



Secondary resistance to sorafenib in two patients with acute myeloid leukemia (AML) harboring FLT3-ITD mutations

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Abstract: Dear Editor,
we report on two patients with acute myeloid leukemia (AML) initially presenting in 2007. Both patients were tested positive for a FLT3-ITD mutation located in the juxtamembrane domain and were therefore treated with the FLT3 tyrosine kinase inhibitor sorafenib (400 mg/ day) after progress of AML following several protocols of conventional chemotherapy. Both patients responded with a significant reduction of peripheral blast counts after 10 to 17 days leading to hematological response for 12 and 14 weeks, respectively. At the time of progression, molecular analysis investigating mutations of both tyrosine kinase domains by sequencing of each individual FLT3-ITD cDNA did not demonstrate any additional mutations. We therefore suggest that the secondary resistance to sorafenib is mediated by other mechanisms than the acquisition of secondary mutations of FLT3 in these patients.

Response to Reviewers: Dear Professor Ganser,

thank you and the reviewer(s) very much for reviewing our Letter to the Editor entitled „Secondary resistance to sorafenib in two patients with acute myeloid leukemia (AML) harbouring FLT3-ITD mutations“.

We carefully read the reviewer's recommendations and would like to explain our changes point by point.

1.) In patient 1, there was a new translocation t(9;18) detectable at early relapse as reported on page 3. After this, there were no further cytogenetic aberrations of AML blasts in patient 1 until progression following sorafenib treatment. In contrast, in patient 2 who initially presented with a partial monosomy 7 we could only detect leukemic blasts without chromosomal aberrations prior to start of sorafenib. These data are now introduced in the paper.

2.) In the meanwhile, we also cloned and sequenced the genomic region containing the internal tandem duplication. The sequencing data confirm that both patients carried a FLT3-ITD located in the JM domain and not in the TKD1 region.

- 3.) Unfortunately, we did not measure plasma concentrations of sorafenib or even intracellular reduction of STAT5 phosphorylation in the blasts of these patients.
- 4.) The FLT3mut/ wildtype ratio was determined in patient 1 (ratio 0,729) as well as in patient 2 at diagnosis (ratio 0,694). Indeed, in patient 1 there was a secondary loss of the FLT3 wildtype allele during AML progression. These aspects are now introduced in the manuscript.
- 5.) Indeed, the benefit of figure 2C is limited. We agree with the reviewer and introduced a schematic presentation of the receptor while the primer sequences are now indicated in the text.
- 6.) The term „relapse" was replaced by the term „progression".
- 7.) We changed the spelling between British and American English.

We would like to submit our revised version and would be happy if our manuscript could be considered for publication in Annals of Hematology.

Yours sincerely,

Sebastian Scholl on behalf of the authors

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Case report

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FLT3-ITD mutations represent the second most frequent molecular aberration in AML leading to a constitutive activity of the class III receptor tyrosine kinase FLT3. FLT3-ITD mutations can be found in 25-30% of all AML patients and are associated with a reduced disease free survival and overall survival [1-3].

The biaryl urea sorafenib (BAY 43-9006, Bayer Schering, Leverkusen, Germany) that has been approved for the treatment of several solid tumors, e.g. renal and hepatocellular carcinoma, is able to significantly inhibit FLT3 including activating FLT3 mutations like FLT3-ITD at a nanomolar concentration [4, 5]. Furthermore, *in vitro* analysis of several FLT3 inhibitors revealed a different spectrum of additional mutations that may confer resistance to these inhibitors [6]. Recently, several clinical studies investigating the feasibility and efficacy of sorafenib in a small cohort of patients with relapsed FLT3-ITD positive AML have been reported including a case report demonstrating even a molecular complete remission following sorafenib treatment in a patient with AML who relapsed after allogeneic stem cell transplantation [5, 7-9]. So far, there is no report on analyses of FLT3-ITD kinase domain sequences in patients with progress of AML after treatment with sorafenib.

