

# FLT3 Internal Tandem Duplication and D835 Mutations in Acute Myeloid Leukemia

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**Introduction:** FLT3 is a member of receptor tyrosine kinases expressed in leukemic cells. Internal tandem duplications (ITDs) and D835 mutations in FLT3 tyrosine kinase receptor have been shown to confer a bad prognosis in acute myeloid leukemia (AML). The aim of the present study was to determine the incidence of both ITD and D835 mutations in the FLT3 gene, in patients with AML from Tg-Mures, Romania.

**Materials and methods:** DNA was obtained from peripheral blood samples. ITDs were investigated by polymerase chain reaction (PCR). D835 mutations were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), with the digestion of restriction endonuclease EcoR V. The amplified and restricted products were finally electrophoresed on agarose gel stained with ethidium bromide.

**Results:** Alterations in the FLT3 gene were detected in 8 patients out of the 23 cases analyzed. These aberrations included ITD in 4 cases, D835 mutations in 2 cases and both types of alteration (ITD + D835) in 2 patients.

**Conclusion:** In this study we demonstrated that FLT3 mutations are frequent molecular abnormalities in AML patients with an incidence of 34.8%. Although our data do not support its value as a prognostic factor in AML patients because of the small cohort, further investigation is required.

**Keywords:** FLT3 internal tandem duplications, D835 mutations, acute myeloid leukemia

## Introduction

The *fms*-like tyrosine kinase 3 (FLT3) gene belongs to the class III receptor tyrosine kinases that induce signals for cell proliferation and is expressed on hematopoietic cells [1]. Many studies have shown that any abnormality in the FLT3 gene plays an important role in the pathogenesis of acute myelogenous leukemia [2].

FLT3 is the most commonly mutated gene in AML with the mutation occurring in approximately 30–40% of AML patients [3]. The most frequent FLT3 mutations found in AML are internal tandem duplications (FLT3-ITD) that have been reported in 20–30% of cases. In AML, FLT3 ITDs have been more frequently detected in patients with a normal karyotype and their presence has been associated with a poor prognosis [4].

According to Peng et al. patients with this abnormality have increased incidence of leukocytosis and decreased overall survival in comparison with patients without this abnormality [2].

In the last years, an additional type of FLT3 mutation has been described. The second type of FLT3 mutation is a point mutation at aspartic acid residue 835. The D835 point mutation has been identified in 6–10% of patients with acute myeloid leukemia [5,6].

Several studies have suggested that D835 mutations are also an adverse prognostic indicator in AML [7]. Both types of mutations (ITD + D835) in AML are rarely found (1–3%) [8,9].

The aim of the present study was to determine the incidence of both ITD and D835 mutations in the FLT3 gene, in patients with AML from Tîrgu Mureş, Romania.

## Materials and methods

### Patients

This study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Tîrgu Mureş, and written informed consent was obtained from all patients.

The study included 23 patients with newly diagnosed acute myeloid leukemia from Hematology Clinic Tîrgu Mureş. The DNA from the blood samples was extracted using the Genomic DNA Purification Kit (ZymoResearch) according to the manufacturer's instructions.

### Detection of ITD

FLT3-ITDs were investigated by polymerase chain reaction (PCR) with the primers 5'-GCAATTTAGGTATGAAA-GCCAGC-3', and 5'-CTTTCAGCATTTTGACG-GCAACC-3' [10]. DNA amplifications were performed with a Mastercycler Gradient Thermal Cycler (Eppendorf) with 2 min of denaturation at 95°C, followed by 30 cycles with denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 2 min at 72°C, followed by 5 min of extension at 72°C. The amplified products were finally electrophoresed on a 2% agarose gel stained with ethidium bromide.

### Detection of FLT3-D835 mutations

Mutations of FLT3-D835 in exon 20 (previously exon 17) were detected by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [11]. The amplification reaction was carried out using 2 min of initial denaturation at 95°C, 35 cycles of 30 sec at 94°C, 30

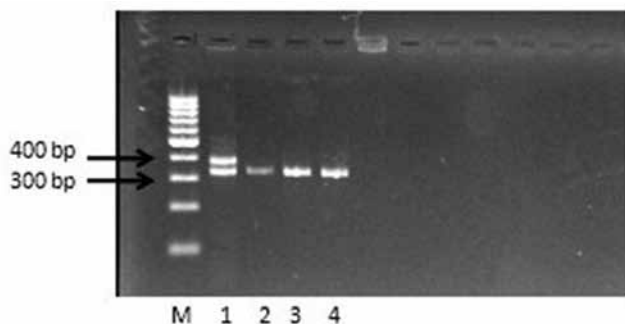


Fig. 1. PCR amplification of genomic DNA for the FLT3 gene. The 329 bp fragment indicates the size of the wild-type FLT3 gene in the absence of ITD (lanes 2-4), whereas additional upper bands are detectable in cases with the ITD mutation (lane 1). M – molecular marker 100 bp DNA ladder

sec at 55°C, and 30 sec at 72°C. The amplified product was digested with EcoR V restriction endonuclease. The restricted products were finally electrophoresed on agarose gel stained with ethidium bromide.

