

Internal Tandem Duplication of FLT3 Gene in Egyptian Pediatric Acute Myeloid Leukemia and Acute Lymphoblastic Leukemia

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ABSTRACT

Objective: To study internal tandem duplication of FLT3 gene in Egyptian pediatric AML and ALL and its correlation to prognostic factors as well as clinical outcome.

Methods: We examined 152 newly diagnosed acute leukemia cases including 69 AML and 83 ALL by genomic PCR. We correlated tandem duplication of FLT3 to age, sex, TLC, FAB, immunophenotypic subtypes and induction of remission.

Results: Tandem duplication was found in 14/69 (20.3%) AML cases: M1 7/24, M2 1/14, M3 4/13, M4 0/8 and M5 1/4. Ten of the cases with duplication were males, 8 cases were in the high-risk age group [< 2 and ≥ 10] and 6 cases had TLC $\geq 50 \times 10^9/L$. Failure to achieve induction remission was 62% in cases with duplication vs. 31.7% in cases without duplication, also 37.5% vs. 14.6% of cases respectively remained refractory to therapy.

Tandem duplication was found in 6/83 (7.2%) ALL cases: 2 cases were precursor-B and 4 were T-ALL. Four cases with duplication were in the high-risk age group and 3 cases had TLC $\geq 50 \times 10^9/L$. Tandem duplication of FLT3 did not show an impact on induction remission rate in ALL cases.

Conclusion: FLT3/ITD in our pediatric AML cases (20.3%) was much higher than that reported in literature (5.3%-11%). It was as high as that reported in adults (20-23%). It was identified as a poor prognostic factor in pediatric AML as regards remission rate. FLT3/ITD was reported for the first time in classical ALL (7.4%). Further study on a larger scale is recommended.

Key Words: Prognostic factors - Pediatric AML and ALL - FLT3 - Molecular study.

INTRODUCTION

FLT3 (fms-like tyrosine kinase-CD 135) also referred to as fetal liver kinase 2 or stem cell tyrosine kinase located on chromosome 13q12 is a member of the class III receptor tyrosine

kinase family that includes the c-kit, c-fms and PDGF receptors [1]. FLT3 is composed of four domains; the extra cellular domains consisting of five immunoglobulin-like structures, the trans-membrane (TM) domain, the juxta-membrane (JM) domain and the tyrosine kinase (TK) domain separated by a kinase insert (KI) followed by a carboxyl tail [2].

FLT3 plays an important role with FLT3 ligand in the early stages of haematopoiesis. Their interaction regulates growth of pluripotent haematopoietic stem cells, early progenitor cells and immature lymphocytes as well as leukemic cells. In haematological malignancies expression of FLT3 gene was observed dominantly both at the m-RNA and protein levels in precursor-B ALL, AML especially those with monocytic component and blastic crisis of CML [3]. It has been demonstrated that FLT3 receptor activation causes proliferation of AML cells in vitro as it appears to both stimulate activation and inhibit apoptosis of the cells.

In recent years internal tandem duplication within JM/TK-1 domains as a somatic mutation of the FLT3 gene (FLT3/ITD) has been reported in about 20-23% of adult AML, 3% of MDS [4,5] about 11-13% in pediatric AML [6,7] but none in CML [7]. However limited data are available about pediatric ALL as only two studies were reported. One reported only 2 cases and they were biphenotypic while the other reported none [7,8].

The duplicated sequences were recognized in exons 11 and 12. Their location and length was different in every case. Notably, the altered FLT3 gene was always transcribed in frame.

In vitro studies proved that mutant FLT3/ITD receptors are dimerised in a ligand independent manner, leading to constitutive activation of the tyrosine kinase moieties that leads to autonomous, cytokine-independent growth in the mutant cells [9]. It was also reported that FLT3 was associated with the progression of acute promyelocytic leukemia and the leukemic transformation of MDS suggesting that FLT3/ITD may be associated with poor prognosis [6]. Several studies reported that FLT3/ITD was associated with poor outcome in adult AML as well as childhood AML.