Patient #1 was a 59-year-old man with AML M2 according to the French American British (FAB) classification presenting with a hyperleukocytosis at primary diagnosis. Cytogenetic and molecular genetic analyses revealed a normal karyotype and the presence of a FLT3-ITD as well as a NPM1 mutation. The patient received an induction chemotherapy consisting of idarubicine and intermediate-dosed cytarabine resulting in a complete hematologic remission of AML. Consolidation chemotherapy consisted of three courses with alternating anthracyclines (idarubicine versus mitoxantrone) combined with intermediate-dosed

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Patient #2 was a 69-year-old male patient AML (FAB M2) diagnosed in 2007. At diagnosis, AML presented with 24% bone marrow blasts and signs of dysplasia. These morphological aspects and the result of cytogenetic analysis (del7q) prompted the assumption of an antecedent myelodysplastic syndrome. The patient underwent an induction chemotherapy consisting of mitoxantrone and intermediate-dosed cytarabine resulting in a complete remission. After two courses of consolidation therapy (mitoxantrone and intermediate-dosed cytarabine) persistent complete hematological remission was demonstrated. Due to the unfavourable karyotype, preparation for an allogeneic stem cell transplantation including a reduced intensity conditioning was initiated. However, the patient unfortunately relapsed within two months after completion of the consolidation therapy. While there was no severe co-morbidity in this patient, conventional chemotherapy was continued using an age-adapted regimen containing cytarabine and cyclophosphamide but bone marrow blasts persisted. Thus, we did not pursuit a curative concept any longer and palliative cytoreductive chemotherapy was started. After failure of three consecutive cytoreductive regimens treatment with sorafenib (200 mg b.i.d.) was commenced. This patient obtained HR as shown by a

continuously decreasing number of peripheral blasts while there was no objective remission in the bone marrow. In detail, WBC declined from 59/nl prior to the start of sorafenib therapy to 1.1/nl at day 16 while a significant reduction of the proportion of peripheral blasts was observed (from 67% to 5%). Importantly, this patient had a persistent transfusion dependent anemia and thrombocytopenia. Treatment with sorafenib resulted in a stable HR for 101 days. At this time, WBC as well as peripheral blasts increased reflecting a secondary resistance to sorafenib treatment. Consecutive therapy with low dose cytarabine only led to a transient disease control. The patient died of a sepsis due to pneumonia and severe infectious colitis.

In both patients, a FLT3-ITD mutation was detected at diagnosis on the level of genomic DNA. We therefore confirmed the stability of these molecular aberrations before the treatment with sorafenib was started. Furthermore, FLT3-ITD expression was detected on cDNA level as well in both patients. At the time of progression during sorafenib therapy, two cDNA fragments were cloned containing the entire sequences of the tyrosine kinase domain (TKD) 1 and 2 of FLT3 in order to elucidate TKD mutations that might confer resistance to the tyrosine kinase inhibitor (Figure 2C). Since sequence analyses of several clones were not able to demonstrate any additional mutation within one of the FLT3-TKD's when both patients became refractory to sorafenib, an additional methodological approach was performed. In detail, direct sequencing of cDNA fragments with primers spanning either TKD1 or 2 did also not reveal further mutations of FLT3-ITD in both patients.

The course of these two patients treated with sorafenib shows not only the response to a monotherapy with this tyrosine kinase inhibitor after failure of different regimens with conventional chemotherapy. We can in particular demonstrate that sequence analysis after cloning of each individual FLT3-ITD allele did not reveal additional mutations within the tyrosine kinase domains. In contrast to *in vitro* studies with inhibitors of FLT3-ITD, we can show that apart from the acquisition of TKD mutations additional mechanisms may be responsible for the secondary resistance to sorafenib in AML patients.

Such target-independent mechanisms of resistance to tyrosine kinase inhibitors have already been demonstrated in the treatment of chronic myelogenous leukemia with imatinib. These mechanisms that are not mediated by mutations of the target gene and include clonal cytogenetic aberrations, activation of growth factor receptors, and drug influx and efflux pumps [10-13]. To our knowledge, there are no data about such mechanisms in AML patients who were treated with a FLT3 inhibitor. Sequence analysis as demonstrated in our patients let

us hypothesise that secondary resistance to kinase inhibitors like sorafenib might also be mediated by such target-independent mechanisms in FLT3-ITD positive AML.

