

Is FLT3 Internal Tandem Duplication an Unfavorable Risk Factor for High Risk Children with Acute Myeloid Leukemia? – Polish Experience

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Abstract: According to the AML-BFM 2004 Interim, a treatment protocol used in Poland since 2005, presence of FLT3 internal tandem duplication (FLT3/ITD) qualifies a patient with acute myeloid leukemia (AML) to a high-risk group (HRG).

The present study was aimed to identify the prevalence of FLT3/ITD in children with AML in Poland and to evaluate its prognostic significance in the HRG patients.

Out of 291 children with *de novo* AML treated in 14 Polish centers between January 2006 and December 2012, samples from 174 patients were available for FLT3/ITD analysis. Among study patients 108 children (61.7%) were qualified to HRG. Genomic DNA samples from bone marrow were tested for identification of FLT3/ITD mutation by PCR amplification of exon 14 and 15 of FLT3 gene. Clinical features and treatment outcome in patients with and without FLT3/ITD were analyzed in the study.

The FLT3/ITD was found in 14 (12.9%) of 108 HRG children. There were no significant differences between children with and without FLT3/ITD in age and FAB distribution. The white blood cells count in peripheral blood at diagnosis was significantly higher ($p < 0.01$) in the children with FLT3/ITD. Over 5-year overall survival rate for FLT3/ITD positive children was worse (42.4%) comparing to FLT3/ITD negative children (58.9%), but the statistical difference was not significant. However, over 5-year survivals free from treatment failures were similar.

The FLT3/ITD rate (12.9%) observed in the study corresponded to the published data. There was no significant impact of FLT3/ITD mutation on survival rates, although further studies are needed on this subject.

Keywords: FLT3/ITD mutation, acute myeloid leukemia, children, treatment result, high risk group.

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INTRODUCTION

Acute myeloid leukemias (AML) are a heterogeneous group of diseases of hematopoietic system that can be classified by morphology, lineage specific antigens and genetics [1]. Despite enormous progress that was achieved over the last few decades in the treatment of pediatric AML, still approximately 35-45% of patients have failures of treatment such as a refractory disease or relapse [2, 3]. New treatment strategies are dramatically needed to improve the survival rates in AML. One has revealed new targets for possible anticancer therapy exploring the knowledge on cancer biology. Precise stratification of patients to adequate risk groups is also believed to improve treatment results [3, 4]. In AML cytogenetic and molecular findings at diagnosis are critical determinants of outcome and allow for stratification of patients to an adequate risk group. In a new BFM treatment protocol (Registry AML-BFM 2012) the allocation to a risk group is based mainly on those genetic fingerprints [3].

According to the AML-BFM 2004 Interim, the treatment protocol used in Poland since 2005, presence of FLT3 internal tandem duplication (FLT3/ITD) qualifies a patient with acute myeloid leukemia to a high risk group (HRG) [5]. It is noteworthy that prognostic significance of some clinical and biological factors are being interpreted differently by different study groups (i.e. the NOPHO does not consider FLT3/ITD as a poor risk factor, while BFM-AML group does) [2].

The FLT3 (Fms-like tyrosine kinase 3, CD135) is a member of the class III tyrosine kinase receptor family, which also includes c-FMS, c-kit, and PDGFR receptors. The FLT3 receptor in normal bone marrow is restricted to hematopoietic stem cells and regulates proliferation, differentiation and survival of stem cells. It is also expressed in many hematologic malignancies including most subtypes of AML, B-cell precursor acute lymphoblastic leukemia (ALL), T-cell ALL, and chronic myeloid leukemia (CML) in blast crisis [6]. There are two different types of FLT3 receptor mutations: internal tandem duplication (FLT3/ITD) in the juxtamembrane region and activation of a loop mutation (FLT3/ALM) in the tyrosine kinase domain. The most common mutation of FLT3 in AML is FLT3/ITD, which promotes cell proliferation and resistance to apoptosis via the constitutive activation of many signaling pathways [6-8]. The FLT3/ITD is one of the most frequent mutations in hematologic malignancies, occurring in CML and

MDS with similar prevalence (5-10%), and AML (15-35%) [3, 8, 9].

