FLT3 Internal Tandem Duplication in 234 children with acute myeloid leukemia (AML): prognostic significance and relation to cellular drug resistance

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ABSTRACT

FLT3 is a receptor tyrosine kinase involved in the proliferation and differentiation of hematopoietic stem cells. FLT3 internal tandem duplications (FLT3/ITD) have been reported in acute myeloid leukemia (AML) and predict poor clinical outcome. We found FLT3/ITD in 11.5% of 234 children with de novo AML. FLT3/ITD positive patients were significantly older, had more frequently normal cytogenetics and FAB M1/M2, and less frequently 11q23 abnormalities or FAB M5. FLT3/ITD positive patients had lower remission induction rates (70% vs. 88%, p=0.01), and lower 5-year probability of event free (pEFS 29% vs. 46%, p=0.0046) and overall survival (32% vs. 58%, p=0.037). Patients with high ratios between mutant and wild-type FLT3, i.e. above the median, did significantly worse in terms of 2-year EFS than FLT3/ITD negative patients (pEFS 20% vs. 61%, p=0.037), whereas patients with ratios below the median did not (pEFS 44% vs. 61%, p=0.26). FLT3/ITD was the strongest independent predictor for pEFS with an increase in relative risk for an event of 1.92 (p=0.01).

We studied cellular drug resistance, using an MTT-based assay, on 15 FLT3/ITD positive and 125 FLT3/ITD negative AML samples, but we found no differences in cellular drug resistance which could explain the poor outcome of FLT3/ITD positive patients.

We conclude that FLT3/ITD is less common in pediatric than in adult AML. FLT3/ITD is a strong and independent adverse prognostic factor, and high ratios between mutant and WT-FLT3 further compromise prognosis. However, poor outcome in FLT3/ITD positive patients could not be attributed to increased in-vitro cellular drug resistance.
INTRODUCTION

The prognosis of children with acute myeloid leukemia (AML) has improved considerably over the past decades, with overall survival rates of 50-60%. However, relapse remains the most important cause of failure, which occurs in 30-40% of children in first complete remission.

The wild-type (WT) FLT3 receptor is a class III receptor tyrosine kinase, expressed on hematopoietic progenitor cells and on the majority of leukemias, such as AML, CML in blast crises and ALL. Recently, an internal tandem duplication of FLT3 has been described (FLT3/ITD), which leads to ligand-independent FLT3 dimerization and constitutive activation through autophosphorylation. This results in a proliferation and survival advantage of the cell. In humans, FLT3/ITD is found almost exclusively in AML.

Recently, several large studies showed that FLT3/ITD occurs in approximately 20-30% of adult AML cases, which, in most but not all studies, is associated with poor clinical outcome. In AML in children, the incidence of FLT3/ITD was reported to be lower than in adults, and was also associated with poor prognosis. However, all pediatric studies published to date included only relatively small numbers of patients. Interestingly, a high mutant versus wild-type FLT3 (WT-FLT3) ratio further compromises prognosis, as has been described both for adults and children with AML.

Differences in prognosis may reflect differences in cellular drug resistance, which we have previously demonstrated in several other studies in childhood leukemia. Cellular drug resistance is also an independent risk-factor in childhood ALL. In AML in children, cellular drug resistance is related to short-term clinical response.

In this study, we retrospectively investigated the incidence and prognostic impact of FLT3/ITD as well as the ratios between mutant and WT FLT3, in 234 children with de novo AML, included in the BFM-AML 87, 93 and 98 studies, or the corresponding BFM-based Dutch ANLL 87 and 94 protocols, all consisting of intensive cytarabine/anthracycline based treatment. This is the largest pediatric cohort published today. In addition, we analyzed whether increased cellular drug resistance could be a mechanism for the poor clinical outcome of FLT3/ITD positive AML.
MATERIALS AND METHODS

Patient samples

We tested either bone marrow or peripheral blood samples from children (0-≤18 years of age). Samples were only taken after informed consent had been obtained and with IRB approval. Two collaborative groups participated in this study: the AML-'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG, Münster, Germany) and the Dutch Childhood Leukemia Study Group (DCLSG, The Hague, The Netherlands). Both Study Groups performed central review of the diagnosis, classification and clinical follow up of the patients. For drug resistance testing, samples were sent to the Research Laboratory of Pediatric Oncology in Amsterdam. These samples were assessed for FLT3/ITD in collaboration with Dr. Meshinchi, Seattle, USA, for patients included in the BFM-AML 87/93 and the corresponding Dutch protocols. A second cohort of patients, included in the BFM-AML 98 protocol, was analyzed for FLT3/ITD by Dr Griesinger, Germany.

