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# Reversal of FLT3 Mutational Status and Sustained Expression of NPM1 Mutation in Paired Presentation, and Relapse Samples in a Patient with Acute Myeloid Leukemia

Milica Radojkovic[1,2], Natasa Tosic[4], Natasa Colovic[1,3], Slobodan Ristic[1,2], Sonja Pavlovic[4], and Milica Colovic[1,3]

[1]Medical Faculty, University of Belgrade, Belgrade, Serbia; [2]Clinical Center Dr Dragisa Misovic, Belgrade, Serbia; [3]Institute of Hematology, Clinical Center of Serbia, Belgrade, Serbia; [4]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

**Abstract.** We report a case of *de novo* acute myeloid leukemia (AML) with unstable FLT3 gene mutations and stable NPM1 mutation. FLT3/D835 and NPM1 (Type A) mutations were detected upon diagnosis. During the relapse, the FLT3/D835 mutation changed to an FLT3/ITD mutation while the NPM1 (Type A) mutation was retained. Cytogenetic analyses showed the normal karyotype at diagnosis and relapse. Our findings raise interesting questions about the significance of these mutations in the leukemogenic process, about their stability during the evolution of the disease, and regarding the selection of appropriate molecular markers for the monitoring of minimal residual disease.

**Key words:** acute myeloid leukemia, FLT3, NPM1, relapse

## Introduction

To date, karyotype abnormalities remain the most important prognostic information in acute myeloid leukemia (AML), allowing the application of appropriate risk-adapted treatment protocols. However, the most common cytogenetic group – comprising 40-50% of adult AML – is normal karyotype AML (NK-AML), associated with intermediate prognosis [1]. Recently, a number of genetic mutations have been identified in this cytogenetic subset of AML [2]. These new genetic markers showed substantial prognostic relevance and reilability for minimal residual disease monitoring.

Two distinct types of FMS-like tyrosine kinase 3 (FLT3) activating gene mutations have been described in patients with AML. More than 20% of FLT3 mutations involve an internal tandem duplication (ITD) in the juxtamembrane domain of the FLT3 gene (FLT3/ITD) [3,4]; an additional 5-10% carry a point mutation in the activation loop of the tirosine kinase domain (TKD). The most frequent FLT3 point mutation occurs in the second TKD, codon 835. Missense point mutations that substitute the aspartic acid in codon 835 (FLT3/D835) have been reported in about 7% of adult AML patients [5]. FLT3/ITD mutations have been associated with leukocytosis, a high percentage of bone marrow blast cells, increased risk of treatment failure, increased risk of relapse after complete remission (CR), and reduced overall survival (OS) [3, 5]. The prognostic impact of FLT3 point mutations is less evident, but it appears that alteration of D835 correlates with worse disease free survival (DFS) [6].

More recently, mutations of the nucleophosmin gene (NPM1), causing cytoplasmic localization of the NPM protein, have been described [2]. Acquired mutations in exon 12 of the NPM1 gene have been reported in 35% of adult AML patients and in up to 50% of NK-AML cases [2, 7]. The presence of NPM1 mutations is associated with favorable outcomes and increased DFS and OS, but only in cases when they are not in tandem with FLT3/ITD mutations [7].

Address correspondence to Milica Radojkovic, MD; Clinical Center Dr Dragisa Misovic, Belgrade, Serbia; Clinic of Internal medicine, NH Milana Tepica 1, 11000 Belgrade, Serbia; tel +381 11 3630618; fax +381 11 3630623; e-mail: radojkov@EUnet.rs

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**WT** QFRYESQLQMVQVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF

**Patient** QFRYESQLQMVQVTGSSDNEYFYVDFREYEYDLKW***GT*****SDNEYFYVDFREYEYDL KWEFPRENLEF**

***Figure 1A.*** Alignment of the wild-type exon 14 encoded sequence of *FLT3* gene (WT) and mutant amino acid sequences in presented case (63bp duplication). The original wild-type and duplicated sequence are shown in underlined and bold text and the italicized, larger letters indicate the novel amino-acids.

## Case Report

A 57-year-old woman noticed weakness, fatigue, and bruising of the skin in February 2004. The year before, she had been treated with tuberculostatics (rifamore, miambutol, isoniasid) for miliary tuberculosis. The patient had suffered from hypertension for 10 years and had absolute arrhythmia. The physical examination showed pale skin, hemorrhagic syndrome in the skin of the lower extremities (petechiae and hematomas), and an enlarged liver (2cm below the right costal margin). On cardiac examination tachycardia and absolute arrhythmia were observed (heart beat: 116/min, TA: 160/100mmHg)