In the present study we determined the prevalence and prognostic significance of FLT3/ITD in children with AML from HRG in Poland, who were treated according the same therapy protocol, and correlated its presence with clinical characteristics and outcome.

PATIENTS AND METHODS

Between January 2006 and December 2012 two hundred and ninety one (291) children (0 – 18 years) with *de novo* AML treated according to AML-BFM-2004 Interim protocol were enrolled at 14 centres of the Polish Pediatric Leukemia/Lymphoma Study Group (PPLLSG). In all children, diagnosis of AML was based upon bone marrow (BM) examination, including cell morphology assessment according to French-American-British (FAB) classification, cytochemistry, and immunophenotyping. Cytogenetic analyses, both classical and fluorescence in situ hybridization (FISH) were mandatory. Out of 291 children with *de novo* AML, 174 (59.7 %) had bone marrow samples at diagnosis available for FLT3/ITD analysis. Among study patients, 108 children (61.7%) were qualified to a high risk group (HRG). In the study we focused on patients from the HRG, therefore children with Down syndrome, as well as M3 FAB, who are stratified to a standard risk group, were not *a priori* analysed. Patients with myelodysplastic syndrome and secondary AML were also excluded. The treatment protocol and risk classification were previously reported and described elsewhere [5]. In brief, the AML-BFM-2004 Interim protocol for HRG consists of two induction courses, first: AIE (ARA-C, Idarubicin, Etoposide) and second: HAM (high doses ARA-C, Mitoxantron). Consolidation therapy includes three courses of chemotherapy: AI (ARA-C, Idarubicin) and haM (ARA-C, Mitoxantron) and HAE (high doses ARA-C, Etoposide). Allogeneic hematopoietic stem cell transplantation (HSCT) from an HLA-identical sibling donor is indicated for all HRG patients in the first remission. Children with leukemic blasts after the second induction phase (HAM) or still in aplasia after second induction are qualified for allo-HSCT from an HLA-matched unrelated donor. Intrathecal treatment during chemotherapy and cranial irradiation is also used as a prophylaxis as well as therapy [5]. Informed consent was obtained from the patient or their caregivers, according to guidelines based on the principles of the revised Declaration of Helsinki. The institutional

review board of Jagiellonian University Medical Faculty approved the study.

The genomic DNA polymerase chain reaction (PCR) assay of exon 14 and 15 of *FLT3* gene was performed to identify FLT3/ITD. DNA was extracted from EDTA stabilized diagnostic bone marrow aspirates by using a DNA extraction kit (MasterPure™ Purification Kit for Blood Version II, Epicentre® Biotechnology, USA) according to the manufacturer's instruction. PCR amplification was performed with primers 11F, 5'-GCAATTTAGGTAT-GAAAGCCAGC – 3' and 12R, 5' – CTTTCAGCATTTTGACGGCAACC – 3' [7]. The PCR reaction was carried out in a mixture of 25 µl containing 100 ng DNA, 200 µM dNTP, 1x Pol Buffer B, 2 U Perpetual Taq Polymerase (Perpetual Taq DNA Polymerase, Eurx, Poland) and 800 nM for each primer. PCR consisting of denaturation at 95 °C for 30 seconds, annealing at 61 °C for 30 seconds and extension at 72 °C for 45 seconds for 30 cycles was performed on T3Thermocycler, Biometra. There was an initial 15-minute denaturation at 95°C and a final extension at 72 °C for 5 minutes. The reaction products were separated on 3% agarose gel (Prona Agarose BIO STANDARD, Prona, Spain) and detected by ethidium bromide staining. A normal *FLT3* amplification product length was 329 bp. The presence of additional high-molecular weight band represented the FLT3/ITD (Figure 1).

