

Importance of early detection and follow-up of FLT3 mutations in patients with acute myeloid leukemia

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Received: 12 March 2007 / Accepted: 28 May 2007 / Published online: 20 June 2007
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Abstract Mutations in the *fms*-like tyrosine kinase 3 (FLT3) gene, such as internal tandem duplication (FLT3/ITD) in the juxtamembrane domain and point mutations in the tyrosine kinase domain, are the most common abnormalities in acute myeloid leukemia (AML). FLT3/ITD and FLT3/D835 mutations were analyzed in 113 Serbian adult AML patients using polymerase chain reaction. Twenty patients were found to be FLT3/ITD positive (17.7%). The mutations occurred most frequently in M5 and M0 subtypes of AML. They were mainly associated with the normal karyotype. All patients harboring FLT3/ITD had a higher number of white blood cells than patients without it ($p=0.027$). FLT3/ITD mutations were associated with lower complete remission (CR) rate ($\chi^2=5.706$; $p=0.017$) and shorter overall survival (OS; Log rank=8.76; $p=0.0031$). As for disease-free survival, the difference between FLT3/ITD-positive and FLT3/ITD-negative patients was not statistically significant (Log rank=0.78; $p=0.3764$). In multivariate analysis, the presence of FLT3/ITD mutations

was the most significant prognostic factor for both OS and CR rate ($p=0.0287$; relative risk=1.73; 95% CI=1.06–2.82). However, in the group of patients with the intermediate-risk karyotype, the mere presence of FLT3/ITD was not associated with inferior clinical outcome. FLT3/D835 point mutation was found in four patients (3.5%) only. Follow-up of the FLT3/ITD-positive patients revealed stability of this mutation during the course of the disease. However, changes in the pattern of FLT3/D835 mutations in initial and relapsed AML were observed. Our results indicate an association of FLT3/ITD with the adverse outcome in AML patients treated with standard induction chemotherapy. Because FLT3/ITD mutation is a target for specific therapeutic inhibition, its early detection could be helpful in clinical practice.

Keywords AML · FLT3 mutations · Prognostic significance · Paired initial and relapsed samples

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Introduction

The *fms*-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase, with a structure that resembles KIT, FMS, and the platelet-derived growth factor receptor [21, 28, 39]. It is predominantly expressed on hematopoietic progenitor cells, but it is also found in a variety of hematological malignancies including acute myeloid leukemia (AML), B-precursor cell acute lymphoblastic leukemia (ALL), a fraction of T cell ALL, myelodysplastic syndrome in leukemic transformation, and chronic myelogenous leukemia in blast crisis [8, 29]. FLT3 plays an important role in stem cell proliferation, differentiation, and survival. In normal hematopoiesis, FLT3 ligand binding to the FLT3 receptor causes dimerization of the receptor,

autophosphorylation, activation of tyrosine kinase, and induction of multiple intracellular signaling pathways, which are involved in cell proliferation and leukemogenesis [10, 15].

The presence of a mutation in the FLT3 gene results in FLT3 ligand-independent kinase activation, leading to increased and uncontrolled cell proliferation [15, 23].

The most common mutation in the FLT3 gene is an internal tandem duplication (FLT3/ITD) of the region coding for the juxtamembrane (JM) domain of the FLT3 receptor [14]. It can be found in approximately 20–30% of adult AML patients [20]. The frequency among children is much lower, approximately 5–22% [43]. The presence of a FLT3/ITD mutation is clearly associated with poor prognosis in both groups of the patients.

The second most common type of FLT3 mutations are mis-sense point mutations in the tyrosine kinase domain (TKD). These mutations also promote constitutive phosphorylation of the receptor [2, 36, 42]. The most frequent are point mutations and deletions of codons 835/836 in second TKD, but there are reports on the presence of a 6-bp insertion between codons 840 and 841 (FLT3-840GS) [36] and two novel point mutations N841I and N841Y [16]. Mis-sense mutations were also identified in the JM domain [37] and in the first TKD of FLT3 [25, 31].