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Figure legends

Figure 1

Time course of peripheral blood counts during sorafenib treatment in patient 1 and 2.

WBC (/nl), leukemic blasts (%), platelets (/nl), hemoglobin (mmol/l)

Figure 2

Detection of FLT3-ITD and sequence analysis in AML patients refractory to sorafenib.

FLT3-ITD could be detected by PCR on the level of genomic DNA (gDNA) as well as using cDNA (A and B, respectively) in leukaemic blasts at relapse prior to start of sorafenib treatment in both patients. M, 1kb marker, “-“, negative control (THP-1 cells), “+”, positive control (MV4-11 cells), #1, patient 1, #2, patient 2. C, sequence analysis to detect additional mutations were performed using cDNA isolated from AML blasts at the time of secondary resistance. Two primer pairs spanning both tyrosine kinase domains (TKD) of FLT3 are marked in bold or underlined (TKD1 and 2, respectively).

Figure 1

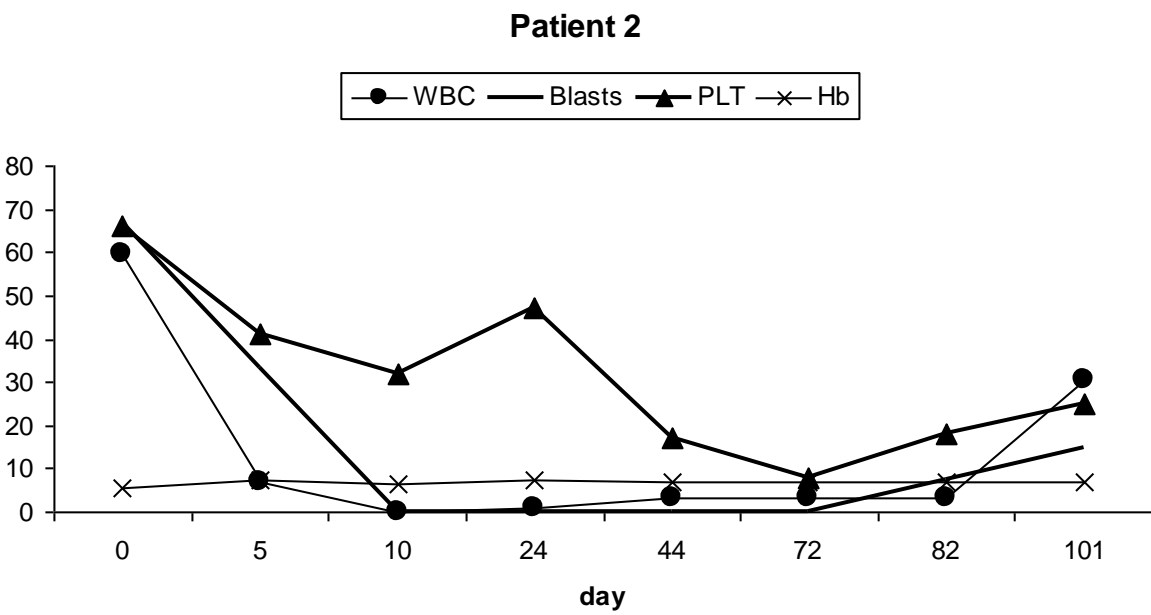
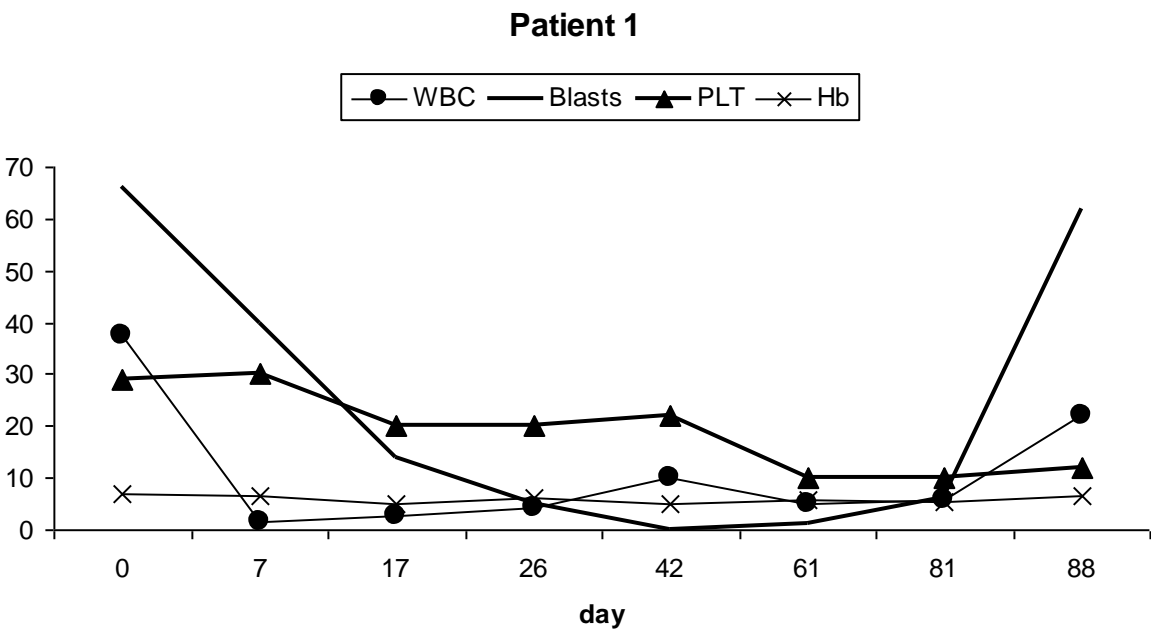
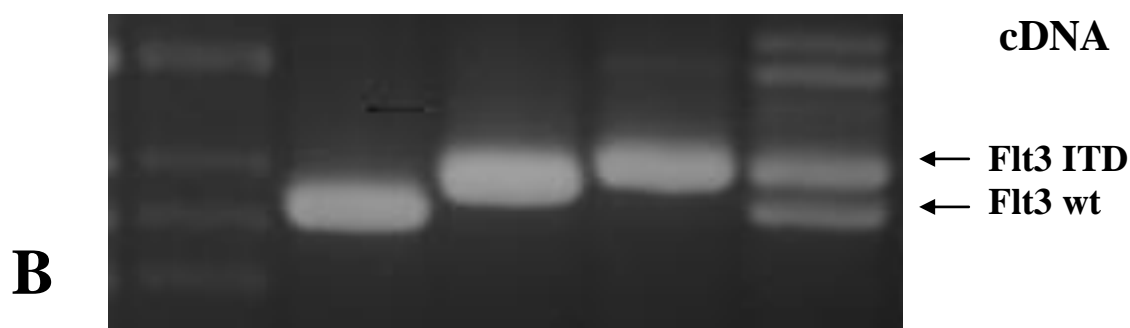
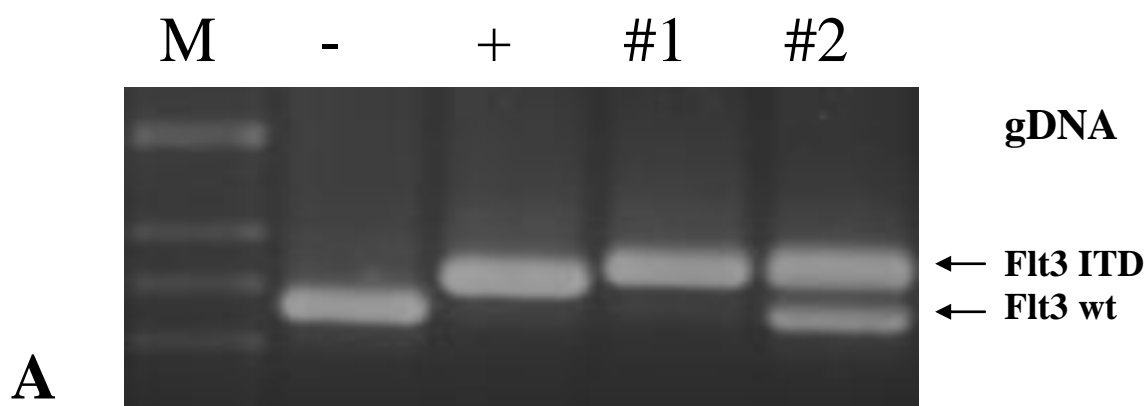