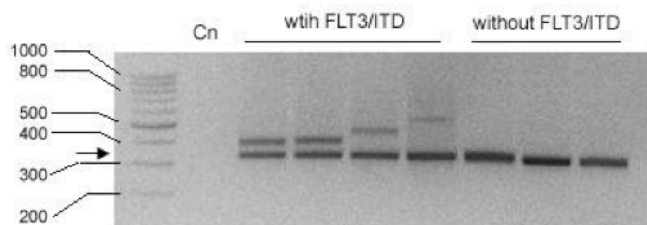


Figure 1: Genomic PCR analysis for FLT3/ITD detection. Seven genomic DNA samples from different pediatric patients were PCR amplified and resolved on 3% agarose gel. A normal *FLT3* amplification product length is 329 bp (arrow points). High-molecular weight bands represent the FLT3/ITD. Cn, non-nucleic acid control.

The Kaplan-Meier method was used to estimate the survival distribution. Differences were compared using a log-rank test. The results were expressed by means of remission rates, event-free survival (EFS), overall survival (OS), and relapse-free survival (RFS). The comparison between the groups with and without FLT3-ITD was performed by nonparametrical tests: the Mann-Whitney U-test and the Kolmogorov-Smirnov test. We assumed in the study that treatment failures include lack of complete remission (CR) during 6

weeks since beginning of the treatment, relapse or progression of AML, early death (during the first 42 days from beginning of the treatment) or death due to AML progression or relapse. For statistical analyses, STATISTICA, version 10, StatSoft Inc. (2011) software packages were used. The observation was completed on December 30, 2012. For all analyses the p values less than 0.05 ($p < 0.05$) were considered statistically significant.

RESULTS

In the HRG patients consisting of 47 girls and 61 boys, the median age was 11.2 years (0.2-17.7 years). The FLT3/ITD was found in 14 (12.9%) of 108 HRG children. In not analyzed standard risk group patients FLT3/ITD was detected in 4 out of 66 children (6%). All four patients had concomitant PML-RARA mutations and M3 AML, therefore those patients were treated according to AML-BFM-2004 Interim protocol as SRG patients irrespective of FLT3/ITD presence. Laboratory and clinical characteristics of patients with and without FLT3 mutations were compared (Table 1). There were no significant differences between children with and without FLT3/ITD in age and FAB distribution ($p > 0.1$). We found a stepwise age-dependent increase in the number of FLT3/ITD positive patients in the group of children aged 1 to 10 years. In infant AML we didn't find FLT3/ITD. The FLT3/ITD mutation was found in three out of 27 (11.1%) patients aged 1 to 5, in four patients out of 12 aged 5 to 10 (33.3%), in seven patients out of 61 (11.4%) aged above 10 years (Table 2). In FLT3/ITD positive patients there was no predominance of any particular FAB subtype, although FAB subtypes M6 and M7 were not represented. The median age of children with FLT3/ITD was 10.6 years, similar to that of patients without FLT3/ITD mutations (11.2 years) ($p > 0.1$). The white blood cells (WBC) count in peripheral blood as well as leukemic blast cells at diagnosis were significantly higher in the children with FLT3/ITD. Patients with FLT3/ITD had a significantly elevated diagnostic WBC count with a median diagnostic WBC count of $88.9 \times 10^9/L$ compared with $22.2 \times 10^9/L$ for the children without FLT3/ITD ($p < 0.01$). Similarly, patients with FLT3/ITD had a significantly higher peripheral blood blast percentage of 79% compared with 43% for patients without FLT3/ITD ($79.14 \pm 23.3\%$ vs $43.6 \pm 32.93\%$, $p < 0.01$).