Mis-sense point mutations that substitute the aspartic acid in codon 835 have been reported in about 7% of AML patients [2, 42]. Although these point mutations, similar to the FLT3/ITD mutations, cause ligand-independent activation of the FLT3 receptor, their impact on the clinical outcome is not clear. In some studies, FLT3/D835 mutations are associated with shorter disease-free survival (DFS) and relative reduction in overall survival (OS) [2, 42]. On the other hand, in one large study by Thiede et al. [38] on 979 AML patients, no significant differences in DFS or OS could be established between patients harboring FLT3/D835 and patients without the mutation. There is a possibility that the less clear impact of the FLT3/D835 mutation on prognosis is due to a low number of patients with this mutation available for analysis or due to its different influence on cell signaling compared to FLT3/ITD [12].

We have examined biological and hematological characteristics of 113 adult AML patients treated in the Institute of Hematology, Clinical Center of Serbia. We have also analyzed the impact of FLT3 mutations on clinical outcome in these patients, who were followed up for 52 months, with some patients following up for 6 years.

Materials and methods

Patients and treatment

Bone marrow or peripheral blood was obtained at diagnosis from 113 patients (66 female and 47 male) after signed

informed consent. Patients with acute promyelocytic leukemia received treatment according to the Pethema/LPA99 protocol (induction therapy: idarubicin 12 mg/m² on days 2, 4, 6, and 8 and all-transretinoic acid [ATRA] 45 mg/m² on day 1 until complete remission [CR]), consolidation with idarubicin, novantrone, and ATRA for four cycles, and maintenance chemotherapy with 6-mercaptopurin, methotrexate, and ATRA [30]. All other AML patients received induction and consolidation chemotherapy with standard doses of daunorubicin and cytarabine according to the protocol 3+7 (Ara-C 200 mg/m² for 7 days in continuous infusion and 3 days doxorubicin 50 mg/m² (cycle I) followed by a second cycle of protocol ADE in which etoposide 200 mg/m² was added for 5 days. After the ADE protocol, the patients received two courses of consolidation therapy of MACE (amsacrine, cytarabine, etoposide) and MIDAC (mitozantrone, cytarabine) according to the MRC10 protocol [40]. Patients who achieved CR were off from the treatment except for four patients: one who underwent an allogeneic stem cell transplant from an unrelated donor and three patients that were transplanted from a matched sibling donor.

Cytogenetic analyses

Cytogenetic G-banding analysis was performed with standard methods. The definition and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature [22]. As cytogenetic finding is thought to be one of the most important prognostic factor for AML patients, they were stratified in three groups: the favorable-risk group consisting of patients with karyotypes of t(8;21), t(15;17), or inv(16), an adverse-risk group defined by -5, del (5q), -7, abnormal 3q, or the presence of a complex karyotype with more than three aberrations, and an intermediate-risk group, which included patients with a normal karyotype and patients with a trisomy of chromosome 8, 21, or 22, alterations in the 11q23 region, del (7q), and del (9q) [11].

Flow cytometry

Immunophenotypic analysis was carried out with flow cytometry FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) using monoclonal antibodies against human common leukocyte antigens CD45, HLADR, CD34, CD33, CD13, CD117 (c-kit), CD15, CD11b, CD11c, CD41, CD61, and glycoporphin A. Analysis was done using the Cell Quest software package (Becton Dickinson). Using flow cytometry and cytological findings, we classified patients in eight subgroups according to French–American–British (FAB) classification.

Detection of FLT3 mutations by PCR

Genomic deoxyribonucleic acid (DNA) was isolated from mononuclear cell preparations using GFX genomic Blood DNA Purification Kit (Amersham Biosciences, GE Healthcare, UK) according to the manufacturer's recommendations.

As the location of FLT3/ITDs is restricted to exons 14 and 15, the polymerase chain reaction (PCR) amplification was carried out as previously described [17], and its products were resolved on a 4% agarose gel stained with ethidium bromide. Each sample displaying an additional PCR product (longer than 325 bp) was considered as the one containing the internal tandem duplication. Longer PCR products were directly sequenced by the same primers used for amplification.

