A NEW AND RECURRENT ACTIVATING LENGTH MUTATION IN EXON 20 OF THE FLT3 GENE IN ACUTE MYELOID LEUKEMIA

Karsten Spiekermann1,3*, Ksenia Bagrintseva1,3, Claudia Schoch2,3, Torsten Haferlach2,3, Wolfgang Hiddemann1,2,3 & Susanne Schnittger2,3*

1Clinical Cooperative Group “Leukemia”
GSF - National Research Center for Environment and Health
2Laboratory for Leukemia Diagnostics
3Department of Medicine III, University Hospital Grosshadern, Ludwig-Maximilians University, Munich, Germany

This work was supported by a grant from the Deutsche Krebshilfe (10-1562)

Category of manuscript: Brief report
Running title: A new activating length mutation in exon 20 of FLT3
Keywords: Acute myeloid leukemia, receptor tyrosine kinase, FLT3, signal transduction
Abstract word count: 145
Total word count: 1176

*Corresponding authors:
Karsten Spiekermann, MD; Susanne Schnittger, PhD
Department of Medicine III, University Hospital Grosshadern, Ludwig-Maximilians University,
Marchioninistraße 15, 81377 Munich, Germany
Tel: ++49-89-7099-417/423
Fax: ++49-89-7099-400
email: spiekermann@gsf.de
susanne.schnittger@med3.med.uni-muenchen.de

Copyright 2002 American Society of Hematology
ABSTRACT

Activating length mutations in the juxtamembrane (JM) domain of the FLT3 gene (FLT3-LM) and mutations in the catalytic domain (FLT3D835/836) of this receptor tyrosine kinase represent the most frequent genetic alterations in acute myeloid leukemia (AML). Here, we describe a six base pair (bp) insertion in the activation loop of FLT3 between codons 840 and 841 of FLT3 (FLT3-840GS) in two unrelated patients with AML. Screening for other activating mutations of FLT3, CKIT and NRAS showed no further genetic alterations in patients carrying the FLT3-840GS. In functional analyses we could show that this mutant is hyperphosphorylated on tyrosine and confers IL-3 independent growth to Ba/F3 cells which can be inhibited by a specific FLT3 PTK inhibitor. Our results show for the first time that in addition to known mutations in the JM and the catalytic domain, further activating length mutations exist in the FLT3 gene.
INTRODUCTION

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, a partial tandem duplication within the MLL gene (MLL-PTD) has been shown to define a subgroup of AML patients with unfavourable clinical outcome ¹.

Activation of the FLT3 receptor tyrosine kinase (RTK) due to length mutations in the juxtamembrane domain (JM) are found in 20-25% of AML ²-⁷. In addition, point mutations and deletions of codons 835/836 of FLT3 which are located in the activation loop (A-loop) of the protein tyrosine kinase (PTK) domain, have been described in about 7% of all AML ⁸,⁹.

FLT3 has a high homology to other class III RTK and plays an important role in the proliferation of hematopoietic progenitors and AML blast cells ¹⁰-¹². Functionally, mutations in the JM region and also in the A-loop result in a constitutively active FLT3 kinase which confers IL-3 independent growth in Ba/F3 cells and activates the MAPK and STAT5 pathways ¹³-¹⁵. In the murine bone marrow transplantation model and in transgenic animals active FLT3 mutants induce a myeloproliferative disease, but not acute leukemia which shows that probably additional genetic alterations are necessary for the clinical phenotype in FLT3 LM positive AML ¹⁶,¹⁷.

In the present study we describe a recurrent activating length mutation in the activation loop of FLT3 in two patients with AML.
PATIENTS, MATERIALS AND METHODS

Patient samples: Bone marrow samples from 359 adult patients with newly diagnosed and untreated AML were analyzed for the new FLT3 mutation. The studies abide by the rules of the local internal review board and the tenets of the revised Helsinki protocol.

Patient #1, a 69 year old female was diagnosed as AML FABM0/M1. The peripheral leukocyte count was 29,700/µl and the percentage of blasts in the bone marrow was 90%. The cytogenetic analyses showed the karyotype: 46,XX,der(7)t(1;7)(q25;q22)[13]/46,XX[2]. After treatment with a high dose AraC based regimen, the patient died 4 weeks later with no response to therapy. Patient #2, a 76 year old male was diagnosed with AML FAB M6 with the following cytogenetic findings: 44,X,-Y,der(7)t(7;8)(q22;?),t(8;11)(q11;q11),+11,der(12)t(12;18)(p12;?),-17,-18[2]/46,XY[11]. In this patient the peripheral leukocyte count was 1,570/µl and the percentage of bone marrow blasts was 20%. A low dose AraC and idarubicin containing chemotherapy resulted in a complete remission, but an early relapse occurred and the patient died after 3 months of diagnosis.