In this study we analyzed tandem duplication of the FLT3 gene in Egyptian pediatric AML and ALL patients. We evaluated its correlation to other prognostic factors as well as remission rate.

PATIENTS AND METHODS

This study was carried out on 152 newly diagnosed pediatric acute leukemia cases, 69 AML and 83 ALL. All cases were presented to pediatric oncology unit of the National Cancer Institute, Cairo University.

AML patients included 39 males and 30 females with age ranging from 0.5-16 years with a median of 8 years. ALL patients included 53 males and 30 females with age ranging from 0.5-17 years with median of 6.5 years. Cases were diagnosed according to standard methods including morphological, cytochemical and immunological evaluation [10].

Bone marrow or peripheral blood cells were collected from patients at presentation. Mononuclear cells were obtained by Ficoll-Hypaque density-gradient centrifugation method and stored at -80°C until use.

Determination of FLT3 mutation:

All samples were analyzed for mutation in exon 11 of the FLT3 gene using genomic PCR method. The use of exon 11 specific primers allowed covering the whole JM and the first part of the TK-1 domain where most of the reported mutations are located. The genomic structure of FLT3 gene and site of primers used are presented in Fig. (1) [7].

High molecular weight DNA was prepared using a standard procedure [11].

Fifty to 100ng of genomic DNA was amplified in a 50 ul-reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM Mg Cl₂, 200 uM of each deoxyribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, 40 pmol of each primer and 6% dimethylsulphoxide. PCR amplification was performed on Perkin-Elmer Cetus thermal cycler. Amplification process consisted of 40 cycles of 30s at 94°C for denaturation, 45s at 50°C for annealing, 1 min at 72 for extension and one cycle of 7 min at 72°C for the final extension [7]. The sequence of the primers used is:

- 11F (Sense) 5' CAATTTAGGTATGAAAGCC-3'
- 11R (Antisense) 5' CAAACTCTAAATTTTCTCT-3'

Eight to 10 ul of the PCR product were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. Size marker ϕ X 174 Hae III was used.

Treatment:

Patients with AML were treated with induction therapy consisting of one course of ADE (Aracytin, Doxorubicin, Etoposide) followed by another two courses as consolidation. Four courses of MIDAC (Mitoxentron and intermediate dose Aracytin) as continuation therapy.

Patients with ALL were treated according to the NCI treatment protocol modified from the study XIII-B of St. Jude Children Research Hospital. It included 6 weeks of induction, 2 weeks of consolidation and 120 days of continuation therapy. Induction therapy consisted of prednisone, vincristine (VCR), daunomycin, asparaginase, etoposide (VP-16), aracytin (Ara-C), in addition to triple intrathecal (IT) therapy for CNS prophylaxis. Consolidation therapy included 2 courses of high dose methotrexate (HDMTX), 6-mercaptopurine (6-MP) and triple IT therapy. Continuation therapy consisted of extended triple IT therapy and 15 cycles of 8-week course of VP-16+cyclophosphamide (CTX), 6-MP+MTX, MTX+Ara-C, dexamethasone (Dex)+VCR, VP-16+Ara-C, 6-MP+HDMTX, VP-16+Ara-C, Dex+VCR. During continuation therapy reinduction was given in the form of VCR, daunomycin, Dex, HDMTX, 6-MP and triple IT therapy. HDMTX was replaced by MTX while VP-16 was substituted by 6-MP after week 54 to minimize late drug effect.

Clinical Evaluation and Follow up:

Bone marrow aspirate was done to evaluate response to chemotherapy (status post induction) following 6 weeks of induction therapy in ALL and following 1st course of ADE in AML. Evaluable cases included 49 AML and 55 ALL. Cases who died before treatment or their data were not available were excluded.

Evaluable patients were followed up to evaluate disease status for a period ranging from 6-48 months with median observation period of 24 months.