However, there were no statistically significant difference between children with and without FLT3/ITD mutations in regard to percentage of bone marrow

Table 1: Characteristics of HRG AML Patients with or without FLT3/ITD

	Total	with FLT3/ITD	without FLT3/ITD	p Value
No. of patients	108	14	94	
Age (year) <i>median (range)</i>	11.2 (0.2 – 17.7)	10.6 (2.0 – 17.7)	11.2 (0.2 – 17.6)	>0.1
Gender female male	47 61	5 9	42 52	
Initial WBC ($\times 10^9/L$) <i>median (range)</i>	28.2 (1.1 – 573)	88.9 (6.4 – 573)	22.2 (1.1 – 570.1)	<0.01
FAB subtype				
M0	17 (15.7%)	4 (3.7%)	13 (12.0%)	>0.1
M1	18 (16.7%)	4 (3.7%)	14 (13.0%)	
M2	16 (14.8%)	3 (2.8%)	13 (12.0%)	
M4	28 (25.9%)	2 (1.9%)	26 (24.1%)	
M5	22 (20.4%)	1 (0.9%)	21 (19.4%)	
M6	1 (0.9%)	0	1 (0.9%)	
M7	6 (5.6%)	0	6 (5.6%)	

WBC – white blood cell count.

Table 2: Frequencies of FLT3/ITD Mutation in Different Age Groups

	<1 y	1-4,9 y	5-9,9 y	>10 y	p Value
No. of patients	8	27	12	61	
No. of FLT3/ITD pos. patients	0	3	4	7	>0.1
Percentage (%)	0	11	33	11	

blasts at diagnosis ($77.6\% \pm 28.2\%$ vs $68.4 \pm 23.2\%$, respectively, $p=0.1$).

The achievement of a complete remission (CR), as well as percentage of blast cells in bone marrow at day 15 of chemotherapy are the measures of early treatment response. In the total of 108 HRG patients, 88 (81.6%) children achieved a CR (Table 3). The complete remission rate was comparable for both groups of patients. Eleven (78.5%) out of 14 patients with FLT3/ITD achieved a CR compared with 77 (82%) out of 94 children without this mutation. The bone marrow blast cells percentage at day 15 above 5% is considered as poor response to treatment. There were no statistically relevant differences in the number of children with bone marrow blast cells >5% at day 15 between patients with and without FLT3/ITD ($p>0.1$).

At least one of treatment failures was observed in seven (50%) patients out of 14 with FLT3/ITD, and in 40 (42%) children out of 94 FLT3/ITD negative

patients, although the statistical difference was not significant ($p=0.6$). Of the 88 patients who achieved a CR, 28 children had a relapse: 5 out of 11 CR patients (45%) from the FLT3/ITD positive group and 23 out of 77 CR patients (30%) from the FLT3/ITD negative group (Table 3).

Over 5-year overall survival rate for FLT3/ITD positive children was worse (42.4%) compared to FLT3/ITD negative children (58.9%), but the difference did not reach statistical significance (Figure 2). However, over 5-year survivals free from treatment failures were similar for patient with and without FLT3/ITD mutation (42.4% vs 45.3%, respectively; $p=0.68$) (Figure 3).