All patients were re-classified according to the BFM-AML risk group classification criteria. ‘Standard risk (SR)’ patients were defined as having favorable morphology (i.e. French-American-British (FAB) M2 with Auer rods, M3 or M4Eo) and blast cell reduction in the bone marrow on day 15 to <5% (not obligatory for FAB M3). All other patients were considered high-risk (HR).32

Treatment protocols

Patients were treated according to several different treatment protocols, including the German AML-BFM 87, 93 and 98 study and the Dutch protocols DCLSG ANLL 87 and 94, which were based on their corresponding BFM counterparts.1, 2, 32-34 As the treatment according to these protocols was very similar, patients were taken together for the analysis of the prognostic significance of FLT3/ITD.

In brief, the AML BFM 87 protocol started with an 8-day induction course, followed by a 6-week consolidation block. Subsequently, 2 intensification courses were given. Intrathecal chemotherapy and/or cranial irradiation were given as CNS prophylaxis [irradiation in approximately half of the patients (randomized)].35 This was followed by maintenance therapy, up to a total treatment duration of 18 months. Sibling donor allogeneic stem cell transplantation (SCT) was advised for high-risk patients in first complete remission (CR).

In the AML BFM 93 study (Figure 1), the patients were stratified according to risk-groups. At diagnosis, patients were randomized between daunorubicin (ADE) and idarubicin (AIE) induction therapy, which did not result in differences in long-term outcome.1 For HR patients, one of the intensification blocks was changed to high-dose cytarabine with mitoxantrone (HAM), which improved treatment outcome compared with
protocol AML BFM 87.° Sibling SCT was advised for HR patients in first CR. SR patients did not receive HAM and outcome was identical to the AML BFM 87 study.

Protocol AML BFM 98 consists of induction with the idarubicin block, followed by HAM. In the consolidation phase patients were randomized for either receiving the 6-week consolidation block followed by 1 intensification block, versus 3 intensive courses of chemotherapy.

Study DCLSG ANLL 87 was similar to AML BFM 87, but no prophylactic cranial irradiation was given, nor maintenance therapy. In protocol DCLSG ANLL 94 all patients received the idarubicin arm in induction, as well as the HAM block for consolidation. No maintenance therapy was given. Patients were transplanted with either an HLA-identical sibling donor or autograft.

Analysis of the FLT3/ITD

A first cohort of patients was tested by Meshinchi (SM) and co-workers, who developed a genomic PCR for the detection of FLT3/ITD.° DNA was obtained from either bone marrow or peripheral blood cytospin slides (prepared after density gradient centrifugation using Ficoll Isopaque) that had been stored in liquid nitrogen. After thawing, extraction of genomic DNA was performed by adding 10µl of water to the leukemic cells. The DNA was PCR amplified (35 cycles) as described before.°°°° Patients that were identified with a FLT3/ITD were further analyzed with Gene Scan analysis to determine the ratio between mutant and WT-FLT3. The two primers used were 5FLT3/ITD, 5’-6FAMGCA ATT TAG GTA TGA AAG CCA GC-3’ and 3FLT3/ITD, 5’-CCT TCA GCA TTT TGA CGG CAA CC-3’. (Applied Biosystems, Foster City, CA).

Polymerase chain reaction (PCR) was carried out in duplicates in a final volume of 50 ul containing genomic DNA (10 and 25 ng), KCl (50mM), Tris-HCl (20mM, pH 8.4), MgCl₂ (3mM), primers (10uM each), nucleotides (0.2mM each), and Platinum Taq DNA Polymerase (2.5 U; Invitrogen). Samples were processed through 35 cycles at 94°C for 5 min, 94°C for 30 s, 66°C for 1 min, and 72°C for 2 min, with a final step for 7 min at 72°C. PCR products were diluted 1:100 with filtered Nanopure water and turned in for analysis. This 1ul of sample was diluted into a 10ul cocktail of formamide and size standard (0.05ul size standard/sample) and run on the 3100 Genetic Analyzer (Applied Biosystems) with the POP-4 Polymer. Data was analyzed using Genescan software.