## Results

We examined the D835 and ITD mutations of the FLT3 gene in a total of 23 patients with acute myeloid leukemia. Alterations in the FLT3 gene were detected in 8 patients among 23 AML cases analyzed. FLT3-ITDs were found in 6 (26.1%) of 23 AML patients and D835 mutations in 4 (17.4%), which was significantly lower than that of ITD mutation. Two patients had both ITD and D835Y point mutations. There was no statistically significant difference in age and gender between the patients with mutation in the FLT3 gene and patients without FLT3 mutation.

According to the FAB classification, FLT3-ITD mutation was frequently found in the M1 type (2 of 6 cases; 33.3%); 1 of 4 (25%) of M4, 2 of 10 (20%) of M2 and in one patient with M3. No patient was positive for FLT3-ITD mutation in M0, M5, M6 or M7. Among the AML patients, the incidence of D835 mutation was as follows: M2 (2/10), M1 (2/6). No patient was positive for D835 mutation in M3, M4, M5, M6 or M7.

## Discussion

Mutations of the FLT3 genes have a great impact on acute myeloid leukemia pathogenesis. These mutations also lead to uncontrolled proliferation of leukemic cells and are usually associated with a poor prognosis [6]. According to Kottaridis et al the FLT3 mutations are secondary events in leukemogenesis and may be a relevant marker for minimal residual disease (MRD) [12]. Different studies showed that cell clones with FLT3-ITD are resistant to chemotherapy and had a proliferative advantage [13]. In the present study we investigated the presence of FLT3-ITD and D835 mutation by PCR-RFLP in AML patients with different FAB subtypes.

In our cohort, the frequency of FLT3-ITD mutation was 28.6% in acute myeloid leukemia, which is higher

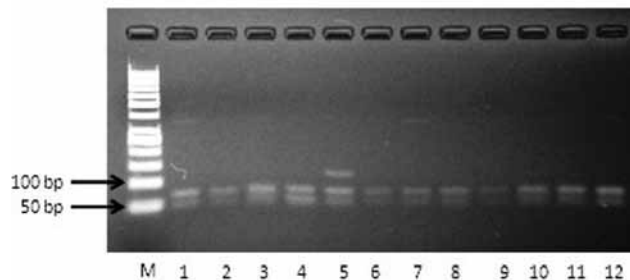


Fig. 1. Results of D835 mutations in the FLT3 gene. The amplified product of wild type was digested to two bands (68 bp and 46 bp) by EcoR V in lane 1-4, 6-12. When amplified products contained D835 mutations, undigested bands (114 bp) were visualized on agarose gel electrophoresis (lane 5). M – molecular size marker 50 bp DNA ladder.

than that reported in a Japanese (23%) [14] and Chinese studies (25%) [2]. In our AML patients, the frequency of FLT3-ITD positivity is similar to the reported frequency of 20–35% in the European studies [4]. In the same cohort of patients, point mutations of the activation loop of the second tyrosine kinase domain (D835) were identified in 17.4%. The incidence of D835 mutations was lower than that of ITD mutations.

The frequency of D835 mutation in our AML patients is higher than that found in previous studies in AML [15].

We think that the different results concerning the frequency of D835 mutation in AML obtained in our study are due to the low number of patients. We only had 23 samples from patients with AML while the other studies had at least 80 cases.

Taken together, constitutive activation of FLT3 was present in 34.8% (8 of 23) of these patients, which indicates that FLT3 is an important target of mutational activation in adult AML. The frequency of FLT3 mutation is similar to that described by Wang et al. (30.8%) [16].

Two patients showed both ITD and point mutations. The frequency of both FLT3 ITD and D835 in our AML patients (8.7%) is higher than that described in previous reports in AML (1–3%) [8,9].

Our data indicate that FLT3-ITD was not equally distributed among the different FAB-types, with a higher frequency in patients with FAB M1, M2, and M3. This confirms the results of other studies [6,16,17].

According to the FAB classification D835 mutation was found in M1, M2 AML patients. The frequency of D835 mutation in our study is comparable with the results of Sheikhha et al. [18].

A number of reports have shown that FLT3 mutation is associated with a poor prognosis in AML patients [19]. According to the literature AML patients with FLT3/ITD mutation have shorter re-mission duration and overall survival [15,20,21].

Whitman et al. found that FLT3 D835 mutation is associated with reduced remission duration relative to FLT3

wild-type patients in their patient cohort with de novo cytogenetically normal acute myeloid leukemia [15].

