward the C lobe of the tyrosine kinase domain (TKD) to generate the activated kinase fold.

The cytoplasmatic juxtamembrane domain is highly conserved between different members of the class III receptor tyrosine kinase (RTK) family. A variety of tumors in animals and humans have been described that harbor activating mutations in the JM domain.[11-14] The most frequently occurring activating mutations in AML, FLT3-ITDs, occur primarily in the JM-Z domain. They represent a heterogenous group of mutations, where a fragment of the JM domain, varying in length from 2 to 204 nucleotides (nt), is duplicated and inserted in a direct head-to-tail orientation always maintaining the reading frame.

Recently, we discovered a novel missense point mutation in the JM domain of FLT3 in the AML cell lines Mono-Mac (MM)–1 and MM-6, changing valine with alanine at position 592.[15] By performing a LightCycler (Roche, Mannheim, Germany) mutational screening of FLT3 in 785 AML patient samples, we were able to identify two other point mutations: F594L in two AML patients and Y591C in 1 AML patient. In addition, Stirewalt et al[16] found additional point mutations in the JM domain of FLT3 (V579A and F590GY591D) in AML patients by using single-stranded conformational polymorphism analyses (polymerase chain reaction [PCR]/SSCPs).

Here, we have studied the functional significance of this new class of activating mutations in patients with AML: PMs that cluster in a 16-aa stretch of the JM domain (FLT3-JM-PMs).

We could clearly demonstrate that FLT3 receptors harboring one of these JM point mutations, when expressed in Ba/F3 cells,

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induced interleukin-3 (IL-3)–independent growth, increased resistance to apoptosis via up-regulation of Bcl-x(L) and constitutive activation of the receptor and STAT5. Treatment with a specific FLT3 phospho-tyrosine-kinase (PTK) inhibitor, PKC412, was able to block IL-3–independent proliferation and resulted in reduced phosphorylation of STAT5. These data clearly define a third class of activating mutations in AML. Patients with AML harboring these mutations may respond to small-molecule FLT3 inhibitors like PKC412.

Materials and methods

Patient samples

All samples of bone marrow or peripheral blood (with at least 70% circulating blast cells), were obtained at diagnosis and were sent to the leukemia diagnostics laboratory, Munich, Germany. All patients gave informed consent before entering the study. The study design adhered to the principles of the Helsinki Declaration and was approved by the ethics committees of the participating institutions. A total of 785 unselected AML samples were analyzed.

Screening method for mutations in the juxtamembrane domain of FLT3

Mononucleated cells were isolated by standard Ficoll-Hypaque density gradient centrifugation. Nucleic acid isolation and cDNA synthesis was performed as described before.[5] Screening for FLT3-V592A mutations was performed using a melting curve–based LightCycler assay with forward primer FLT3JM-F: CAATTTAGGTATGAAAGCCAG, reverse primer FLT3JM-R: TGATCCTAGTACCTT; and hybridization probes FLT3JM-S (sensor) TTCATATTCTCTGAAATCAACGTAGAAGT-FL, and FLT3JM-A (anchor) LC-Red640-TCATTATCTGAGGAGCCGGTCACCT-P. The PCR reaction was carried out in a 20-μL reaction volume with each 0.5 μM forward and reverse primer, 0.75 μM Hyb-Probes (Metabion, Martinsreid, Germany) 4 mM MgCl2, and 2 μL LightCycler-FastStart DNA Master Hyb-Probes (Roche Diagnostics, Mannheim, Germany). LightCycler data were analyzed using LightCycler 3.0 software (Roche Diagnostics) and the second derivative maximum method. Each 20-μL reaction contained 2 μL cDNA, an equivalent of about 3000 cells. Amplification was performed with 40 cycles using 50°C annealing temperature. Final melting curve analysis was started at 35°C up to 85°C with slope of 0.2°C/sec and continuous detection with channel F2/F1.

Reagents and cell lines

Low-passage murine Ba/F3 cells were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and were maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 10% WEHI-conditioned medium as a source of murine IL-3 when indicated. PKC412 was kindly provided by Novartis Pharma (Basel, Switzerland).

Cell proliferation of Ba/F3 cells

Cells were seeded at a density of 4×10^4/mL in the presence or absence of IL-3 or FLT3 ligand (Promocell, Heidelberg, Germany) as indicated. Viable cells were counted at indicated time periods in a standard hemacytometer after staining with trypan blue. Figures show mean values and standard deviations from three independent experiments.

Apoptosis analysis

Assessment of apoptotic cells was carried out by annexin V/7-aminoactinomycin D (7-AAD) staining as recommended by the manufacturer (annexin V phycoerythrin [PE] apoptosis detection kit; Becton Dickinson, Heidelberg, Germany) using a FacsCalibur flow cytometer (Becton Dickinson, San Jose, CA). Determination of the DNA content of cell nuclei was performed by propidium iodide (PI) staining as described previously.[17]

Receptor dimerization experiments, immunoprecipitations, and Western blot analyses

Experiments were performed as described previously.[15,18] The following antibodies were used: anti–phospho-STAT5-Tyr694, anti–phospho-AKT-Ser473, anti-AKT, anti–phospho-p44/42 mitogen-activated protein (MAP) kinase (Thr202/Tyr204), anti-p44/42 MAP kinase (all from New England Biolabs, Frankfurt, Germany), anti-STAT5 (sc-835), anti–Bcl-x(L) (sc-8392), anti-FLT3 (S18, sc-480), anti-PY (PY99) (all from Santa Cruz Biotechnology, Heidelberg, Germany), and anti-β-actin (A-5441; Sigma, Munich, Germany).