A complete blood cell count and peripheral blood smear showed hemoglobin of 85g/L, white blood cell (WBC) and platelet (Plt.) counts of $2.8\times10^9$/L and $32\times10^9$/L, respectively, with 9% myeloblasts, 2% myelocytes, 29% segmented, 8% eosinophils, 36% lymphocytes, and 16% monocytes in differentia leukocyte formula. The bone marrow aspirate was hypercellular, with diffuse infiltration of leukemic myeloblasts (69%), of which 10% were positive for myeloperoxidase. Immunophenotyping of bone marrow mononuclear cells showed positive expression of CD34 (52%), CD117 (54%), CD13 (58%), CD33 (62%), and CD7 (49%), which suggested acute myeloid leukemia M5a lineage with CD7 antigen coexpression. A cytogenetic analysis showed that the karyotype of the bone marrow cells was normal (46XX). Laboratory analyses were normal except for a slightly elevated lactate dehydrogenase of 488 IU/L (normal range 160-410 IU/L).

Radiographic finding in the lungs was normal. Ultrasound examination of the abdomen showed normal structure and size of the liver and spleen. After installation of a central venous line, the patient received chemotherapy according to protocol ADE in the following doses: doxorubicin 90 mg iv on days 1, 3, 5; cytosine arabinoside 180 mg bid over 8 days by a continuous infusion; etoposide 200 mg iv on days 1-5. After a period of iatrogenic aplasia, the patient achieved complete remission in May 2004. According to MRC 10 protocol [8], after establishing remission the patient received one course of consolidation of the ADE protocol, one course of MACE chemotherapy (amsacrine, cytarabine, etoposide), and then one cycle of MIDAC therapy (mitoxantrone, cytarabine). Remission lasted until the beginning of April 2005, when the disease recurred. Laboratory analysis of peripheral blood showed pancytopenia (Hb. 58 g/l, WBC 1.9 $\times10^9$/L, Plt. $8\times10^9$/L), elevated LDH of 541 IU/L. In normocelular bone marrow 19% blasts were present with the same characteristics as at diagnosis. Cytogenetic findings were normal 46XX. In the relapse, the patient was treated with cytosar and vepesid without achieving remission. During the recurrence, the number of leukocytes and the percentage of blasts in peripheral blood increased. The peripheral blood findings were as follows: Hb 75 g/l, WBC 40.0$\times10^9$/L, Plt. 26$\times10^9$/L, 42% myeloblasts, and 24% monocytes. The patient was treated with palliative cytostatic therapy and occasional red cell and platelet transfusions until August 2006.

Molecular analysis at the time of diagnosis detected FLT3/D835 and NPM1 (Type A) mutations. In contrast, the analyses of AML relapse cells showed a loss of the FLT3/D835 mutation and emergence of the FLT3/ITD mutation while the NPM1 (Type A) mutation was retained.

***Detection of the FLT3/ITD mutations.*** To detect FLT3 mutations, genomic DNA was isolated from bone marrow using QIAamp DNA Blood Mini

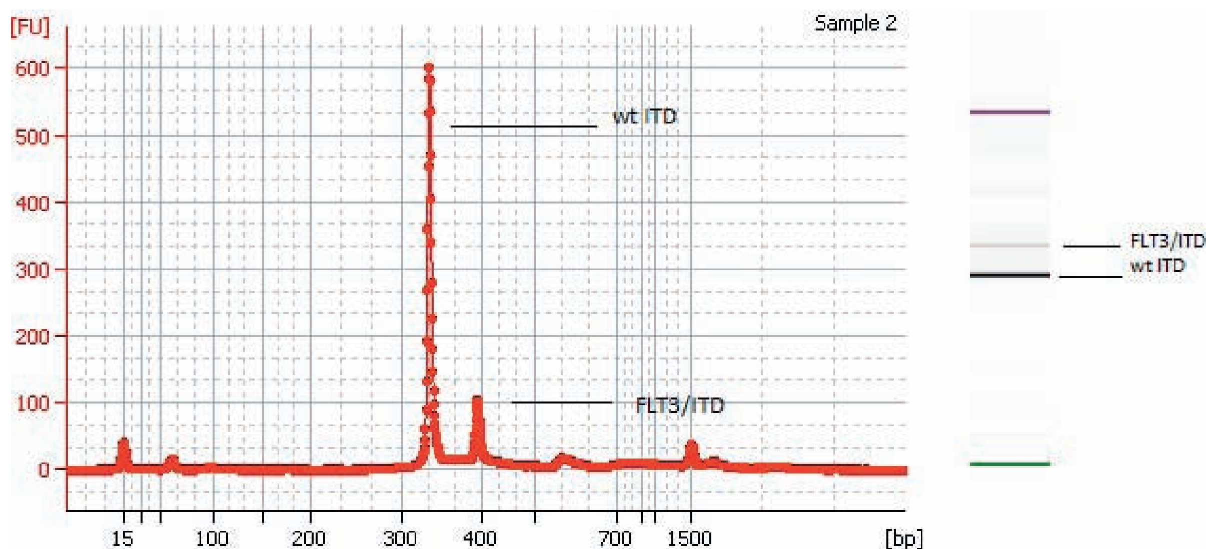


***Figure 1B.*** Relative mutant to wt level of FLT3/ITD mutations (ITD/wt) was determined using Lab-on-Chip technology (Agilent Technology). The ITD/wt ratio was very low at 0.41.