In our study, we observed that AML patients with FLT3 mutation had a shorter survival than those with wild-type FLT3 alleles. Four of our eight patients with FLT3 mutations died within the first year after diagnosis of AML. Taking into account our results we consider that any of the mutations in FLT3 gene confer a poor prognosis in AML.

## Conclusion

Taken together, these data confirm that FLT3 mutation also occurs in a significant percentage of Romanian AML patients.

Our study shows that approximately one third of patients with AML had mutations in the tyrosine kinase receptor. Although our data do not support its value as a prognostic factor in AML patients because of the small cohort, further investigations are required.

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## References

- Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, Toiron Y, et al. – Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. *Blood* 1993, 82: 1110–1119.
- Peng HL, Zhang GS, Gong FJ, Shen JK, Zhang Y, Xu YX, Zheng WL, Dai CW, Pei MF, Yang JJ – Fms-like Tyrosine Kinase (FLT) 3 and FLT3 Internal Tandem Duplication in Different Types of Adult Leukemia: Analysis of 147 Patients. *Croat Med J* 2008, 49(5): 650–659.
- Gregory TK, Wald D, Chen Y, Vermaat JM, Xiong Y, Tse W – Molecular prognostic markers for adult acute myeloid leukemia with normal cytogenetics. *J Hematol Oncol* 2009, 2: 2–23.
- Moreno I, Martín G, Bolufer P, Barragán E, Rueda E, Román J, et al. – Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica* 2003, 88(1): 19–24.
- Gilliland DG, Griffin JD – The roles of FLT3 in hematopoiesis and leukemia. *Blood* 2002, 100: 1532–1542.
- Zaker F, Mohammadzadeh M, Mohammadi M – Detection of KIT and FLT3 Mutations in Acute Myeloid Leukemia with Different Subtypes. *Arch Iran Med* 2010, 13(1): 21–25.
- Kiyoi H, Naoe T – Biology, clinical relevance, and molecularly targeted therapy in acute leukemia with FLT3 mutation. *Int J Hematol* 2006, 83(4): 301–308.
- Bacher U, Haferlach C, Kern W, Haferlach T, Schnittger S – Prognostic relevance of FLT3-TKD mutations in AML: the combination matters—an analysis of 3082 patients. *Blood* 2008, 111(5): 2527–2537.
- Chen W, Jones D, Medeiros LJ, Luthra R, Lin P – Acute myeloid leukaemia with FLT3 gene mutations of both internal tandem duplication and point mutation type. *Br J Haematol* 2005, 130(5): 726–728.
- Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, Kuriyama K, et al. – Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. *Leukemia* 1997, 11: 1447–1452.
- Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodere Y, Miyawaki S, et al. – Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood* 2001, 97: 2434–2439.
- Kottaridis PD, Gale RE, Langabeer SE, Frew ME, Bowen DT, Linch DC – Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood* 2002, 100: 2393–2398.
- Tiesmeier J, Müller-Tidow C, Westermann A, Czwalinna A, Hoffmann M, Krauter J, et al. – Evolution of FLT3-ITD and D835 activating point mutations in relapsing acute myeloid leukemia and response to salvage therapy. *Leuk Res* 2004, 28: 1069–1074.
- Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. – Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood* 1999, 93: 3074–3080.
- Moreno I, Martín G, Bolufer P, Barragán E, Rueda E, Román J, et al. – Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica* 2003, 88(1): 19–24.
- Whitman SP, Ruppert AS, Radmacher MD, Mrózek K, Paschka P, Langer C, Baldus CD, Wen J, Racke F, Powell BL, Kolitz JE, Larson RA, Caligiuri MA, Marcucci G, Bloomfield CD – FLT3 D835/1836 mutations are associated with poor disease-free survival and a distinct gene-expression signature among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking FLT3 internal tandem duplications. *Blood* 2008, 111(3): 1552–1559.
- Wang L, Lin D, Zhang X, Chen S, Wang M, Wang J – Analysis of FLT3 internal tandem duplication and D835 mutations in Chinese acute leukemia patients. *Leuk Res* 2005, 29: 1393–1398.
- Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, et al. – Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood* 2002, 100(1): 59–66.
- Sheikhha MH, Awan A, Tobal K, Liu Yin JA – Prognostic significance of FLT3 ITD and D835 mutations in AML patients. *The Hematology Journal* 2003, 4: 41–46.
- Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. – The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukaemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood* 2001, 98: 1752–1759.
- Bullinger L, Döhner K, Kranz R, Stirner C, Fröhling S, Scholl C, Kim YH, Schlenk RF, Tibshirani R, Döhner H, Pollack JR – An FLT3 gene-expression signature predicts clinical outcome in normal karyotype AML. *Blood* 2008, 111: 4490–4495.
- Gagy E, Horváth E, Bődör C, Timár B, Matolcsy A, Pávai Z – Prognostic significance and detection of the Internal Tandem Duplication of the FLT3 gene in acute myeloid leukemia. *Rom J Morphol Embryol* 2006, 47(4): 331–337.