DNA constructs and vectors

The FLT3-ITD-W51 construct contained a 7-aa duplicated sequence (REYEYDL) inserted between aa 601/602 of human FLT3-WT; the FLT3-ITD-NPOS construct contained a 28-aa duplicated sequence (CSSDNEYFYVDFREYEYDLKWEFPRENL) inserted between aa 611/612 of human FLT3-WT; and the FLT3-ITD-W78 construct contained a 22-aa duplicated sequence (GLVQVTGSSDNEYFYVDFREYE) inserted between aa 598/599 of human FLT3-WT. The FLT3-ITD constructs were kindly provided by Dr G. Gilliland (Howard Hughes Medical Institute and Brigham and Women's Hospital Harvard Institutes of Medicine, Harvard Medical School, Boston, MA). All FLT3 constructs were subcloned in the MSCV-IRES-EYFP retroviral expression vector (kindly provided by R. K. Humphries, The Terry Fox Laboratory, University of British Columbia, Vancouver, BC, Canada). The Bcl-x(L) construct was kindly provided by S. J. Korsmeyer (Howard Hughes Medical Institute, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA).

In vitro mutagenesis

The FLT3-JM point mutations (FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D) and the FLT3-TKD point mutations (FLT3-D835Y and FLT3-D835V) were introduced into the FLT3-WT construct using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturers instructions. The correct sequence of all constructs was confirmed by nucleotide sequencing.

Transient transfection of 293T cells

One day before transfection, 293T cells were seeded into 6-well plates at a density of 3×10^5/mL. Transient transfections were then carried out using the calcium-phosphate coprecipitation method with a total of 2 μg plasmid DNA per well. Eighteen hours after transfection, 2 mL fresh medium was added, the cells were allowed to grow for another 30 hours, and the retroviral supernatant was used for transduction of Ba/F3 cells.

Stable transduction of Ba/F3 cells

Ba/F3 cells (2×10^5) were seeded in 1 mL growth medium and subsequently transduced with 200 μL retroviral supernatant in the presence of polybrene (8 μg/mL). The fluorescence-activated cell-sorting (FACS)–Vantage system equipped with a Turbo-Sort device (Becton Dickinson, San Jose, CA) was used to highly purify EGFP/EYFP-positive pool cells 48 hours after transduction. Expression of CD135 was performed as described previously.[15]

Results

Detection of point mutations in the juxtamembrane domain of FLT3 in patients with AML

By sequencing the complete cDNA of FLT3 in the AML cell lines MM-1 and MM-6, we previously have found a new activating point mutation substituting valine with alanine at position 592 in the

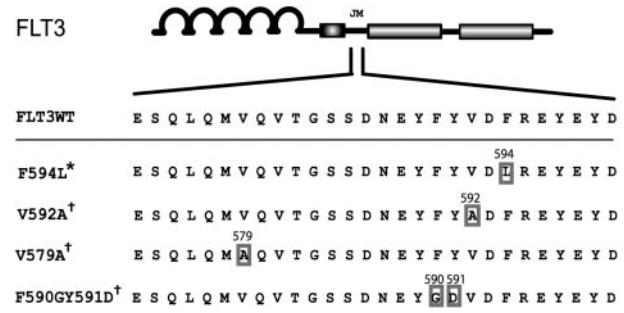

Figure 1. Localization of point mutations in the JM domain of FLT3 (FLT3-JM-PMs) found in patients with AML. The top panel shows the FLT3-WT juxtamembrane protein sequence from aa 573 to 600. Below are the four point mutants found in our study (*) and the study of Stirewalt et al[16] (†) in patients with AML.

functionally important JM domain of FLT3.[15] These results encouraged us to perform a screen for point mutations at position 592 in AML patient samples using a melting curve–based LightCycler assay in a total of 785 samples. Two different point mutations were identified in three patients: Y591C in a patient and F594L in two patients. A recently published study by Stirewalt et al[16] using PCR/SSCP screened for mutations in FLT3 in exons 14 and 15 in patients with AML. They detected two novel missense point mutations in exon 14, V579A and F590GY591D, as well as V592A in about 2% of patients with AML (Figure 1).[16]

These data show that a third class of mutations, point mutations clustering in the JM domain of FLT3 (FLT3-JM-PMs), exist in patients with AML.

So far, the biologic and clinical relevance of these findings have not yet been investigated. We hypothesized that these mutations, which are located in the functionally important JM region, may play an important role in the pathogenesis of AML.

Point mutations in the JM domain of FLT3 confer IL-3–independent growth to the pro–B-cell line Ba/F3

The acquisition of either ITD or TKD mutations in the *FLT3* gene was shown to induce constitutive activation of the receptor- and ligand-independent cell growth in different cell lines.[7,8,19-21] To characterize the functional significance of point mutations in the JM domain, we selected four of the described JM point mutations and introduced them into the FLT3-WT cDNA (Figure 1). We stably transduced the pro–B-cell line Ba/F3 with FLT3-V592A, FLT3-V579A, FLT3-F594L, FLT3-F590GY591D, and FLT3-WT. To directly compare these FLT3-JM-PM cell lines with known activating mutations, we also stably transduced three different FLT3-ITD constructs (FLT3-W51, FLT3-NPOS, and FLT3-W78)[22] and two different FLT3-TKD constructs (FLT3-D835Y and FLT3-D835V).[22] Surface expression levels of FLT3 were confirmed by CD135 antibody staining and FACS analysis (data not shown).