Complete remission (CR) was defined as a normocellular BM containing less than 5% blast cells and showing evidence of normal maturation of other marrow elements.

Disease-free survival (DFS): included time to relapse measured from the end of induction for patients who achieved CR (induction deaths and non-responders were excluded).

Statistical Analysis:

Patients data were tabulated, processed using (SPSS) statistical package for Windows. Qualitative data were expressed as frequency and percentage and quantitative data were expressed as mean \pm SD and median. *t*-student test and chi-square test were used for comparative analysis. Kaplan-Meier analysis was used for survival of patients.

RESULTS

This study included 69-de novo AML and 83-de novo ALL cases.

Frequency of FLT3/ITD:

An extra PCR band (mutant band) in addition to the 133-bp wild band was found in 20.3% (14/69) of AML cases and 7.2% (6/83) of ALL cases. The sizes of the mutant bands are variable. Representative samples of FLT3/ITD+ AML and ALL cases are shown in Fig. (2).

The frequency of tandem duplication of FLT3 gene in relation to FAB classification in cases of AML and to the phenotype in ALL cases is presented in Table (1). In AML cases, the highest frequency was associated with M3 and M1 FAB subtypes, lesser in M2 and none in M4. In ALL cases, the highest frequency was associated with T-early phenotype.

FLT3 and clinical features:

Clinical features of all FLT3/ITD+ and FLT3/ITD- AML & ALL cases are shown in Table (2).

FLT3/ITD in AML:

No statistically significant difference was encountered between FLT3/ITD+ and FLT3/ITD- cases except for age where FLT3/ITD+ had a higher mean age (11.3 \pm 5 vs. 7.5 \pm 4.69 years, *p* 0.01). About 57% of FLT3/ITD+ cases were in the high-risk age group (< 2 or \geq 10 years). FLT3 mutation was found in 28.6% of patients with age \geq 10 years compared to 14.6% for those less than 10. The clinical and laboratory findings of FLT3/ITD+ cases are detailed in Table (3).

FLT3/ITD in ALL:

No statistically significant difference was encountered between FLT3/ITD+ and FLT3/ITD- cases. About 66.6% of FLT3/ITD+ were in the high risk age group. FLT3 mutation was found in 8.5% of patients with age \geq 10 years compared to 6.25% for those less than 10. The clinical and laboratory of FLT3/ITD+ cases are detailed in Table (4).

Clinical outcome and follow up:

AML: 8/14 of FLT3/ITD+ and 41/55 FLT3/ITD- cases were evaluable. Table (5) shows status post induction of evaluable AML patients. FLT3 duplication had an impact on remission rate as only 25% of FLT3/ITD+ patients achieved CR vs. 48.7% of FLT3/ITD-. Remission failure was 62% vs. 31.7%. Also follow up of the patients showed that 3/8 (37.5%) of FLT3/ITD+ cases remained refractory to therapy compared to 6/41 (14.6%) of FLT3/ITD- cases. Though there are differences between the two groups no statistical significance was encountered.

Three-year disease free survival (DFS) was 21% vs. 0% for FLT3/ITD- and FLT3/ITD+ cases respectively (Fig. 3).

ALL: 5/6 of FLT3/ITD+ and 50/77 of FLT3/ITD- cases were evaluable.

Table (5) shows status post induction of the evaluable ALL patients.

The remission rate and DFS (Fig. 4) were comparable in FLT3/ITD+ and FLT3/ITD- ALL patients.

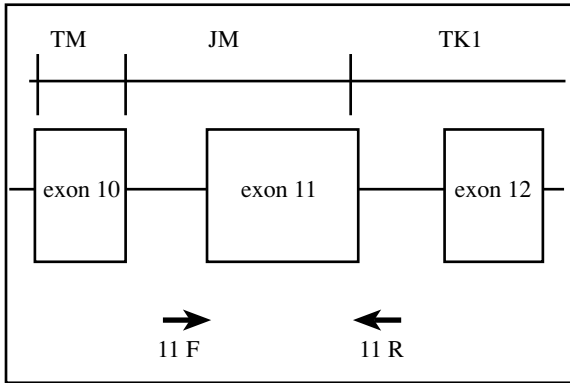


Fig. (1): Genomic structure of FLT3 gene and primers used for PCR.