A second cohort of patients was tested by Griesinger (FG) and co-workers. Total RNA was extracted from 1x10⁷ cells using the “RNEASY Minikit” (Qiagen). For the amplification of the ITD, the protocol of Nakao et al., using the reported primers R5 and R6, was used.°°°° Samples were rated ITD positive, if the ratio between FLT3/ITD and WT-FLT3 exceeded ≥0.03.°°°°

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To study possible differences between the genomic and RT-PCR detection methods 10 samples were sent (blinded) from the laboratory of FG to the laboratory of SM, to determine the FLT3/ITD status. There was not a single discrepancy between the 2 methods, as has also been reported by Schnittger et al.18

Drug resistance testing

The cellular drug resistance studies were performed by Zwaan and co-workers. Mononuclear cells were isolated by density gradient centrifugation, and if necessary blast cell enrichment was performed as described before.27 To measure cellular resistance cells were seeded in 96-well microculture plates and cultured for 4 days. A panel of 14 different drugs was tested, including those frequently used in AML treatment.27 The control cell survival (CCS) was assessed by culturing cells without adding drugs. After 4 days MTT was added, which can be converted by viable cells into a colored formazan product and measured spectrophotometrically at 562 nm. The optical density (OD) is linearly related to the number of viable cells. Results were considered evaluable only if the control wells contained ≥70% leukemic cells, and if the mean control OD exceeded 0.05 arbitrary units. The LC50 value, which is the drug concentration that kills 50% of the leukemic cells, was used as a measure of resistance. Part of the data on drug resistance in AML have been published elsewhere, but not in relation to FLT3/ITD.27,36

Statistical analysis

To assess outcome the following parameters were used: complete remission rate (CR), event-free survival (EFS), disease free survival (DFS), overall survival (OS) and cumulative incidence of relapses. CR was defined as less than 5% leukemic cells in a bone marrow aspirate, no evidence of leukemia at any other site and hematological recovery, according to the CALGB criteria.37 Early death was defined as death before or during the first 6 weeks of treatment. EFS was defined as the time from diagnosis until the date of the first adverse event (relapse, death of any reason or second malignancies); or in case no events occurred, until the date of last follow-up. Patients who did not attain a complete remission were considered failures at time-point zero. DFS was defined as EFS for remission patients only. OS was defined as the time from diagnosis until death of any cause or last follow-up. Probabilities of EFS (pEFS) and OS (pOS) were estimated by the methods of Kaplan and Meier with standard errors according to Greenwood, and were compared using the log-rank test. The cumulative incidence functions of relapse were constructed by the method of Kalbfleisch and Prentice for patients who
achieved a complete remission, and compared with Gray’s test. Prognostic factors were examined by multivariate Cox regression analysis.

For statistical comparisons of categorical variables the Chi-square test was used. To assess differences in the distribution of LC50 values the non-parametric Mann-Whitney U test was used.

P-values of $\leq 0.05$ were considered statistically significant (2-tailed test).
RESULTS

Patient characteristics

We attempted to assess the FLT3/ITD status of 273 children with newly diagnosed AML included in the aforementioned protocols between 02/1990-08/2001. For 8 patients this could not be determined due to low DNA recovery from cytospin slides. Excluded were patients with mixed lineage leukemia (n=2) and MDS (n=3). One patient was not treated with curative attempt and was excluded as well. Samples from 13 children with Down syndrome (DS) and AML, and 12 children with secondary AML, were excluded from the prognostic analysis, but the samples were assessed for FLT3/ITD. Therefore, we successfully evaluated 234 patients for the prognostic significance of FLT3/ITD, which is the study population described here. This included 165 samples from the 934 (18%) de novo childhood AML patients included in the AML-BFM SG trials, as well as 69 samples from 171 (40%) patients included in the DCLSG trials over that time-period. Eighty (80) patients were classified as SR patients, and 154 as HR, according to the AML-BFM risk group criteria. Patient characteristics are given in Table 1. The study population included 20 infants (defined as age <1 year).

We analyzed the potential of selection bias for patients included in this study from AML-BFM SG trials. There were no significant differences in AML-BFM risk group distribution (SR 36% vs. 30%; p=0.20), cytogenetics (good risk cytogenetics 21% vs. 28%; p=0.14), sex (52% boys vs. 56%, p=0.30) or age (median 8.9 vs. 8.2 years, p=0.08), between the 165 AML-BFM SG patients included in this study vs. the 769 that were not included in this study. However, included patients had a higher WBC at diagnosis (30 vs. 17x10^9/l, p=0.0001). This did not result in significant differences in clinical outcome between the 2 groups regarding 5-year pEFS (49 vs. 51%; p=0.76) and pOS (62 vs. 60%; p=0.60).

Of the study population of 234 samples, drug resistance testing was performed in 173 samples. In a total of 39 (22.5%) samples the MTT-assay was not successful, hence we present drug resistance data from 134 children with AML. Patient characteristics are given in Table 1. Patients with a successful drug resistance assay had a significantly higher WBC at diagnosis (median 39.9x10^9/l vs. 13.8x10^9/l, p=<0.001) and were older than children without a successful drug resistance assay (median 8.7 vs. 3.9 years, p=0.009). There was no significant difference for sex (p=0.80).