Overexpression of all FLT3-JM-PMs, but not FLT3-WT, induced IL-3–independent growth in Ba/F3 cells (Figure 2A). In detail, the growth rates of FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D were 10%, 11%, 7%, and 18% of the average growth rate of the 3 FLT3-ITD cell lines, respectively. Also, the FLT3-TKD–expressing cells displayed significantly higher growth rates than the FLT3-JM-PM–expressing cells. However, among the 4 different FLT3-JM-PM–expressing cells there was no consistent significant difference in terms of transforming activity. To analyze whether the autonomous growth of FLT3-JM-PMs might be further stimulated by exogenous ligand, all FLT3-JM-PM cell lines were grown in the presence of 50 ng FLT3 ligand (FL)/mL. Viable cells were counted after 72 hours by trypan blue exclusion. The proliferation rates of FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D were 412%, 286%, 197%, and 248% compared with the growth of FLT3-WT cells grown under identical conditions, respectively (Figure 2B).

Taken together, point mutations in the JM domain of FLT3 have transforming potential in Ba/F3 cells, and these mutant receptors confer a signficant proproliferative activity in response to FL.

FLT3-JM-PM–expressing Ba/F3 cell lines are resistant to induction of apoptosis after IL-3 withdrawal

To gain a deeper insight into the phenotype of FLT3-JM-PM–expressing Ba/F3 cells, we analyzed whether they exhibit antiapoptotic activity. Two different assays were performed to identify cells undergoing apoptotic cell death: staining of cells with annexin V and 7-AAD after 48 hours, and staining of nuclei with propidium iodide after 24 hours of growth factor withdrawal. FLT3-WT cells rapidly underwent apoptosis after IL-3 withdrawal in contrast to FLT3-ITD– and FLT3-TKD–expressing cells, which were protected from induction of apoptosis and served as a positive control (Figure 3).

We could clearly demonstrate that the percentage of apoptotic cells in FLT3-V592A–, FLT3-V579A–, FLT3-F594L–, and FLT3-F590GY591D–expressing cell lines grown without IL-3 was significantly lower compared with FLT3-WT after 48 and 24 hours (Figure 3). In the absence of IL-3, the percentage of apoptotic cells in cell lines expressing FLT3-WT was 26% and 80% after 24 and

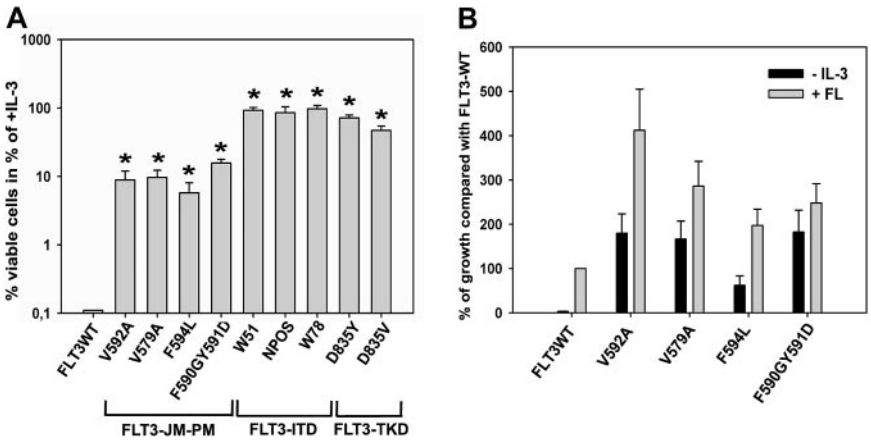

Figure 2. FLT3-JM-PMs induce IL-3–independent growth in Ba/F3 cells and hyperproliferation in response to FL. (A) Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD constructs (W51, NPOS, W78), FLT3-TKD constructs (D835Y, D835V) or one of the FLT3-JM-PM mutants (FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D) were seeded at a density of 4×10^4 cells/mL in the absence or presence of IL-3. Viable cells were counted after 72 hours. The growth of cells in the presence of IL-3 was defined as 100% (control). All FLT3-JM-PM mutants showed a significantly higher proliferation rate compared with FLT3-WT (*$P < .05$), although not as high as FLT3-ITD or FLT3-TKD. SD is indicated. (B) FLT3-WT and mutant-expressing Ba/F3 cells were seeded at a density of 4×10^4 cells/mL in the absence or presence of human recombinant FL (50 ng/mL). Viable cells were counted after 72 hours by trypan blue exclusion. The cell number of FLT3-WT cells after 72 hours was defined as 100%. SEM is indicated.