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 [42]. The PCR products digested with *EcoRV* (Biolabs, England) were resolved on the 8% polyacrylamide gel.

Statistical analysis

Data are presented as medians with range or as absolute numbers with percentages. OS was calculated from the first day of therapy to death. CR duration was calculated from the date of CR to relapse. DFS for patients who had achieved CR was measured from the date of CR to relapse or death. OS and DFS curves were computed according to the Kaplan–Meier method using the SPSS computer software and the rates of survival were compared by log-rank test. Relative risks (RR) and 95% confidence intervals (CI) were estimated with the multivariate Cox's regression model.

Results

Prevalence and association of FLT3 mutations with FAB subtypes and biological characteristics

Of 113 AML patients studied, 20 had the FLT3/ITD mutation (17.7%). The FLT3/D835 mutation was detected only in 4 of 113 (3.5%) patients (Table 1). One patient with FAB subtype M5 showed both FLT3/ITD and FLT3/D835 mutations. The patients ranged in age from 16 to 80 years with a median age of 50 years. There was no statistically significant difference in age and gender between the patients harboring mutations in the FLT3 gene and patients with no FLT3 mutation.

Patients were classified according to the FAB criteria [4]: 12 M0 (11%), 15 M1 (13%), 23 M2 (20%), 13 M3 (12%), 1 M3 var (1%), 23 M4 (20%), 17 M5a (15%), 2 M5b (2%), 2 M6 (2%), and 2 M7 (2%), and three patients had biphenotypic leukemia (2%; Table 1). The majority of FLT3 mutations were detected in patients with M0 6 of 12 (50%), M5 8 of 19 (40.3%), M3 3 of 14 (21.4%), M2 4 of 23 (18.0%), and M4 3 of 23 (12.5%; Table 1).

Of the 113 patients, cytogenetic analysis was successful in 99 patients. They were divided in three risk groups according to cytogenetics (favorable, intermediate, and adverse).

Sixty patients had abnormalities associated with intermediate risk, 25 were stratified in the favorable-risk group (14 patients t(15;17) and 11 inv (16)), and 14 were with adverse-risk karyotypes. The rate of FLT3 mutations was higher among patients with the intermediate-risk karyotype (13 of 21; 62%) compared to patients with the adverse-risk

Table 1 Laboratory and clinical data of patients stratified according to the FLT3 mutations

	FLT3/ITD ⁺	FLT3/D835 ⁺	FLT3 negative
Total number	20	4	89
Median age in years (range)	49.1 (22–72)	48 (45–53)	50.7 (16–80)
Number of patients <60 years old	12/20	0/4	58/89
Gender (M/F)	9/11	2/2	36/53
Mean WBC × 10 ⁹ /l (range)	67.3 (2.1–287)	48.0 (1.7–100)	28.0 (0.8–210)
FAB type			
Biphenotypic	0	0	3
M0	6	0	6
M1	0	0	15
M2	3	1	19
M3	3	0	11
M4	1	2	20
M5	7	1	11
M6	0	0	2
M7	0	0	2
Cytogenetic risk groups ^a			
Favorable	4	1	20
Intermediate	10	3	47
Adverse	3	0	11

^a Cytogenetic stratification was performed according to the criteria described in "Materials and methods."

karyotype (3 of 21; 14%). In the intermediate-risk group, 12 patients had the normal karyotype, and one had trisomy 8.

White blood cell (WBC) counts were significantly higher in patients harboring FLT3/ITD (mean $67.3 \times 10^9/l$) than in patients without it (mean $28 \times 10^9/l$; $p=0.027$). There was not a significant difference in the mean number of WBCs between patients with the FLT3/D835 mutation and patients with no FLT3 mutations.

There was a statistically significant increase in percentage of bone marrow blasts depending on the presence of the FLT3/ITD mutation in subtypes M3, M4, and M5 ($p=0.018$; Table 2).