Analysis of FLT3/ITD

From the 234 evaluable patients, 27 patients (11.5%) were positive for FLT3/ITD, divided in 9 SR and 18 HR patients. Patients with FLT3/ITD were significantly older than children without this mutation (median
13.4 vs. 8.8 years, p<0.001). No FLT3/ITD was found in the 20 infants (age < 1 year) included in this study (p=0.09, infants vs. older children). In children aged ≥1-<10 years the frequency of FLT3/ITD was 5.7%, whereas in children ≥10 years of age the frequency rose to 19.4% (p=0.002). There was no significant difference in WBC (median 50.3 vs. 30.5x10⁹/l, p=0.26), when WBC was analyzed as a continuous variable. When we divided the patients in those with a WBC <50x10⁹/l vs. those with a WBC ≥50x10⁹/l, again we found no significant differences between patients with and without a FLT3/ITD (WBC ≥50x10⁹/l in 52% vs. 38% respectively, p=0.17). There was no difference in sex distribution between FLT3/ITD positive and negative patients (p=0.16).

The FAB-type classification of FLT3/ITD positive patients is shown in Table 1. The number of FAB M1/M2 cases was 59% in FLT3/ITD positive cases versus 39% in the FLT3/ITD negative group (p=0.04, Chi square test, M1/M2 vs. non-M1/M2). FAB M5 represented only 7% of the FLT3/ITD positive cases, versus 23% in the FLT3/ITD negative group (p=0.04, M5 vs. non-M5). In addition, no FLT3/ITD occurred in the 21 FAB M4Eo cases (p=0.08, M4Eo vs. non-M4Eo).

The FLT3/ITD positive group contained only 2 patients with “good risk” cytogenetics (of the 22 patients of whom cytogenetic data were available), versus 40 of the 188 FLT3/ITD negative patients (p=0.37, Chi square test). Twelve FLT3/ITD positive patients were found to have a normal karyotype (54.5%), versus 42 (24.7%) FLT3/ITD negative patients (p=0.003). FLT3/ITD did not occur in the cytogenetic subgroups with 11q23 abnormalities (p=0.02), inv(16) (p=0.20), or abnormalities of chromosome 5/7 (p=0.30).

No FLT3/ITD were detected in the 13 Down syndrome AML patients (not included in the study population). Of the 12 patients with secondary AML (also excluded from the study population) 2 were positive for FLT3/ITD.

**Genescan analysis of the mutant to wild-type ratio**

From 21/27 patients with FLT3/ITD we successfully determined the mutant to WT-FLT3 ratio. Failures were due to lack of cells or DNA recovery. Blast percentages of the cytospin slides (prepared after blast cell enrichment as described earlier) used to determine the ratios was median 92% (range 84-98%, 1 outlier had 70% blasts with a ratio of 0.3). The ratio’s varied from 0.14-3.90, with a median value of 0.69. There was no significant correlation between blast cell count and ratios (rho=0.2, p=0.38). Patients with a ratio below or equal to the median had a lower WBC (39.7x10⁹/l) than patients with a ratio above the median (61.5x10⁹/l), but this
difference was not statistically significant (p=0.23). Also, there were no age differences between the 2 groups (p=0.55).

Clinical outcome and prognostic significance of FLT3/ITD

The CR rate was 70% for FLT3/ITD positive patients versus 88% for patients without FLT3/ITD (p=0.01). The Kaplan-Meier estimate for 5-year probability of EFS (pEFS) was 46% (SE 4%) for FLT3/ITD negative patients versus 29% (SE 9%) for patients with FLT3/ITD (p=0.0046; Figure 2a). Considering overall survival (OS), the estimated 5-year pOS of FLT3/ITD negative patients was 58% (SE 4%) compared with 32% (SE 12%) for FLT3/ITD positive patients (p=0.037). The probability of DFS at 5 years (for patients in CR only) was 53% (SE 5%) for FLT3/ITD negative versus 41% (SE 12%) for FLT3/ITD positive patients (p=0.09). Censoring SCT in first CR did not change the results considering DFS [FLT3/ITD negative 57% (SE 5%) vs. FLT3/ITD positive 42% (SE 15%), p=0.43]. The 5-year cumulative incidence of relapses for patients in CR was 39% (SE 5%) for FLT3/ITD negative and 47% (SE 15%) for FLT3/ITD positive patients (p=0.17). Median follow-up for patients still under observation was 3.3 years (range: 0.5-10.0 years) for FLT3/ITD negative patients versus 2.2 years (range 0.6-9.4 years) for FLT3/ITD positive patients.