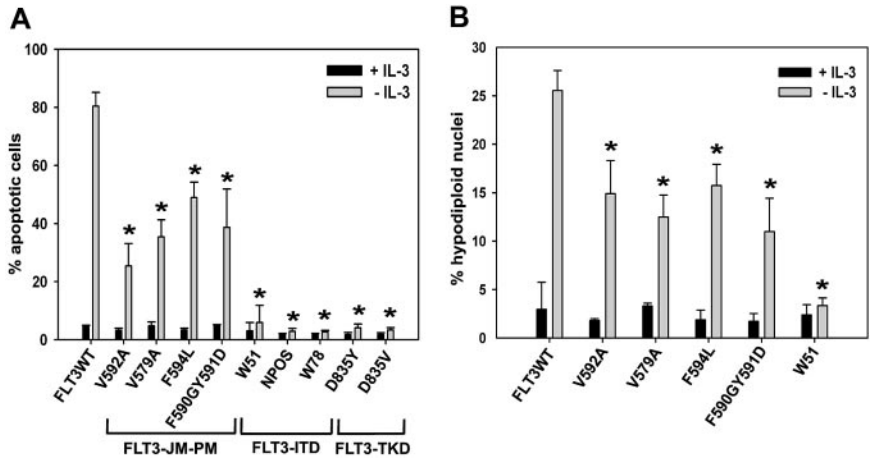

Figure 3. Point mutations in the JM domain of FLT3 induce resistance to apoptosis after IL-3 withdrawal. (A) Ba/F3 cells stably transduced with FLT3-WT, FLT3-ITD (W51, NPOS, W78), FLT3-TKD (D835Y, D835V) or FLT3-JM-PM (FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D) were seeded at a density of 1×10^5 cells/mL and grown for 48 hours in the presence or absence of IL-3. Cells were then analyzed by flow cytometry after staining with annexin V–PE and 7-AAD. All FLT3-JM-PMs showed a significantly lower percentage of apoptotic cells compared with FLT3-WT after IL-3 withdrawal (*$P < .05$). FLT3-ITDs and FLT3-TKDs protected cells from undergoing apoptosis. (B) Cells were cultured in the presence or absence of IL-3 for 24 hours and analyzed by flow cytometry after staining of nuclei with propidium iodide. FLT3-JM-PMs showed a significantly lower percentage of hypodiploid nuclei compared with FLT3-WT (*$P < .05$). SD is indicated.

48 hours, respectively. In contrast, only 11% to 15% (24 hours) and 25% to 49% (48 hours) apoptotic cells were found in FLT3-JM-PM–expressing cells. Addition of IL-3 protected all cell lines from undergoing apoptosis (data not shown).

In summary, these data clearly show that the point mutations in JM of FLT3 constitutively activate antiapoptotic signaling pathways of FLT3, although the antiapoptotic activity of these mutants is significantly lower compared with FLT3-ITD and FLT3-TKD mutants.

FLT3-JM-PM receptors are constitutively autophosphorylated on tyrosine residues

We next examined whether the acquisition of point mutations in the JM domain of FLT3 results in constitutive activation of the FLT3 receptor. Cell lysates of unstimulated and FL-stimulated FLT3-JM-PM–expressing Ba/F3 cells as well as FLT3-ITD– and FLT3-TKD–expressing cells were prepared and subjected to immunoprecipitation with FLT3 antibody followed by immunoblotting with anti–phospho-tyrosine antibody. In contrast to the FLT3-WT receptor, all FLT3-JM-PM receptors were constitutively phosphorylated on tyrosine residues (Figure 4A).

Densitometric analysis revealed that the ratios of phosphorylated to total FLT3-JM-PM receptors were 20% for FLT3-V592A–expressing cells, 28% for FLT3-V579–expressing cells, 10% for FLT3-F594L–expressing cells, and 14% for FLT3-F590GY591D–expressing cells, and considerably lower compared with the ratio of phosphorylated FLT3-ITD (42%-60%) and FLT3-TKD (41%-66%) receptors (Figure 4B). We further performed cross-linking experiments to analyze constitutive oligomerization of FLT3-JM-PM receptors. Unstimulated and FL-stimulated cells were incubated in bis(sulfosuccinimidyl)suberate (BS^3) cross-linking buffer for 30 minutes before cell lysis. In the absence of FLT3 ligand, FLT3-JM-PM receptors showed a higher rate of constitutive oligomerization compared with FLT3-WT receptors (data not shown).

Taken together, the acquisition of FLT3-JM-PMs leads to direct activation of the receptor. FLT3-JM-PMs show increased autophosphorylation and constitutive oligomerization in the absence of FLT3 ligand. The extent of receptor activation is nevertheless weaker compared with FLT3-ITD and FLT3-TKD receptor mutants.

FLT3-JM point mutants induce constitutive STAT5 activation

STAT5 is an important downstream target of the constitutively activated FLT3 receptor and is probably responsible for most of its transforming potential in vitro and in vivo.[15,20,23] Activation of STAT5 results in altered expression of several genes regulating cell cycle, apoptosis, and proliferation.[24] To investigate the activation of the STAT5 signaling pathway, we prepared crude cell lysates of serum-starved Ba/F3 cells transduced with either FLT3-WT, FLT3-JM-PM (FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D) or FLT3-ITD/FLT3-TKD constructs. Lysates were analyzed by immunoblotting with a specific antibody against phospho-STAT5. We could clearly demonstrate that the expression of FLT3-JM-PMs in Ba/F3 cells induces a significantly stronger STAT5 activation compared with FLT3-WT (Figure 5A). The ratio of phosphorylated STAT5 to total STAT5 in FLT3-JM-PM cells ranged from 20% to 35% and was considerably weaker than in FLT3-ITD–expressing cells (49%-61%) and FLT3-TKD–expressing cells (56%-66%) (Figure 5B). We further investigated