Impact of FLT3 mutations on clinical outcome in patients with AML

CR was obtained in 8 of 24 (33.3%) patients with FLT3 mutations. The rate of CR in those patients was significantly lower when compared with the patients who had no FLT3 mutations (CR 54 of 89, 60.7%). This difference was statistically significant ($\chi^2=5.706$; $p=0.017$).

Median duration of remission in the whole group of patients was 12 months (mean 17.5), vs 10 months (mean 13.5) in FLT3/ITD-positive patients. There was no statistically significant difference in DFS between FLT3/ITD-negative and FLT3/ITD-positive patients (mean=13.75; median=10.00; Log rank=0.78; $p=0.3764$; Fig. 1).

Median survival was 4 months (95% CI 2.09–5.91) for patients with FLT3/ITD mutation comparing to 12 months (95% CI 8.79–15.21) for the patients with no FLT3/ITD mutation.

The OS curve (Fig. 2) suggested a significantly shorter OS in AML patients carrying the FLT3/ITD mutation comparing to those without it. The difference was statistically significant (Log rank=8.76; $p=0.0031$).

In multivariate analysis, the presence of the mutation was the most significant prognostic factor for both OS and CR rate ($p=0.0287$; RR=1.73; 95% CI=1.06–2.82).

As the intermediate-risk disease represents the largest group of patients lacking additional prognostic markers, we have analyzed these patients separately. No statistical significance in DFS was observed between FLT3/ITD-

Table 2 Percentage (%) of bone marrow blasts in AML subtypes depending on the presence of FLT3 mutations

	FLT3/ITD and FLT3/D835 positive (%)	FLT3/ITD and FLT3/D835 negative (%)
M0	83	86
M2	57	66
M3	95	85
M4	92	74
M5	82	70

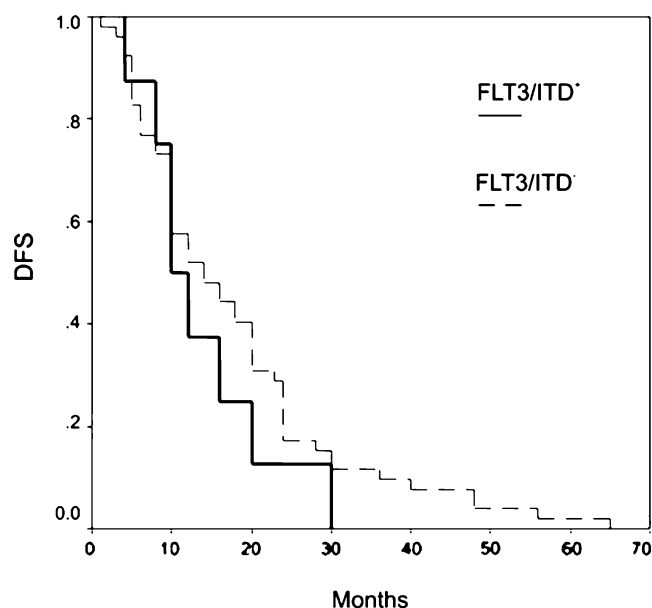


Fig. 1 Kaplan–Meier curves of the effects of FLT3/ITDs on DFS of all AML patients included in the study. *Solid line*, FLT3/ITD-positive patients; *dashed line*, FLT3/ITD-negative patients

negative and FLT3/ITD-positive patients (Log rank=0.29; $p=0.5904$; Fig. 3). In this group of patients, OS was shortened in the patients with the FLT3/ITD mutation compared to the ones with no FLT3/ITD (5 vs 12 months), but this difference did not reach statistical significance ($p=0.0723$ by the log-rank test; Fig. 4). Multivariate analysis showed that the FLT3/ITD state is not an independent prognostic factor for DFS or OS in the intermediate risk group of AML patients ($p=0.6176$ and $p=0.0878$, respectively).