Subsequently, we divided the patients according to the BFM criteria into SR and HR subgroups. The difference in 5-year pEFS between FLT3/ITD negative and positive patients was significant in the subgroup of SR patients [pEFS=61% (SE 8%) versus 22% (SE 14%) respectively; p=.0002], but not within the HR group [pEFS=39% (SE 5%) versus 34% (SE 12%); p=0.22]. The difference in 5-year pOS between FLT3/ITD negative and positive patients was significant in the subgroup of SR patients [pOS=73% (SE 7%) versus 22% (SE 14%) respectively; p<0.0001], but again not in the HR group [pOS=50% (SE 5%) versus 53% (SE 12%) respectively; p=0.78].

Within the subgroup of normal cytogenetics the difference in 5-year pEFS was 59% (SE 11%) for FLT3/ITD negative (n=42) versus 39% (SE 15%) for FLT3/ITD positive patients (n=12; p=0.06), with a difference in 5-year pOS of 77% (SE 8%) for FLT3/ITD negative versus 56% (SE 15%) for FLT3/ITD positive patients (p=0.18).

We next studied the prognostic impact of the mutant to WT-FLT3 ratios. Patients with high ratios had a relatively short follow-up (i.e. the 2 patients with high ratios that did not yet suffer from an event), therefore 2-year EFS data were used for comparison instead of 5-year EFS data. There was no significant difference in CR rate (50% vs. 73%, p=0.28) or 2-year pEFS between patients with a ratio above (n=10) versus those with a ratio
below or equal (n=11) to the median [pEFS 20% (SE 13%) vs. 44% (SE 15%); p=0.41], but numbers were small (depicted in Figure 2b). When we compared the FLT3/ITD negative samples with ITD positive samples with a ratio ≤0.69, there was no significant difference in CR rate (88% vs. 73%, p=0.14) or 2-year pEFS [61% (SE 3%) vs. 44% (SE 15%); p=0.26]. However, the FLT3/ITD positive samples with a ratio >0.69 did significantly worse than the ITD negative samples, both considering CR rate (50 vs. 88%, p=0.001) as well as 2-year EFS [pEFS 20% (SE 13%) vs. 61% (SE 3%); p=0.0037].

We evaluated several prognostic variables for pEFS in a multivariate Cox regression model. No significant prognostic relevance was found for: a) treatment according to Dutch versus German treatment protocols (p=0.55), b) FLT3/ITD analysis performed in the USA or in Germany (p=0.66) and c) interaction between location and FLT3/ITD [i.e. different prognostic impact of FLT3/ITD in the two cohorts] (p=0.27). To further determine which factors were independent prognostic factors for poor outcome (pEFS), we included the well-known prognostic factors in BFM-studies such as the BFM-risk group classification (SR or HR) and WBC (<50x10⁹/l or ≥50x10⁹/l), but also FLT3/ITD status and SCT in 1st CR as a time-dependent variable. FLT3/ITD strongly predicted for poor outcome (p=0.01), with an increase in relative risk for events of 1.92 (95% confidence interval (CI) 1.16-3.17). In addition, the AML-BFM risk group classification strongly predicted for EFS (p=0.007, relative risk 1.79 [95% CI 1.17-2.72]), as well as WBC larger than or equal to 50x10⁹/l (p=0.016, RR 1.58 [95%CI 1.09-2.30]). When cytogenetic subgroups [favorable cytogenetics defined as inv(16), t(8;21) and t(15;17)] were added to this model instead of the AML-BFM risk group classification, we again found FLT3/ITD to be the strongest independent predictor of outcome (p=0.022, RR 1.86). The mutant to WT-FLT3 ratio did not have independent prognostic significance, but when we added a ratio above 0.69 to the model we found this had an independent adverse prognostic significance (relative risk of 2.5, p=0.016).

Within the group of FLT3/ITD positive patients we compared patients in continuous complete remission with those with events. However, there were no significant differences in WBC (p=0.87) or age (p=0.63) or sex distribution (p=0.55) between these 2 groups. In addition, the FAB-type distribution was similar.

**MTT-assay**

The MTT-assay was successfully performed in 15 of the 20 (75%) FLT3/ITD positive and in 119/153 (78%) of the FLT3/ITD negative samples (Chi-square, p=0.78). In the FLT3/ITD positive subgroup failures were due to lack of cells before culture (n=1), transport longer than 48 hours (n=1), and low blast percentage after 4 days of culture (n=3). In the FLT3/ITD negative subgroup MTT-assay failures were due too: lack of cells before
culture (n=14), low blast percentage after 4 days of culture (n=14), low OD (n=5) and infection of the culture (n=1). There were no significant differences in OD/10^5 cells (p=0.42) or CCS (p=0.37) between FLT3/ITD positive and negative samples.