Cytogenetics: Cytogenetic G-banding analysis was performed with standard methods 18,19.

PCR: Isolation of mononucleated leukemic cells, mRNA/DNA extraction, reverse transcription and PCR was performed as described previously 5. RT-PCR was carried out using primers from exon 20 of the FLT3 gene: e20forward: 5´-CCGCCAGGAACGTGCTTG-3´ (corresponding to nts 2395-2412 of XM39994.1) and e20reverse: 5´-ATGCCAGGGTAAGGATTCAACC-3´ (corresponding to nts 2632-
2610 of XM39994.1) resulting in a 238 bp amplification product. Genomic PCR was
carried out with primers 20F: 5´-CCAGGAACGTGCTTGTCA-3´ (corresponding to nts
76543-76526 of AL591024.14) and 20R: 5´-TCAAAAATGCACCACAGTGAG-3´
(corresponding to nts 76349-76369 of AL591024.14) generating a 195 bp
amplification product in normal controls.

**Screening for further mutations:** Screening for MLL-PTD, FLT3-LM, NRAS-
mutations, FLT3D835 and CKITD816 were performed as has been described
previously 1,5,20.

**Antibodies:** The following antibodies were used: anti-FLT3/flk2 (S18, sc-480, Santa
Cruz, Heidelberg, Germany), anti-PY (PY99, Santa Cruz).

**In vitro mutagenesis:** The FLT3-840GS mutation, found in clinical samples, was
introduced into the full length human FLT3 wildtype cDNA using the QuikChange
Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the
manufacturer’s instructions.

**Cell culture and protein analyses:** Cell proliferation of Ba/F3 cells, transient
transfection of BOSC23 cells, transduction of Ba/F3 cells, flow cytometric analysis of
FLT3 expression, immunoprecipitation and Western-blot analysis were performed as
described previously 21.
RESULTS AND DISCUSSION

We used a PCR-based method to screen for mutations of codons D835/836 in patients with AML. After gel electrophoresis of the amplification products from exon 20 we observed an additional PCR fragment which was slightly larger than the PCR product of the wildtype allele in two patients (figure 1B). RT-PCR was repeated more than ten times with different aliquots of RNA as well as cDNA and always gave the same results. Sequencing revealed an insertion of 6 nucleotides between the codons S840 and N841 just five amino acids (AA) downstream of D835. These nucleotides generated a BamHI restriction site which was confirmed by BamHI digestion and gel electrophoresis (figure 1C). The mutation resulted in the insertion of a glycine and a serine between AA 840 and 841 of FLT3 (figure 1D). For patient 1 a DNA sample was available and the mutation could be shown also at the genomic level by PCR, confirming the presence of the mutation (Figure 1B). The respective mutation was not detected in further 357 unselected AML pts. Additional screening for frequent genetic mutations in these two patients showed no further activating mutations of FLT3, NRAS, CKIT or MLL (figure 1A).

We generated FLT3-840GS and FLT3-ITD expressing Ba/F3 cells. In Western-blot analyses, the FLT3-840GS and the FLT3ITD receptors were hyperphosphorylated compared to the FLT3WT receptor (figure 2B). As shown in figure 2A, the FLT3-840GS as well as FLT3ITD expressing Ba/F3 cells grew factor-independently, whereas mock- and FLT3WT expressing cells were unable to proliferate in the absence of IL-3.
The detailed analysis of the growth characteristics of FLT3-840GS Ba/F3 cells showed a significantly slower growth rate compared to the FLT3ITD expressing cells. For maximal proliferation, the FLT3-840GS expressing cells required additional IL-3 at concentrations as low as 0.1 ng/ml (figure 2D).

We next asked whether the FLT3-840GS mutant is sensitive to the growth inhibitory activity of SU5614, a FLT3 PTK inhibitor (unpublished data). Our results clearly show that SU5614 induced a growth inhibition of the FLT3-840GS as well as of the FLT3ITD transduced Ba/F3 lines in the absence, but not in the presence of IL-3 (data not shown).