Figure 2



C

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1861 tttcagagaa tatgaatatg atctcaaattg ggagtttcca agagaaaatt tagagtgttg
1921 gaaggtacta ggatcagggtg cttttggaaa agtgatgaac gcaacagctt atggaattag
1981 caaaacagga gtctcaatcc aggttgccgt caaaatgctg aaagaaaaag cagacagctc
2041 tgaaagagag gcactcatgt cagaactcaa gatgatgacc cagctgggaa gccacgagaa
2101 tattgtgaac ctgctggggg cgtgcacact gtcaggacca atttacttga tttttgaata
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2221 ttggacagag attttcaagg aacacaattt cagtttttac cccactttcc aatcacatcc
2281 aaattccagc atgcctgggt caagagaagt tcagatacac ccggactcgg atcaaatctc
2341 agggcttcat gggaattcat ttcactctga agatgaaatt gaatatgaaa accaaaaaag
2401 gctggaagaa gaggaggact tgaatgtgct tacatttgaa gatcttcttt gctttgcata
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2701 atatggaata ttactgtggg aaatcttctc acttggtgtg aatccttacc ctggcattcc
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2941 gatgtatcag aatgtggatg gccgtgtttc ggaatgtcct cacacctacc aaaacaggcg

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Figure legends

Figure 1

Time course of peripheral blood counts during sorafenib treatment in patient 1 and 2.

WBC (/nl), leukemic blasts (%), platelets (/nl), hemoglobin (mmol/l)

Figure 2

Detection of FLT3-ITD and sequence analysis in AML patients refractory to sorafenib.

FLT3-ITD could be detected by PCR on the level of genomic DNA (gDNA) as well as using cDNA (A and B, respectively) in leukaemic blasts at progression prior to start of sorafenib treatment in both patients. M, 1kb marker, “-“, negative control (THP-1 cells), “+”, positive control (MV4-11 cells), #1, patient 1, #2, patient 2. C, Schematic illustration of FLT3 receptor tyrososine kinase with its different domains: EC, extracellular domain; TD, transmembrane domain; JM, juxtamembrane; TKD, tyrosine kinase domain; KI, kinase insert. The arrows indicate the primers used for cloning TKD1 (TKD1fw GACCGGCTCCTCAGATAATGAG, TKD1rv CAGGTCTCTGTGAACACACGAC) and TKD2 (TKD1fw TCCAGCATGCCTGGTTCAAGAG, TKD1rv GTGAGGACATTCCGAAACAC), respectively.