FLT3/ITD positive samples appeared to be significantly more resistant to 2-chlorodeoxyadenosine than the FLT3/ITD negative cases (median 2.0 fold, p=0.006). Although FLT3/ITD positive samples were 1.8 fold more resistant to etoposide than FLT3/ITD negative samples, this difference did not achieve statistical significance (p=0.27). For the other drugs tested, including cytarabine and the anthracyclines, no significant differences in cellular resistance were found between FLT3/ITD positive and negative samples. When we compared samples with a mutant to WT-FLT3 ratio above versus those with a ratio below or equal to the median no statistically significant differences in drug resistance were found. In addition, there were no significant differences when we compared these subgroups with the ITD negative samples.

We previously reported on differences within childhood AML considering FAB-type and cytogenetic subgroups and cellular drug resistance.27, 36 Given the differences in FAB-type distribution between FLT3/ITD positive and negative cases, we also analyzed if there were differences in drug resistance within the FAB M1/M2 subgroup with regards to FLT3/ITD status. However, no significant differences were found. In addition, when we analyzed differences in cellular drug resistance within the 'normal cytogenetics' subgroups, again no significant differences between FLT3/ITD positive and negative cases were found.
DISCUSSION

A FLT3/ITD was found in 11.5% of the children with newly diagnosed AML included in this study, which is the largest pediatric series to date. The occurrence of FLT3/ITD appeared to be age-dependent, i.e. no cases were found in infant AML, 5.1% in children 1-10 years of age, whereas in older children (≥10-18 years of age) the frequency rose to 19.4%. FLT3/ITD was not equally distributed among the different FAB-types, with a significantly higher frequency in patients with FAB M1/M2. Considering the cytogenetic subgroups, a normal karyotype was found in 54.5% of the FLT3/ITD positive patients; significantly higher than the 22.3% found in FLT3/ITD negative patients. Moreover, no FLT3/ITDs were found in the subgroup of AML with 11q23 abnormalities (p=0.02). Although FLT3/ITD has been described to occur frequently in acute promyelocytic leukemia (APL), we could not assess this due to the low number of patients with APL included in our study. 

Although not included in the study population for determination, we found no FLT3/ITD in the 13 DS AML patients analyzed. When we take all the reported pediatric studies together, the incidence of FLT3/ITD in newly diagnosed childhood AML cases is 12.8%, with 52% classified as FAB M1/M2 and 45% in the ‘normal cytogenetics’ subgroup (data not shown). 

The frequency of FLT3/ITD positivity is lower in children than the reported frequency of 20-35% in the adult series. It has to be noted that also for other cytogenetic abnormalities differences in frequencies between adults and children have been reported. For instance, the ‘normal cytogenetics’ subgroup comprises approximately 15-30% of children with AML and approximately 40-50% of adults with AML. As FLT3/ITD mainly occurs in this subgroup, this may add to the lower frequency observed in children. There are no data yet about the frequency of point mutations of FLT3 in children with AML.

FLT3/ITD positive patients did significantly worse in terms of CR rate, event free and overall survival than FLT3/ITD negative patients. The poor clinical outcome of FLT3/ITD positive patients could mainly be attributed to induction failures. However, FLT3/ITD positive patients who achieved CR seemed to have a lower DFS, although this did not reach statistical significance. It has to be noted that the median follow-up time of the FLT3/ITD positive patients at risk was shorter (2.1 years) than for FLT3/ITD negative patients (3.3 years), hence longer follow up might affect the relapse rates reported here. Meshinchi et al. also reported a significant difference in CR rate, and only 1 of the 6 FLT3/ITD positive patients achieving remission was a long-term survivor (4 relapsed, 1 died due to toxicity). This differs from the results found in 2 adult series, which both observed an increased relapse rate and no significant differences in CR rate or resistant disease. In part, these differences may be explained by differences in definitions of CR (especially regarding hematological recovery).
as well as differences in the induction regimens. We also analyzed the prognostic significance of the mutant versus WT FLT3 ratios. High ratios have been described to be more predictive regarding poor clinical outcome than the mere presence of FLT3/ITD by itself.23-25 Patients with a ratio above the median did not do significantly worse than patients with a ratio below or equal to the median in terms of CR rate or 2-year event free survival, but numbers were small. We subsequently compared the FLT3/ITD positive patients with higher ratios and those with lower ratios with the ITD negative patients, and showed that patients with higher ratios have the worst clinical outcome. This confirms the results of other studies.23-25 However, we feel this should not lead to the conclusion that patients with ratios below the median have a similar prognosis than patients without any FLT3/ITD. It is of interest that the ratios described by Thiede et al. for adult AML have a much wider range (0.03-32.56) than we found (0.14-3.9) in our patient cohort.25 Especially patients with a ratio above 2.0 did worse in their series, which we observed in only 1 patient. Larger series are needed to study if the ratios in pediatric AML differ from those in adults.