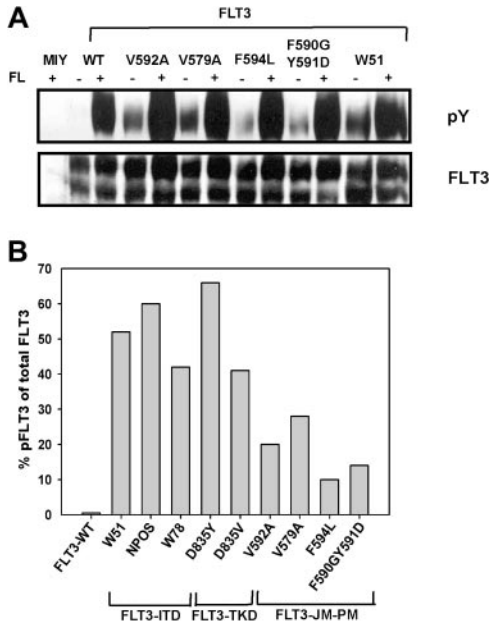

Figure 4. FLT3-JM-PM receptors are constitutively autophosphorylated on tyrosine residues. (A) Lysates of FL-stimulated (100 ng FL/mL for 5 minutes) and unstimulated, serum-starved FLT3-WT–expressing cells, FLT3-JM-PM–expressing cells (FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D), and FLT3-W51–expressing cells were subjected to immunoprecipitation with FLT3 antibody followed by immunoblotting with phospho-tyrosine antibody. Blots were stripped and reblotted with FLT3-antibody. (B) FLT3-ITD–expressing cells (W51, NPOS, and W78) and FLT3-TKD–expressing cells (D835Y and D835V) were analyzed as described in panel A and densitometric analysis was performed using TINA 2.0 software to quantify the percentage of phospho-FLT3 of total FLT3.

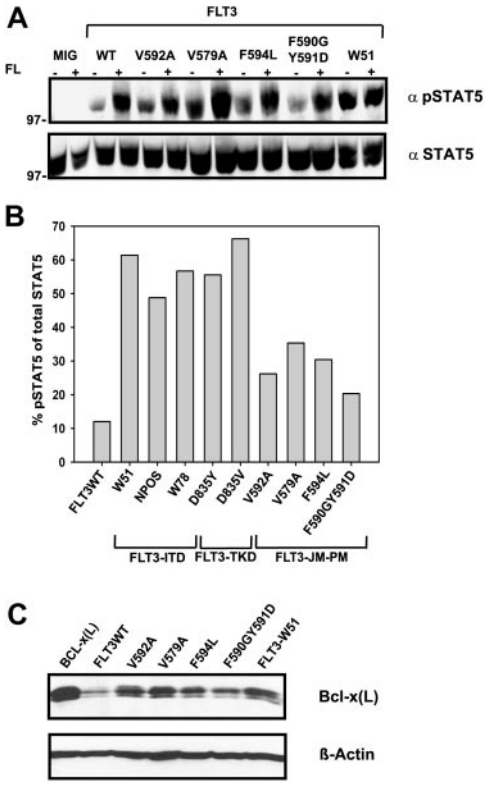

Figure 5. FLT3-JM-PM mutants expressed in Ba/F3 cells show constitutive activation of STAT5 and up-regulation of Bcl-x(L). (A) FLT3-WT, FLT3-W51, FLT3-V592A, FLT3-V579A, FLT3-F594L, FLT3-F590GY591D, or mock-transduced cells were starved for 24 hours in the presence of 0.3% FBS and stimulated with 100 ng FL/mL for 5 minutes. Crude-cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted on a nitrocellulose membrane. Blots were incubated with anti–phospho-STAT5 antibody, stripped, and reblotted with anti-STAT5 antibody. (B) FLT3-ITD–expressing cells (W51, NPOS, and W78) and FLT3-TKD–expressing cells (D835Y and D835V) were analyzed as described in panel A, and the films were subjected to densitometric analysis to quantify the percentage of phospho-STAT5 in relation to total STAT5 amount in unstimulated cells. (C) Crude-cell lysates were subjected to Western blot analysis using a monoclonal antibody against Bcl-x(L). Bcl-x(L) overexpressed in Ba/F3 cells served as a positive control. Equal protein loading in all lanes was confirmed by immunoblotting using an anti–β-actin antibody.

the FL-independent activation of MAP kinase (MAPK) and the activation of serine/threonine protein kinase AKT as a marker for activation of phosphatidylinositol 3-kinase (PI3K)–dependent pathways. Immunoblotting with phospho-AKT-Ser473–specific antibody revealed a slightly increased basal level of phosphorylation of AKT in FLT3-JM-PM cells compared with FLT3-WT (data not shown). Immunoblotting with phospho-MAPK antibody showed the same level of phosporylation of MAPK in FLT3-JM-PM cells as in FLT3-WT cells (data not shown). Since AKT activation is only slightly increased in FLT3-JM-PM cells and MAPK activation level is not changed compared with FLT3-WT, we conclude that the prominent proliferative signal is mediated by STAT5.

As the JM point mutants induce antiapoptotic signaling when expressed in Ba/F3 cells, we hypothesized that this might be due to the up-regulation of an important STAT5 antiapoptotic downstream target, Bcl-x(L). Cell lysates were subjected to Western blot analysis and immunoblotted with specific monoclonal antibody against Bcl-x(L). We found a significant up-regulation of Bcl-x(L) in FLT3-V592A–, FLT3-V579A–, FLT3-F594L–, and FLT3-F590GY591D–expressing cells compared with FLT3-WT (Figure 5C).

Thus, the most important signaling pathway downstream of FLT3, STAT5, is constitutively activated by the FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D receptor mutants and induces up-regulation of the antiapoptotic protein Bcl-x(L).