Figure 1

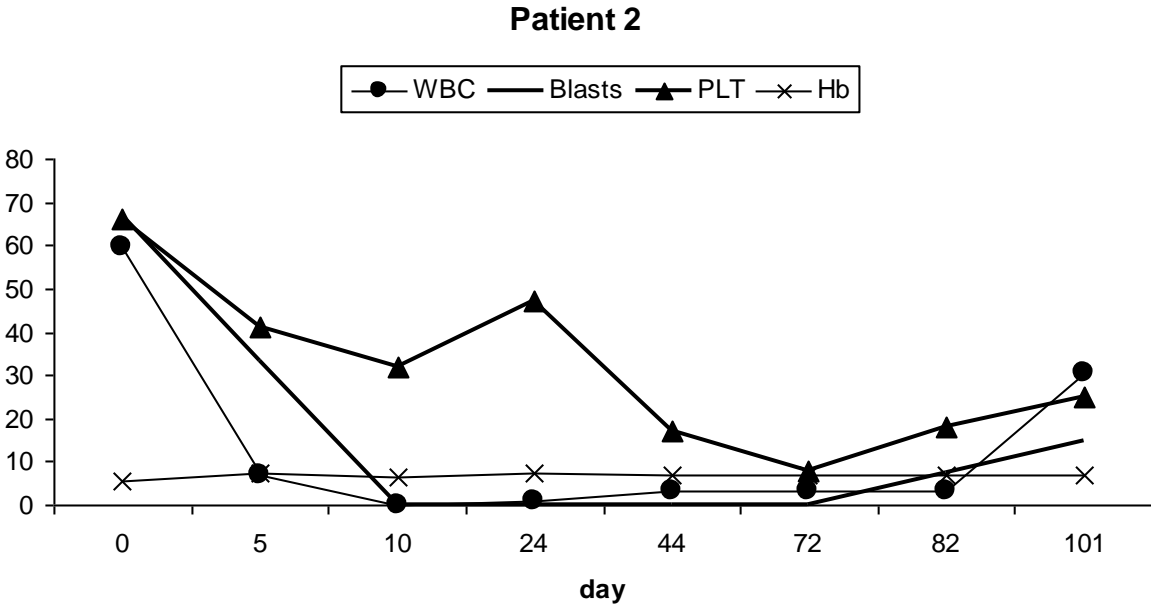
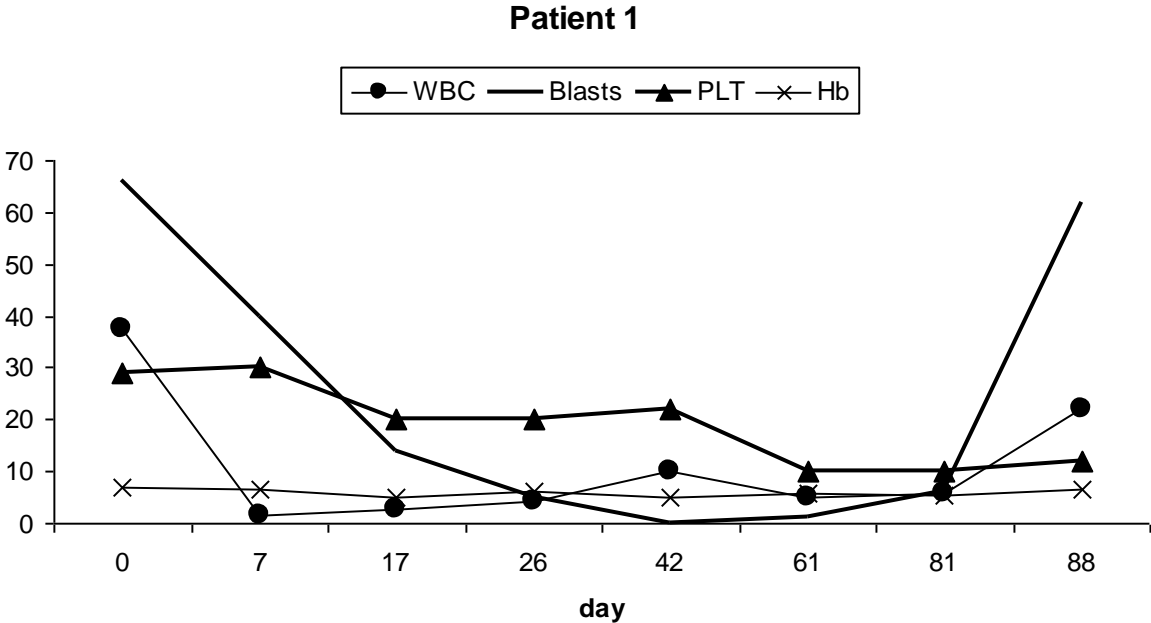


Figure 2