FLT3/ITD was an independent factor for pEFS in our study, and was a stronger predictor than AML-BFM risk group classification and high WBC at diagnosis. This confirms the results of Meshinchi et al. and Kondo et al. for AML in children.19, 22 Meshinchi et al. suggested that stem-cell transplant (SCT) might improve outcome in children with FLT3/ITD positive AML.19 In this study, multivariate analysis did not show any prognostic benefit of SCT in first CR. This is in line with earlier publications from the AML-BFM group, in which no clear benefit of SCT in 1st CR in childhood AML is found.3, 43, 44 Larger prospective studies are needed to further evaluate the role of SCT for patients with FLT3/ITD.

FLT3/ITD positive samples were significantly more in-vitro resistant to 2-chlorodeoxyadenosine (2-CdA) than FLT3/ITD negative samples. This might be related to the lower number of FAB M5 samples in the FLT3/ITD positive subgroup. In earlier studies, FAB M5 samples show enhanced sensitivity to 2-CdA, both in-vivo45 as well as in-vitro [CM Zwaan et al., unpublished observations, 2001]. However, the difference in prognosis between FLT3/ITD positive and negative patients reported here was not related to differences in sensitivity to 2-CdA, as patients were not treated with this drug. Although FLT3/ITD positive samples were more resistant to etoposide, this difference was not statistically significant. FLT3/ITD positive samples did not show enhanced in-vitro resistance to the other drugs, including those frequently used in AML treatment. This suggests that the bulk of AML cells can be killed just as effectively in FLT3/ITD mutated versus non-mutated samples. We cannot exclude however, that a small but resistant subclone might be responsible for the poorer
prognosis, as this cannot be picked up by the MTT-assay. In addition, the MTT-assay is a non-clonogenic assay, and in an earlier study we found no predictive value of cellular resistance to cytarabine and daunorubicin for long-term clinical outcome.\textsuperscript{36}

Other explanations for the poor prognosis of FLT3/ITD than cellular resistance may exist, such as enhanced re-growth potential of residual disease, resulting in an enhanced relapse rate. This is further supported by the myeloproliferative disease which developed in mice after introduction of FLT3/ITD in their stem cells.\textsuperscript{17} Obtaining control of this regrowth mechanism might be achieved by the novel FLT3/ITD inhibitors that have been developed recently, and awaits further testing in clinical trials.\textsuperscript{46}

One important potential selection bias of our study has to be mentioned. We only studied a relatively small subset (18\%) of patients included in the AML-BFM SG clinical trials. However, further analysis did not reveal clinically relevant differences regarding initial patient data and clinical outcome, between the patients from the entire cohort included in the AML-BFM studies and the patients tested for FLT3/ITD that were presented here, with the exception of a higher WBC in the latter group. This may have resulted in a slight overestimation of the frequency of FLT3/ITD in our patients, as this is associated with a higher WBC.

In conclusion, FLT3/ITD can be found in 10-15\% of children with AML, with an increasing frequency with increasing age. Despite that, in older children the frequency is still lower than the 20-30\% reported for adults with AML. FLT3/ITD is not equally distributed among FAB-types or cytogenetic subgroups, and occurs more frequently in the FAB M1/M2 and ‘normal cytogenetics’ subgroups. It is associated with significantly worse clinical outcome, and it was the strongest independent predictor for event-free survival. In addition, a high mutant to WT-FLT3 ratio further compromises prognosis. We have no evidence for differences in cellular drug resistance between samples with and without FLT3/ITD, but numbers in the FLT3/ITD positive subgroup were small. Further clinical studies will have to demonstrate the effectiveness of FLT3/ITD inhibitors to improve the prognosis of this subgroup of children with poor prognosis AML.
ACKNOWLEDGEMENTS

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REFERENCE LIST


LEGENDS OF FIGURES

Figure 1. AML-BFM 93 treatment protocol.
In the AML BFM 93 study patients were stratified according to risk-groups [standard risk (SR) or high risk (HR)] following induction therapy. At diagnosis, patients were randomized between induction therapy with daunorubicin (ADE) or idarubicin (AIE). SR patients were then treated with a 6 week consolidation block followed by an intensification course. For HR patients, 2 intensification blocks were given. The first (HAM) was given either before or after the 6-week consolidation block, the second one (HD-A+VP) as the last intensification block. Sibling SCT was advised for HR patients in first CR.