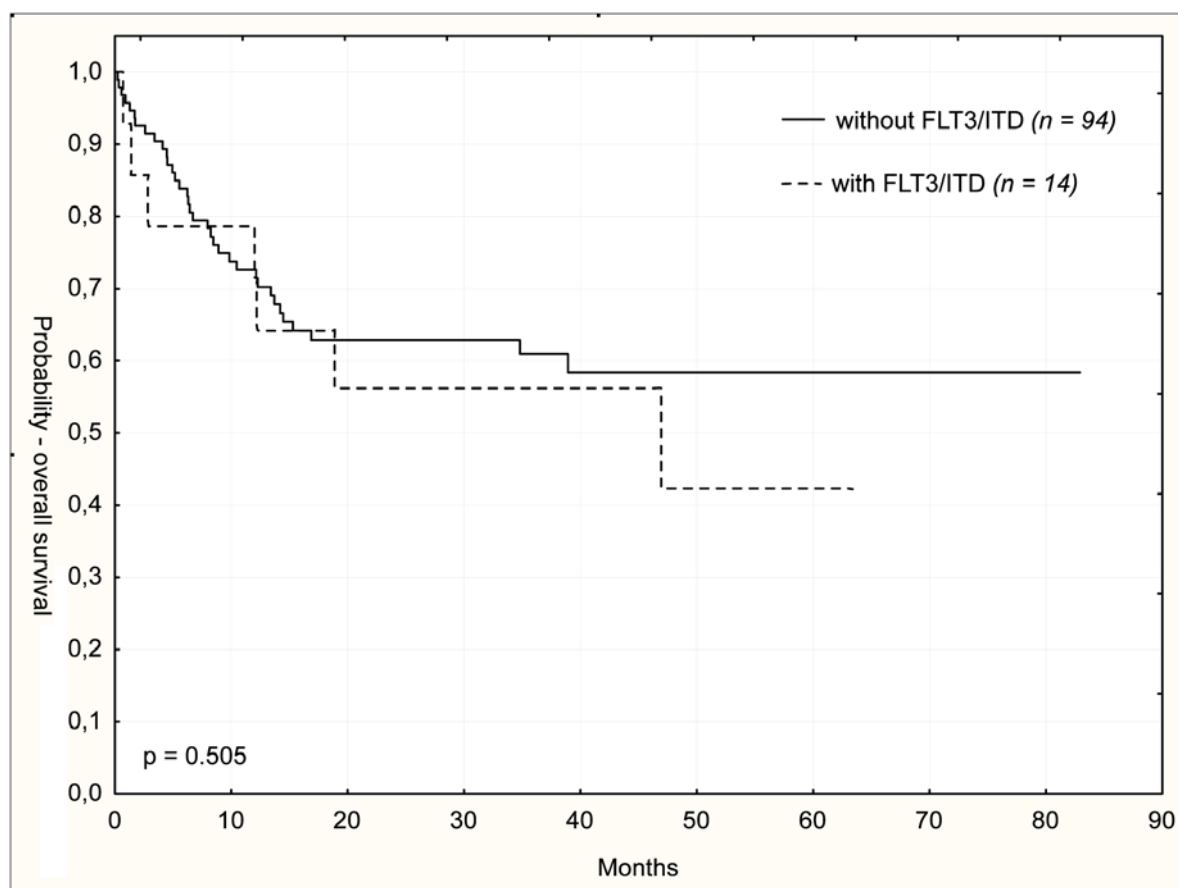
DISCUSSION

The aim of the study was to identify the prevalence of FLT3/ITD in children with AML in Poland and to evaluate its prognostic significance in the HRG patients.

Table 3: Treatment Results of HRG AML Patients with or without FLT3/ITD.

	Total	with FLT3/ITD	without FLT3/ITD	p Value
Blast cells in BM at day 15 of chemotherapy				
<= 5 %	77/108 (71.3 %)	10/108 (9.3 %)	67/108 (62.0 %)	>0.1
> 5 %	25/108 (23.1 %)	3/108 (2.8 %)	22/108 (20.4 %)	
no data	6/108 (5.6 %)	1/108 (0.9 %)	5/108 (4.6 %)	
Treatment results				
CR	88/108 (81.8%)	11/14 (78.5%)	77/94 (81.9%)	
5-year overall survival	55.6%	42.4%	58.9%	0.505
Probability of treatment failures 5 – year free survival	45.6%	42.4%	45.3%	0.681
Treatment failures				
lack of CR	20/108 (18.5%)	3/14 (21.4%)	17/94 (18.0%)	
relapse of AML	28/88 (31.8%)	5/11 (45.4%)	23/77 (29.8%)	
progression of AML	4/108 (3.7%)	1/14 (7.1%)	3/14 (3.2%)	
early death	7 (6.5%)	2 (14.3%)	5 (5.3%)	
death due to AML progression or relapse	24/108 (22.2%)	4/14 (28.6%)	20/94 (21.3%)	

BM – bone marrow; CR – complete remission; OS - overall survival.