The FLT3 PTK inhibitor PKC412 induces growth arrest and inhibits tyrosine phosphorylation of STAT5 in FLT3 JM point mutants

PKC412 was inititally developed as a vascular endothelial growth factor receptor (VEGFR) inhibitor and was shown to block the activity of FLT3-WT and mutant FLT3 receptors.[25,26] To analyze the inhibitory activity of PKC412 against FLT3-JM-PMs, we treated the FLT3-V592A–, FLT3-V579A–, FLT3-F594L–, and FLT3-F590GY591D–expressing cells with different concentrations of PKC412 up to 50 nM. PKC412 showed a strong growth inhibitory effect on FLT3-V592A–, FLT3-V579A–, FLT3-F594L–, and FLT3-F590GY591D receptor–expressing cells in the absence but not in the presence of IL-3. The inhibitory concentration of 50% (IC_{50}) of PKC412 was significantly lower in FLT3-JM-PM–expressing cells (< 1 nM) and FLT3-D835Y–expressing cells (< 1 nM) compared with the FLT3-W51 mutant (5 nM; Table 1).

Furthermore, we treated serum-starved FLT3-V592A–, FLT3-V579A–, FLT3-F594L–, FLT3-F590GY591D–, and FLT3-W51–expressing cells for 1 hour with 50 nM PKC412. Immunoblotting with pSTAT5 antibody showed a significant reduction in the amount of phosphorylated STAT5 in FLT3-V592A, FLT3-V579A, FLT3-F594L, FLT3-590G591D, and FLT3-W51, confirming that STAT5 activation is directly mediated by the activated FLT3 receptor (Figure 6).

These data clearly show that the proliferative signal of FLT3-JM-PM receptors can be blocked by PKC412 and that FLT3-JM-PM–expressing cells are considerably more sensitive to this compound than FLT3-ITD–expressing cells.

Discussion

Since 1996, when the first description of activating FLT3 mutations was published, extensive research has been performed to characterize the functional relevance of these mutations in AML.[3] Although the *FLT3* gene has been studied in detail in AML, we and others were recently able to identify a new class of mutations in patients with AML, the FLT3-JM-PMs.[15,16] According to Stirewalt et al,[16] who performed a SSCP analysis of the entire JM region, the frequency of these point mutations in the JM region was about 2%.

Table 1. FLT3 JM point mutants expressed in Ba/F3 cells are sensitive to the FLT3 PTK inhibitor PKC412

Mutation class	IC_{50} PKC412, nM
FLT3-ITD	
W51	5.0
FLT3-TKD	
D835Y	0.8
FLT3-JM-PM	
V592A	0.7
V579A	0.8
F594L	0.9
F590GY591D	0.7

FLT3-WT, FLT3-W51, FLT3-D835Y, and the FLT3-JM-PM mutants FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D expressing Ba/F3 cells were seeded at a density of 4×10^4 cells/mL in the absence or presence of different concentrations of PKC412 (0 to 50 nM) and counted after 72 hours. All cell lines were also cultured in the presence of IL-3 and 50 nM PKC412 to confirm nontoxicity of PKC412 to Ba/F3 cells. The IC_{50} was calculated from three independent experiments.

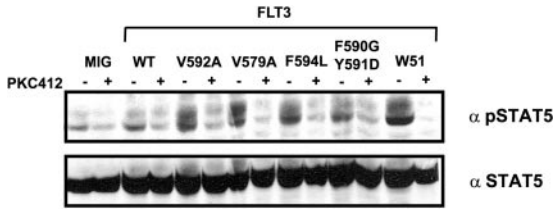

Figure 6. PKC412 inhibits autophosphorylation of STAT5 in Ba/F3 cells expressing JM point-mutated FLT3 receptors. Mock cells and cells expressing FLT3-WT, FLT3-W51, FLT3-V592A, FLT3-V579A, FLT3-F594L, and FLT3-F590GY591D were starved for 24 hours and treated with 50 nM PKC412 1 hour before cell lysis. Cell lysates were subjected to Western blot analysis using polyclonal anti–phospho-STAT5 antibody. Blots were stripped and reblotted with polyclonal anti-STAT5 antibody.

In the present study, we focused mainly on the V592A mutation that we found in MM-1 and MM-6 cell lines. Our LightCycler-based screening assay covers approximately 5 to 6 amino acids surrounding V592A and underestimates the frequency of FLT3-PMs in the JM domain.

Our results show that these JM point mutations in the *FLT3* gene induce factor-independent growth and hyperresponsiveness to FL, and have an antiapoptotic activity in hematopoietic cells. The FLT3-JM-PM receptors are autophosphorylated and show a higher constitutive oligomerization rate compared with FLT3-WT receptors. We could show that all FLT3-JM-PMs activate STAT5 and up-regulate Bcl-x-(L). The activation of the receptor and STAT5, respectively, is lower in FLT3-JM-PM–expressing cells compared with FLT3-ITD– and FLT3-TKD–expressing cells, and we propose that these differences are mainly responsible for the weaker phenotype of FLT3-JM-PMs. A selective FLT3 PTK inhibitor, PKC412, was able to abrogate the factor-independent growth and down-regulated the activation of STAT5. Compared with the two known classes of FLT3 mutations (FLT3-ITD and FLT3-TKD), FLT3-JM-PM–expressing cells showed a weaker phenotype in terms of their proliferation rate, antiapoptotic activity, FLT3 receptor activation, and activation of STAT5 (summarized in Table 2).

The reason for the weaker transforming potential might be that the FLT3-JM-PMs, in contrast to FLT3-ITDs, induce only subtle alterations in the protein structure of the autoinhibitory JM domain. All FLT3-JM-PM–expressing cells showed hyperresponsiveness to FLT3 ligand, with a 2 to 4 times higher proliferation rate compared with FL-stimulated FLT3-WT cells. As FLT3 ligand is often coexpressed in primary AML blast cells, this might reflect more precisely the "in vivo" situation. Furthermore, we could demonstrate that a single point mutation in the JM domain is sufficient to constitutively activate the receptor and increase its spontaneous dimerization rate.