Kit (Qiagen, Gremany). The PCR amplification was carried-out as previously described [9] and its products were resolved on 4% agarose gel stained with ethidium bromide. Each sample displaying an additional PCR product (longer than 325bp) was considered as indicating the presence of internal tandem duplication. Additional bands were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, Germany) and were directly sequenced. Sequencing analysis of the duplication revealed in-frame duplication of 63 bp in exon 14, involving the juxta-membrane domain of FLT3 **(Figure 1A)**. Relative mutant to wt level of FLT3/ITD mutations (ITD/wt) was determined using Lab-on-Chip technology (Agilent Technology). The ITD/wt ratio vas very low at 0.41 **(Figure 1B)**.

***Detection of FLT3 /TKD mutations.***

Analysis of the FLT3/D835 mutation was carried out as follows: exon 20 of the FLT3 gene was amplified by genomic DNA PCR as previously reported [10]. The PCR products digested with EcoRV (Biolabs, England) were resolved on the 8% polyacrilamide gel.

***Detection of NPM1 gene mutations.*** For the screening of NPM mutations, we amplified genomic DNA corresponding to exon 12 of the NPM1 gene using PCR and direct sequencing method as previously described [2].

## Discussion

We report a case of 57-year old female patient with *de novo* AML-M5a, initially presenting with normal cytogenetic, FLT3/D835, and NPM1 (Type A) mutations. When relapse occurred FLT3/D835 mutation was lost, and a new FLT3/ITD mutation emerged, while the NPM1 mutation and normal cytogenetic was retained.

Several studies confirmed the instability of FLT3 mutations in AML, most of them focusing on the FLT3/ITD mutations. Their common conclusion is that changes in the FLT3 mutation pattern are not rare, and that they may reflect the outgrowth of a mutant clone or evolution of a new leukemic clone [11]. In a large study of 3082 AML patients, from the 13 cases that were positive for FLT3/D835 mutation at diagnosis, 9 (69%) lost the mutation at relapse [12]. Shih LY *et al.* [13] found that 8 of 13 AML patients carrying FLT3/D835 mutations at diagnosis did not have detectable mutations at relapse, and in one of them the loss of the FLT3/D835 mutation was followed by the gain of the FLT3/ITD mutation. It seems that the loss of FLT3/D835 mutations during the relapse of the

disease are not such rare events and may be associated with gain of FLT3/ITD mutations.

The changes in the FLT3 mutational status can be explained by a few possible events. First, along with the dominant FLT3/D835 subclone, a FLT3/ITD oligoclone may have been present upon diagnosis, though below the limits of detection. Malignant cells bearing the FLT3/D835 mutation may be lost during chemotherapy, due to eradication of the leukemic clone carrying the FLT3/D835 mutations. On the other hand, the FLT3/ITD subclone can expand after therapy, eventually mediating relapse. In the recently published data by McCormick SR *et al.* [14], FLT3 mutational status differed at relapse and diagnosis in 22% of the patients, with a trend towards the loss of FLT/TDK mutations and gain of FLT3/ITD mutations, which may be important for the development of AML relapse.

Secondly, the gain of FLT3/ITD as de novo mutation may represent the result of a genotoxic effect of treatment that increases the incidence of DNA replication error and induces new mutations due to chemotherapy [15]. The third and least likely explanation of the new FLT3 abnormalities at the time of relapse is that they could represent another event in the process of leukemogenesis due to unstable leukemic clones [10].

Contrary to the FLT3 mutations, the NPM1 mutations show remarkable stability during the course of the disease [16, 17]. There are some reported cases in which the same NPM1 mutation was retained even in the late relapse of the disease (<5 years) [18]. Detecting at the time of late relapse the same genetic alteration as at diagnosis strongly suggests that NPM1 mutations are needed for leukemia growth and survival and that they play a critical role in leukemogenesis.

In conclusion, our report of stable NPM1 and rare FLT3/D835 to FLT3/ITD mutational change during the course of the disease offers more compelling clinical evidence of oligoclonality in AML and of the selection of chemoresitant subclones in the relapse of the disease. In addition, the instability of FLT3 mutations vs. the stability of NPM1 mutations has great implications for the monitoring of minimal residual disease in AML.

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