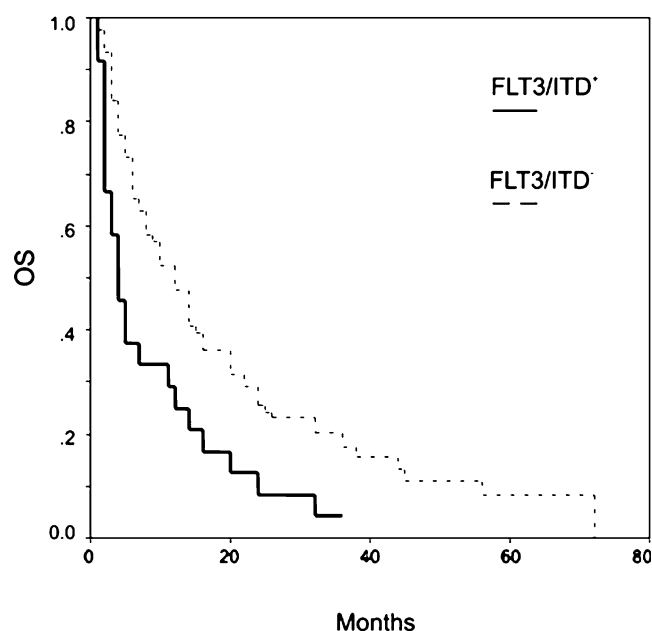


Fig. 2 Kaplan–Meier curves of the effects of FLT3/ITDs on OS of all AML patients included in the study. *Solid line*, FLT3/ITD-positive patients; *dashed line*, FLT3/ITD-negative patients

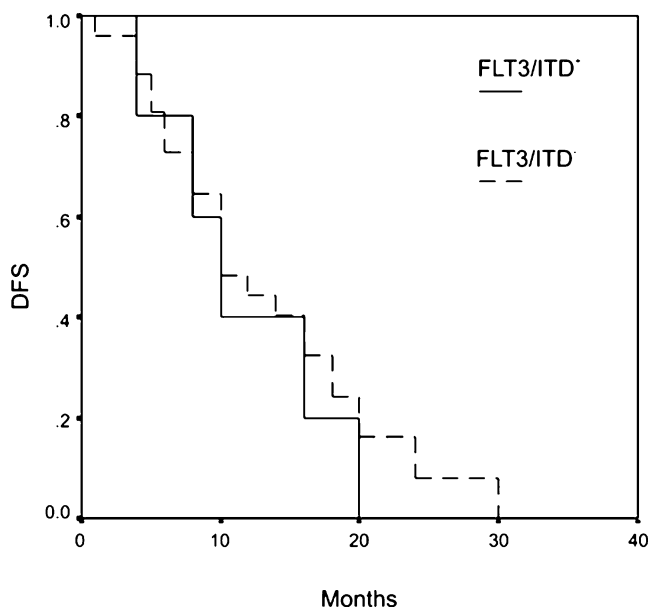


Fig. 3 Kaplan–Meier curves of the effects of FLT3/ITDs on DFS of AML patients with the intermediate-risk karyotype. *Solid line*, FLT3/ITD-positive patients; *dashed line*, FLT3/ITD-negative patients

Because of the limited number of patients with the FLT3/D835 mutation (only four cases), reliable conclusions on survival data could not be drawn.

One patient with AML, subtype M5, with hyperleukocytosis ($185 \times 10^9/l$) had both FLT3/ITD and FLT3/D835 mutations at the same time. That patient did not achieve CR and died 4 months after diagnosis.

Patients harboring FLT3 mutations were followed up in this study. Most of those who achieved CR (8 of 24) maintained the same FLT3 status at presentation and relapse (six of eight). All of the FLT3/ITD-positive patients preserved the same pattern of the mutation during the course of the disease. However, one patient with the FLT3/D835 mutation and *inv(16)* lost the FLT3/D835 mutation at relapse (after 7 months). This patient survived for 14 months with palliative treatment [7]. Another patient with a M5a subtype of the disease and normal cytogenetics was positive for the FLT3/D835 mutation at the diagnosis and lost this type of mutation during relapse (after 8 months) but acquired two different FLT3/ITD (duplications of 63 bp and 46 bp) mutations. The patient survived for 26 months.