The data presented here clearly indicate that activating mutations in the FLT3 gene do not only occur within the juxtamembrane domain and in codons D835/836 but also in other positions of the A-loop of the catalytic domain. The A-loop represents a hot spot region for activating mutations in class III RTK which have been described for cKIT (D816) and FLT3 (D835/836)\(^8\). These mutations induce a conformational change of the A-loop which results in the opening of the catalytic pocket and a constitutive active kinase activity. Although no structural data on the catalytic domain of FLT3 are available which would allow the detailed structure-function analysis, the close proximity to the AA D835/836 suggests a similar mechanism of kinase activation by the FLT3-840GS mutant.

Activating mutations of the FLT3 gene provide an essential anti-apoptotic and pro-proliferative signal in primary AML cells and cell lines\(^{13,14,22}\). Our results clearly indicate that the FLT3840-GS mutant is hyperphosphorylated on tyrosine residues and induces IL-3 independent growth in Ba/F3 cells. These in vitro data underline the pathophysiologic role of this mutant for the leukemic phenotype in patients with AML.
Although the FLT3-840GS is probably a rare mutation, it clearly shows that activating mutations other than the FLT3-LM in the JM domain and FLT3D835/836 in the kinase domain exist in AML. These findings are of significant clinical importance since activating FLT3 mutations could represent selective and specific molecular target structures for therapeutical strategies using PTK inhibitors in AML.²³,²⁴
ACKNOWLEDGEMENTS

The authors thank Inga Böll for help with mutational screening, cloning and sequencing of constructs and Karin Schmieja, Ruth Schwab, Gudrun Mellert, Claudia Tschulik and Tanja Skorupinski for excellent technical assistance. We greatly acknowledge all of more than 300 physicians for sending the patient samples.
REFERENCES


17. Baldwin BR, Tse KF, Small D. Transgenic mice expressing a constitutively activated FLT3 receptor display a myeloproliferative disease phenotype. Blood. 2001;98:801a


Figure 1: Detection of the FLT3-LM in exon 20 in AML. A. Clinical data and alterations of other molecular markers in the two patients carrying the FLT3-840GS mutation. FLT3-LM, length mutations in the JM-region of FLT3; FLT3D835, mutations in codons 835/836; CKITD816, point mutations in codon 816; NRAS, activating point mutations in codons 12, 13 and 61; MLL-PTD, MLL-partial tandem duplication; B. Detection of the length mutation in exon 20 after conventional agarose gel electrophoresis of PCR-products from cDNA and genomic DNA. M: Molecular weight standard, H2O: water control, C: Patient without exon 20 mutation, P1/2: patient 1/2 with the length mutation in exon 20; C. Confirmation of the mutation by BamHI digestion after polyacrylamide gel electrophoresis. M: molecular weight standard, MT: patient with mutation, WT: two patients without mutation, H2O: water control; D. Schematic presentation of the FLT3-840GS mutation.
Figure 2: The FLT3-840GS mutant is hyperphosphorylated and induces IL-3 independent growth in Ba/F3 cells. A. Ba/F3 cells transduced with either pMSCV-EYFP-IRES-FLT3-840GS or empty vector (pMSCV-EYFP-IRES, mock) were grown in the absence or presence of IL-3 as indicated. Results represent means ± SD of three independent experiments; B. Lysates from Ba/F3 cells were immunoprecipitated with αFLT3-antibody and analyzed by Western blot using an antiphosphotyrosine (αPY) antibody, stripped and reblootted with αFLT3 antibody; C. Ba/F3 cells expressing either FLT3WT, FLT3ITD, FLT3-840GS or empty vector were incubated with a mouse isotype-matched control antibody (open histograms) or CD135-phycoerythrin (filled histograms) antibody. Viable cells were analyzed using a FacsCalibur flow cytometer; D. Ba/F3 cells expressing FLT3WT, FLT3ITD or FLT3-840GS were grown for 72h in the absence or presence of different concentrations of IL-3 as indicated. The growth of FLT3ITD expressing Ba/F3 cells at 72h was defined as 100%. Results represent means ± SEM of three independent experiments.
A new and recurrent activating length mutation in exon 20 of the flt3 gene in acute myeloid leukemia

Karsten Spiekermann, Ksenia Bagrintseva, Claudia Schoch, Torsten Haferlach, Wolfgang Hiddemann and Susanne Schnittger