**Figure 2: Overall survival (OS) for children with and without FLT3/ITD mutation.**

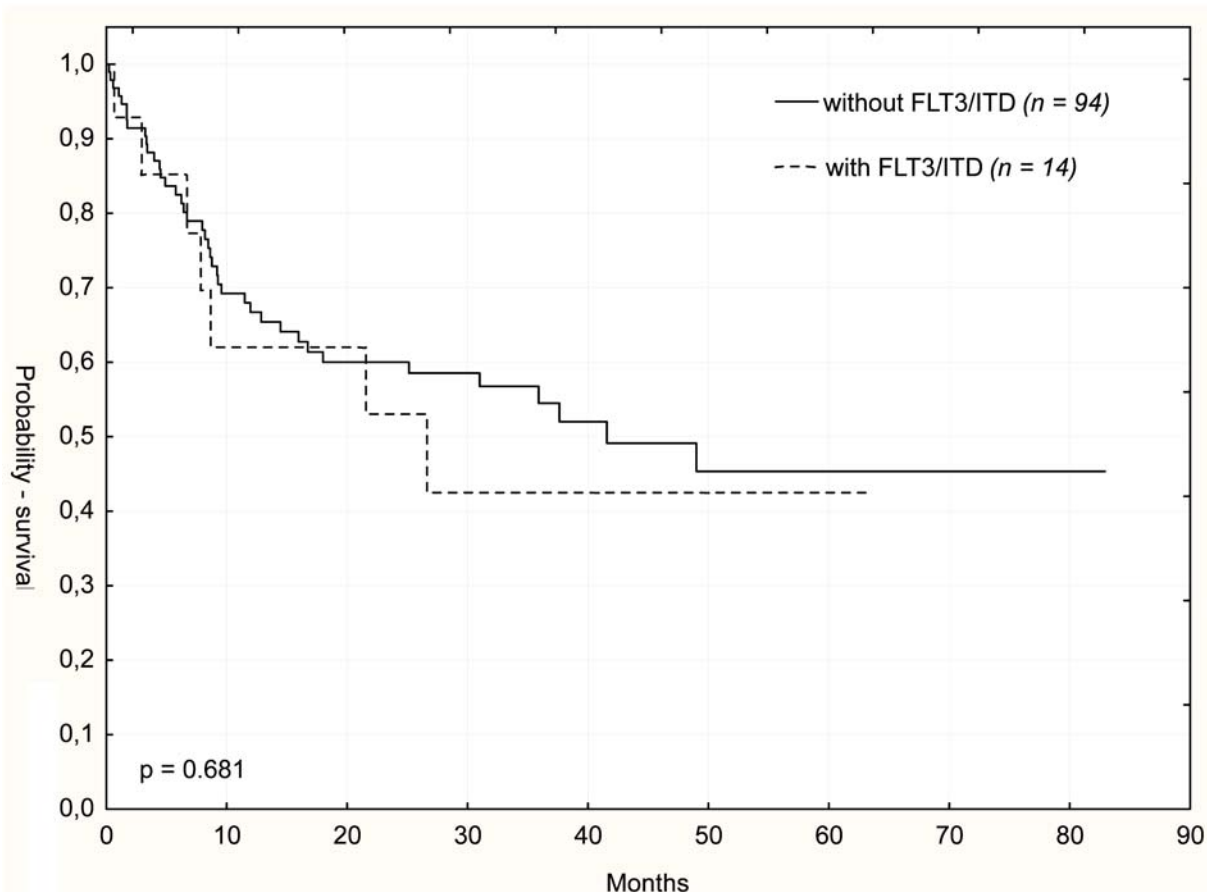


Figure 3: Probability of treatment failures free survival for children with and without FLT3/ITD mutation.