R1=first randomization, R2 is second randomization, y=year, SR=standard risk, HR=high risk, ADE=Ara-C (cytarabine), daunorubicin, etoposide. AIE=Ara-C, idarubicin, etoposide. HAM = high-dose Ara-C and mitoxantrone, HD-A = high-dose Ara-C, VP=etoposide.

Figure 2a. Five-year probability of event-free survival (EFS) in relation to FLT3 internal tandem duplication (FLT3/ITD)
Children with FLT3/ITD positive AML (n=27) have significantly poorer 5-year pEFS [46% (SE4%)] when compared with children without this particular mutation [n=207; pEFS 29% (SE9%); p log rank=0.0046].

Figure 2b. Two-year probability of event-free survival related to the ‘mutant to WT-FLT3 ratio’.
The FLT3/ITD mutated patients were divided in 2 groups according to the mutant to WT-FLT3 ratio. Children with ratios below or equal to the median have a similar outcome as FLT3/ITD negative patients (p=0.26), whereas patients with ratios above the median have significantly worse outcome than the ITD negative patients (p=0.037). The difference between patients with ratios below or equal to the median vs. those with high ratios was not significant (p=0.41), but numbers were small.
Table 1. Patient characteristics of the 234 children with de novo AML included in this study. A FLT3 internal tandem duplication (FLT3/ITD) was detected in 27 samples. In addition, the clinical characteristics of the subgroup of 134 children (15 with and 119 without FLT3/ITD) with a successful cellular drug resistance assay are given.

<table>
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<tr>
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<th>Study population</th>
<th>FLT3/ITD Positive patients</th>
<th>Patients with a successful drug resistance assay</th>
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<tr>
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<td>27 (=11.5%)</td>
<td>134 (incl.15 FLT3/ITD pos.)</td>
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<tr>
<td>WBC at diagnosis,</td>
<td>30.5 (11.8-94.2)</td>
<td>50.3 (21.2-117.3)</td>
<td>39.9 (15.2-117.4)</td>
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<tr>
<td>Median &amp; (P25-P75) (x10^9/l)</td>
<td>8.8 (2.8-13.2)</td>
<td>13.4 (9) (10.7-15.2)</td>
<td>8.7 (3.2-13.3)</td>
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<td>Sex (male: female)</td>
<td>126: 108</td>
<td>18: 9</td>
<td>68: 66</td>
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<tr>
<td>Age at diagnosis</td>
<td>8.8</td>
<td>13.4 (#)</td>
<td>8.7 (**)</td>
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<tr>
<td>Median &amp; (P25-P75, in years)</td>
<td>2.8-13.2</td>
<td>10.7-15.2</td>
<td>(3.2-13.3)</td>
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<td>HR</td>
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<tr>
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Legends belonging to table 1.

WBC = white blood cell count, P25-P75 = 25th and 75th percentile, m = male, f = female,
FAB = French-American-British morphology classification of AML

(2) FLT3/ITD positive patients were significantly older than FLT3/ITD negative ones (median 13.4 vs. 8.8 years, p<0.001). In addition, the frequency of FLT3/ITD was age dependent (see text, results section).

(3) samples with a normal karyotype were found in 54.5% of FLT3/ITD positive samples vs. 24.7% of FLT3/ITD negative samples (p=0.003), and no FLT3/ITD was found in any of AML samples with 11q23 abnormalities (p=0.02).

(##) FAB M1/M2 was found in 59% of FLT3/ITD positive patients vs. 39% in the FLT3/ITD negative group (p=0.04). FAB M5 was found in 7% of FLT3/ITD positive versus 23% in negative samples (p=0.04).

(**) patients with a successful drug resistance assay were significantly older (8.7 vs. 3.9 years, p=0.009) and had a higher WBC at diagnosis (39.9 x10⁹/l vs. 13.8 x10⁹/l, p<0.001), compared with those without a successful assay
Figure 1.
Figure 2a.

Log-Rank p = .0046

- FLT3 neg. (N=207, 96 events)
- FLT3 pos. (N=27, 19 events)
Figure 2b.

- FLT3 neg. $\hat{p} = .61$, SE = .03 (N=207, 96 events)
- FLT3 pos. Ratio $\leq .69$ $\hat{p} = .44$, SE = .15 (N=11, 7 events)
- FLT3 pos. Ratio $> .69$ $\hat{p} = .20$, SE = .13 (N=10, 8 events)
FLT3 internal tandem duplication in 234 children with acute myeloid leukemia (AML): prognostic significance and relation to cellular drug resistance