Discussion

Mutations in the FLT3 gene are one of the most frequent somatic alterations in AML patients [18, 42]. A number of reports have shown that some of these mutations are an independent adverse prognostic factor in AML. This has led to the general belief that FLT3 mutations are an important molecular target for the development of specific therapeutics against the constitutively activated FLT3 receptor. Different treatment protocols reported in various

studies of prognostic significance of FLT3 mutations in patients with AML were useful in designing treatment protocols used in several ongoing or planned trials. Therefore, early detection of FLT3 mutations became a standard diagnostic procedure for AML patients in several clinical centers.

We have investigated the prevalence of FLT3/ITD and FLT3/D835 mutations in a cohort of 113 AML patients treated in the Institute of Hematology, Clinical Center of Serbia, and their possible prognostic significance. The incidence of the FLT3/ITD mutation in our cohort of AML patients (17.7%) is marginally lower than the ones found in UK (27%) [18], Dutch (22%) [27], and German studies (23.5%) [32]. The incidence rate of the FLT3/ITD mutation in this study corresponds with the report of Yamamoto et al. (18.9%) [42]. The rate of the FLT3/D835 mutation (3.5%) was lower than in other series [2, 42]. It is possible that it is due to either a slightly different distribution of FAB subtypes of AML patients in this study compared to the others, or a limited number of patients.

Schnittger et al. [32] and Kiyoi et al. [17] have found that the incidence of FLT3/ITD mutations in patients with *t(15;17)* are 28.6 and 20.3%, respectively. It seems that there is an increased incidence of FLT3 mutations in patients with promyelocytic leukemia–retinoic acid receptor rearrangement.

In our study, the highest rate of FLT3/ITD mutations was detected in FAB M5 (seven patients) and M0 patients (six patients). This confirms the results of some other studies [13, 38]. FLT3 is persistently expressed during the process of monocyte differentiation, and the FLT3-L is required for complete differentiation of monocytes from CD34+ cells [9, 26]. According to these facts, we can presume that

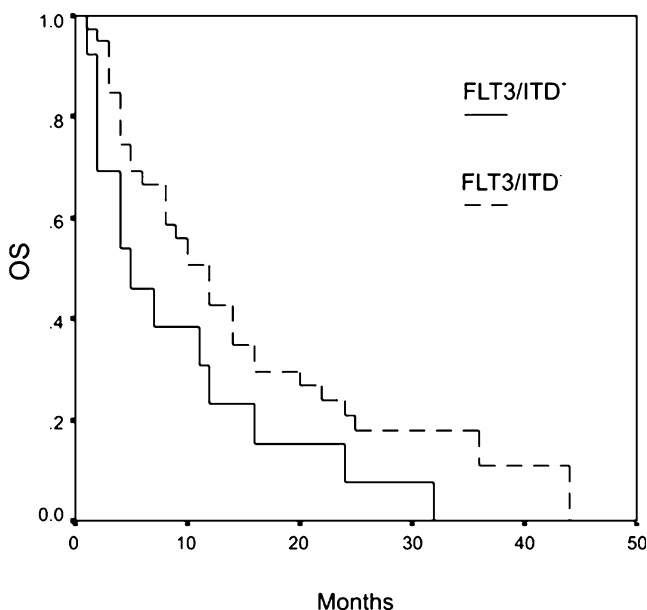


Fig. 4 Kaplan–Meier curves of the effects of FLT3/ITDs on OS of AML patients with the intermediate-risk karyotype. *Solid line*, FLT3/ITD-positive patients; *dashed line*, FLT3/ITD-negative patients

constitutive activation of FLT3 might be associated with monocyte differentiation.