STAT5, the most important downstream target of FLT3, is strongly associated with tumor development and progression.[27] For example, it directly induces the expression of antiapoptotic proteins, like Bcl-x(L), which play an essential role in resistance against apoptotic cell death.[28,29] Bcl-x(L) is overexpressed in a high percentage of blast cells from patients with AML and confers a poor prognosis in these patients.[30] Our results clearly show that FLT3-JM-PMs activate STAT5 and induce up-regulation of Bcl-x(L), and suggest that this pathway is critical for the transforming capacity of activated FLT3 mutants.

The success of the small-molecule tyrosine-kinase inhibitor imatinib (Novartis) in the treatment of chronic myelogenous leukemia (CML) has given much encouragment for the development of other molecularly targeted cancer therapeutics. Due to the fact that *FLT3* is one of the most frequently mutated genes in AML, it is a promising target for the treatment of AML. Small-molecule FLT3 PTK inhibitors have been developed, and are able to induce apoptosis in cell lines with FLT3-activating mutations and prolong the survival of mice expressing mutant FLT3.[31-34] We analyzed the inhibitory activity of PKC412, a compound which is now tested in phase 2 clinical trials in AML, on Ba/F3 cells expressing FLT3-V592A, FLT3-V579A, FLT3-F594L, FLT3-F590GY591D, FLT3-W51, and FLT3-D835Y. Our data clearly show that FLT3-JM-PM–induced cell growth can be inhibited by PKC412. In addition, we were able to show down-regulation of autoactivated STAT5 after treating cells with PKC412, providing direct evidence for the efficacy of this compound to abrogate mitogenic signaling pathways of the receptor. Moreover, the FLT3-JM-PMs are considerably more sensitive to PKC412 than FLT3-ITDs, suggesting that patients with JM point mutations in FLT3 should respond to even lower doses of PKC412.

Our results show that a single amino acid change in the autoinhibitory JM domain is sufficient to activate the transforming potential of FLT3. To structurally analyze the putative effect of the point mutations, we mapped the mutations on the crystal structure of the FLT3 JM domain (Figure 7).[10] From the FLT3 crystal structure it is obvious that these point mutations cluster at a core interaction site of the JM domain with the remainder of the molecule. It is therefore likely that these mutations reduce the stability of the JM domain in the autoinhibitory conformation. For instance, F594 and V592 together form a small hydrophobic contact face of the JM domain with the kinase domain. F594L and V592A mutations topologically perturb this hydrophobic contact face, likely resulting in a destabilized interface between JM domain and kinase domain. Likewise, mutations of F590 to glycine and of Y591 to aspartate might destabilize the autoinhibitory conformation of the JM domain by adding more backbone flexibility, but also by removing the stabilizing contacts of the tyrosine side chain with the rest of the molecule. The effect of Y591D on the activity of the molecule is also consistent with the idea that phosphorylation of the tyrosine pair Y589 and Y591 sterically prohibits the observed autoinhibitory conformation of the JM domain. Finally, V579 stabilizes the inserted β strand of JM-B by a hydrophobic interaction with the rest of the molecule. This stabilization is likely

Table 2. Comparison of the phenotype of FLT3-JM-PM– to FLT3-ITD/TKD–expressing Ba/F3 cells

	FLT3-WT	FLT3-ITD	FLT3-TKD	FLT3-JM-PM
IL-3-independent proliferation*	−	+++	++	+
Antiapoptotic activity†	−	+++	+++	+
Receptor autophosphorylation‡	−	++	++	+
STAT5 autoactivation§	−	+++	+++	+

*+++ indicates 80%-100%; ++, 50%-79%; +, 5%-49%; and −, less than 5% viable cells in percentage of IL-3$^+$ growth after 72 hours (Figure 2A).
†+++ indicates less than 10%; ++, 10%-24%; +, 25%-50%; and −, more than 50% apoptotic cells after 48 hours (Figure 3A).
‡+++ indicates more than 70%; ++, 40%-70%; +, 10%-39%; and −, less than 10% phospho-FLT3 of total FLT3 (Figure 4B).
§+++ indicates 50% or more; ++, 36%-49%; +, 16%-35%; and −, 15% or less phospho-STAT5 of total STAT5 (Figure 5B).

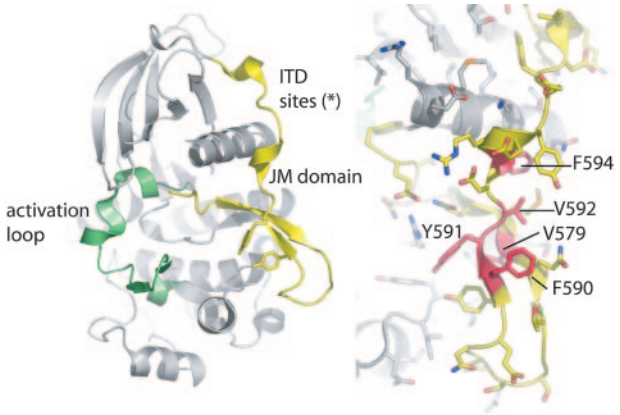

Figure 7. Structural mapping of JM point mutations. (Left) Ribbon model of the crystal structure of the FLT3 kinase domain (Protein Data Bank [PDB] accession no. 1RJB) with green activation loop and yellow JM domain. The positions of internal tandem duplications (ITDs) in JM-Z, leading to FLT3 activation, are indicated. *Some ITDs are found in the tyrosine kinase domain and are not indicated. (Right) Close view of the mutation sites in the JM domain (yellow). The structure is shown as a ribbon backbone, with side chains shown as color-coded sticks. Point mutations identified in this study are depicted in red and are annotated. The clustering and location of the point mutations suggest that they reduce the stability of the observed inhibitory conformation of the JM domain. Structural analysis of FLT3-JM-PMs was done with PyMol (DeLano Scientific, San Carlos, CA).

reduced by mutating this residue to alanine, which would create a cavity in the molecule. Taken together, the effect of the JM-PMs supports a model in which the JM domain rearranges upon activation of the kinase. This rearrangement could be normally promoted by phosphorylation of Y589 and Y591, but in the case of activating mutations, also by destabilization of the autoinhibitory conformation of the JM domain. In this respect, the mutations might promote tyrosine phosphorylation by rendering the JM domain more accessible for autophosphorylation.