The FLT3/ITD rate (12.9%) observed in the study corresponded to the previously published data [3, 6, 8, 9, 10]. Many studies have shown that presence of FLT3/ITD is an independent prognostic factor for poor outcome in AML [11, 12-14]. The prognostic significance of FLT3/ITD in the presented study was less pronounced than in other studies. There were comparable remission rates in both groups (78.5% vs 82%), although relapse rate was higher in FLT3/ITD positive children. Of the 88 patients of HRG who achieved a CR, 28 children had a relapse: 5 out of 11 CR patients (45%) from the FLT3/ITD positive group and 23 out of 77 CR patients (30%) from the FLT3/ITD negative group. Over 5-year overall survival rate for FLT3/ITD positive children was worse (42.4%) compared to FLT3/ITD negative children (58.9%), but the difference did not reach statistical significance. We could not demonstrate clearly prognostic impact of FLT3/ITD in our study, probably because we didn't analyze the presence of ITD-allelic ratio (mutant to wild type allelic ratio), which was shown to be strongly connected with a poor outcome. High allelic ratio of ITD is associated with poor prognosis [3, 4, 8, 13]. Accompanying translocations, such as t(5;11) or

presence of additional mutation of WT1 or NPM1, can also modify the prognostic value of the FLT3/ITD [3, 4, 8, 15-19]. In study by de Jonge *et al.* patients with mutated NPM1 and FLT3/ITD had unfavourable prognosis if they had a WBC count greater than $100 \times 10^9/L$ or ITD allelic ratio was greater than 1. Patients with a WBC count below $100 \times 10^9/L$ and ITD allelic ratio of 1 or less were considered to have favourable prognosis [20].

We have demonstrated the association of FLT3/ITD with leucocytosis and increased peripheral leukemic blasts cell count, which is consistent with most of the previous reports [7-9, 16]. The FLT3 mutations (both FLT3/ITD and FLT3/ALM) has been also found to be associated with leucocytosis in acute promyelocytic leukemia [21].

The frequency of FLT3/ITD increases with the patient's age, it is rare in infant AML, reaches 5-10% in age 5 to 10 years, about 20% in young adults, and increases above 35% in patients older than 55 years [3, 8, 9]. Our current study showed also a stepwise age-dependent increase in the number of FLT3/ITD

positive patients, although there were no significant differences between children with and without FLT3/ITD in age distribution ($p>0.1$) (Table 2). The increase in prevalence of patient with FLT3/ITD mutation from 11% in patients aged 1 to 5 to 33% in children aged 5 to 10, and subsequent decline to 11% in children above 10 years, was probably a result of a small number of patients, especially in children aged 5 to 10 years. Meshinchi *et al.* suggested that age-associated increase in the prevalence of FLT3/ITD mutation can explain the pathology of FLT3/ITD in evolution of AML. Previously acquired, early preleukemic hit leads to a minor clone development with maturation arrest. Such population maybe quiescent until FLT3/ITD is acquired, and the probability of this increases with age of the patients [8].

The prognostic value of FLT3/ITD in pediatric AML remains to be explained. Early studies showed a poor outcome of patients with present FLT3/ITD [7, 11], however later, larger studies suggested a more modest impact on outcome [22, 23]. Some studies demonstrated that the presence of FLT3/ITD was an independent prognostic factor for poor outcome in AML [12- 14]. However, differences in the treatment outcome were reported depending on FLT3/ITD mutant to wild-type ratio (ITD allelic ratio), length of FLT3, as well as on an accompanying translocations such as t(5;11) or presence of additional mutation of WT1 or NPM1 [3, 4, 8, 13, 15, 20, 23, 24].

There are much greater hopes for FLT3/ITD mutations. Not only is it considered a genetic fingerprint guiding the intensity of treatment in children, but also it could be used as a marker for monitoring of minimal residual disease [18, 25] or a target point for new drugs [26, 27]. Furthermore, the FLT3 tyrosine kinase is believed to be the most reasonable target protein in AML. Currently several potent FLT3 kinase inhibitors are being tested in clinical studies (Lestaurtinib - Levis, Midostaurin - Stone), however sorafenib has been the most extensively investigated first generation FLT3 inhibitor so far [26-29].

In conclusion, the present study demonstrates no significant impact of FLT3/ITD mutation on survival rates in HRG children. The observed differences in treatment outcome for children with FLT3/ITD positive AML suggest that biologic and clinical differences exist between various types of FLT3/ITD mutations. Larger prospective studies are needed to evaluate further the role of FLT3/ITD and its potential use as targetable lesion in future therapies.

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