We have detected both FLT3/ITD and FLT3/D835 mutations in favorable and intermediate-risk cytogenetic categories. FLT3/ITD was also detected in an adverse-risk category. Cytogenetic analyses of 21 patients with FLT3 mutations showed that 12 of them had the normal karyotype (57%). These results are similar to most published series [1, 20, 32]. It is well known that the expression of the constitutively activated FLT3 receptor in transgenic mice displayed a myeloproliferative disease phenotype only [3]. These findings suggest that at least one additional mutation besides the one in the FLT3 gene is indispensable for leukemic transformation. Therefore, it is essential to further investigate molecular alterations in patients with AML and the normal karyotype.

Our study demonstrates that the presence of FLT3/ITD mutation at diagnosis predicts the poor outcome of induction chemotherapy and attainment of CR. Moreover, it was the most significant factor predicting for OS in our patients ($p=0.0287$). Decreased OS has been reported in numerous studies of AML patients harboring the FLT3/ITD mutation, including the meta-analysis of Yanada et al. [41]. However, in several studies, the presence of the FLT3/ITD mutation did not effect OS. According to the authors explanations, it could be due to a higher percentage of elderly patients or patients with therapy-related AML, myelodysplastic syndrome, or AML with preceding myelodysplasia included in the study, short follow-up, or the application of more intensive induction chemotherapy [5, 20, 35].

Several studies have shown that OS in the group of AML patients with an intermediate-risk karyotype is not different between those with and without FLT3/ITD mutation [32, 38]. Additionally, in the study of Schnittger et al. [32], the multivariate analysis showed that the FLT3/ITD state is not an independent prognostic factor for DFS or OS in the intermediate risk group of AML patients. The results of our study are similar to those observed by Schnittger et al. [32].

We have followed up our group of FLT3-positive patients to investigate whether the mutation can be used as a marker of minimal residual disease. Most of them (75%) maintained the same FLT3 status at presentation and relapse, including all of the FLT3/ITD-positive patients. We have found that only two patients with the FLT3/D835 mutation lost the mutation at relapse of the disease. At the same time, one of them acquired the FLT3/ITD mutation at relapse. Heterogeneous patterns of FLT3/D835 mutations at presentation and relapse have also been reported in AML patients [19, 33, 34].

Opposite to our findings, Kottaridis et al. found that 5 of 20 (25%) of the patients who had been FLT3/ITD positive at the presentation of the disease had lost their mutation at relapse. The same findings were published by Nakano et al.

[24]. This loss of the FLT3/ITD mutation was not confirmed in the large study of Schnittger et al. [32], who reported that all 25 patients who could be analyzed at diagnosis and at relapse carried the FLT3 mutation at both time points. Our study is in concordance with these results.

We, however, observed that 50% of the FLT3/D835-positive patients lost the mutation in relapse. This finding is in concordance with the report of Shih et al. [34]. Moreover, one patient did not only lose the FLT3/D835 mutation at relapse but gained two different FLT3/ITD mutations. In a recent study by Cloos et al. [6], the authors suggested that the acquisition of FLT3/ITD in relapsed AML can be explained by oligoclonality in the samples from an initial diagnosis. A lot of subclones are undetectable by the PCR assay. We can hypothesize that in the case of this patient, there were at least three subclones present (FLT3/D835-positive and two minor FLT3/ITD subclones under the detection limit). During initial therapy, the D835-positive clone was eradicated only.

Our results suggest that the FLT3/ITD but not the FLT3/D835 mutation can be used as a valid marker of minimal residual disease. However, this should be treated with cautiousness because of the limited number of analyzed cases.

We suggest that an early detection of FLT3 mutations should become a standard diagnostic procedure for AML patients because they are a molecular target for specific therapeutics. In a number of ongoing clinical trials, conventional chemotherapy is combined with FLT3 inhibitors in an attempt to realize a synergistic effect against leukemia cells harboring the FLT3 mutation. The success of these trials will finally determine the importance of FLT3 mutations for clinical practice.

Acknowledgments This study is supported by grant 145 061 from the Ministry of Science and Environmental Protection of Serbia. The experiments performed in this study comply with the current laws of Serbia.

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