The FLT3-ITDs, which are primarily located in the JM-Z region, confer a strong transforming potential to hematopoietic cell lines, but the mechanisms by which these duplicated sequences of FLT3-ITD mutants change the conformation of the FLT3 protein structure remain unknown. It was proposed that the increased length of the JM-Z offsets the position of JM-S, likely disturbing the ideal orientation of JM-S to position JM-B in its binding site.[10] It is difficult to say what effect a length increase in JM-Z has on the overall integrity of the protein structure, but the close location to JM-B suggests that these mutations might completely prevent formation of an autoinhibitory state. From a structural point of view, the presence of additional residues in JM-Z could easily prevent the formation of structurally observed folds of JM-S in the autoinhibitory state. Although FLT3-ITDs can involve the same amino acids in the JM domain as those affected by point mutations, they probably alter the protein conformation in a different way. Compared with FLT3-ITDs, the point mutations represent a much more subtle change in the structural chemistry of this region and might still allow formation of an autoinhibitory complex, albeit with reduced stability. A full (FLT3-ITD) versus partial (FLT3-JM-PM) interference with the autoinhibitory state could explain the much stronger phenotype of ITD mutations compared with point mutations.

The cytoplasmatic juxtamembrane domain is highly conserved between different members of class III receptor tyrosine kinases. A variety of malignancies have been described which harbor activating mutations in the juxtamembrane domain of KIT.[11-14,35-37] In 10% to 20% of all gastrointestinal tumors (GISTs), gain-of-function single amino acid substitutions of the JM region were reported, and two possible hotspot regions, comprising codons 550 to 560 and 567 to 576, were suggested.[38,39] Among others, one frequently affected position is KIT-V560. This residue is homologous to V579 in FLT3 that was found to be mutated in one AML patient (FLT3-V579A). Furthermore the second region, aa 567 to 576 in KIT, is partially homologous to FLT3 and comprises all other 3 reported point mutations (FLT3-V592A, FLT3-F590GY591D, and FLT3-F594L). The clustering of these mutations in the JM domains of both KIT and FLT3 supports their specific biological significance. Further confirmation on the functional relevance of the JM domain was provided by 2 investigators who performed a detailed mutational screening.[40,41] Ma et al[40] analyzed the region from M552 to I563 in the JM of the KIT receptor, and Irusta et al[41] analyzed the region from R529 to W561 in JM of platelet-derived growth factor receptor β (PDGFRβ). Single amino acids were mutated to alanine, and the constitutive tyrosine phosphorylation of the receptor was analyzed by both groups. The V560A mutation in KIT, homologous to V579A in FLT3, as well as V550A mutation in PDGFRβ, homologous to V592A in FLT3, resulted in autophosphorylation of the receptor. Mutation of I537A and D551A in PDGFRβ, corresponding to FLT3 positions V579 and F594, displayed a strong and weak autophosphorylation, respectively. These results clearly show that point mutations targeting this region lead to destabilization of the autoinhibitory JM conformation and finally to activation of the receptor.

In conclusion, we were able to define a new class of activating FLT3 mutations, point mutations that cluster in the JM region. The FLT3-JM-PMs confer a transforming potential to hematopoietic cells in vitro that was weaker than that of FLT3-ITD and FLT3-TKD mutant receptors. Our data suggest that patients carrying FLT3-JM-PMs might respond to treatment with selective FLT3 inhibitors.

Acknowledgments

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Transformation by oncogenic mutants and ligand dependent activation of FLT3-WT requires the tyrosine residues 589 and 591

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ABSTRACT

Mutations in the protein tyrosine kinase FLT3 can be found in 30% of AML patients and are associated with an inferior prognosis. To characterize the critical domains and residues of active FLT3 receptor mutants, we performed a detailed structure-function analysis of putative auto-phosphorylation tyrosine (Y) residues in the FLT3-D835Y background. For this purpose, all Y residues in the juxtamembrane domain (Y566, Y572, Y589, Y591, Y597, and Y599), interkinase domain (Y726, Y768) and carboxyl terminus (Y955, Y969) of the FLT3-D835Y construct were successively mutated to Phenylalanine (F) and the transforming activity of these mutants was analyzed in IL-3-dependent Ba/F3 cells. The mutation of all these Y residues resulted in complete loss of the transforming potential of FLT3-D835Y, which further can be attributed to complete loss of STAT5 activation at molecular level. Re-introduction of single Y residues revealed the critical role of Y589 and Y591 in reconstituting IL-3 independent growth of FLT3-TKD. Combined mutation of Y589 and Y591 to F also completely abrogated ligand-dependent stimulation of FLT3 wild type and the transforming activity and STAT5 activation of FLT3-ITD constructs. In summary, our study underlines the relevance of distinct Y residues for the mutated and wild type FLT3 receptor.