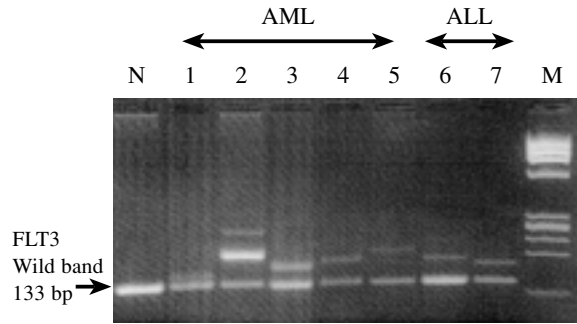


Fig. (2): PCR products of some FLT/ITD+ AML and ALL cases.
 N: normal peripheral lymphocytes Lanes 1-5 AML cases
 M: Size marker ϕ X174 Hae III Lanes 6,7 ALL cases

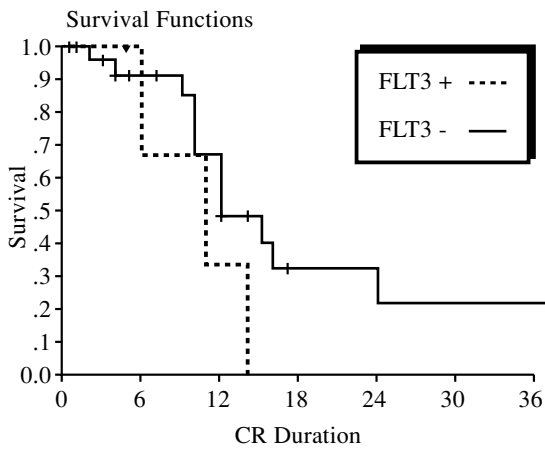


Fig. (3): DFS of AML patients in relation to FLT3/ITD.

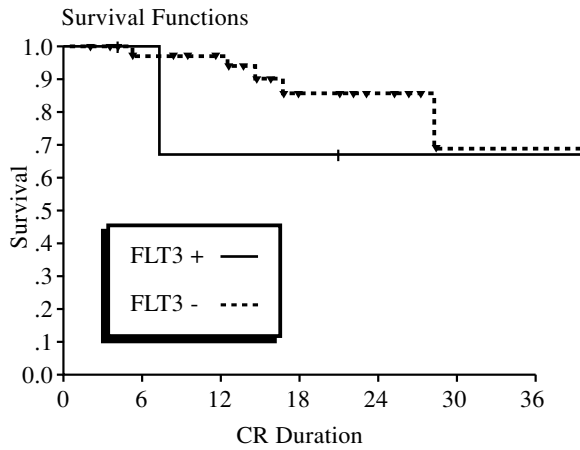


Fig. (4): DFS of ALL patients in relation to FLT3/ITD.

Table (1): Frequency of FLT3/ITD in AML & ALL patient.

Diagnosis	No. of patients	Tandem duplication
<i>*AML:</i>	69	14 (20.3%)
M1	24	7 (29.1%)
M2	14	1 (7.1%)
M3	13	4 (30.7%)
M4	8	0 (0%)
M5	5	1 (20%)
<i>ALL:</i>	83	6 (7.2%)
Precursor-B	50	2 (4%)
<i>T-ALL:</i>	33	4 (12.1%)
T-early	11	3 (27.2%)
T-intermed.	16	1 (6.2%)
T-late	6	0

*FAB subtype was not available in 5 AML cases.

Table (2): Clinical features of FLT3/ITD+ and FLT3/ITD- AML and ALL cases.

	AML		ALL	
	FLT3/ITD+ (n=14)	FLT3/ITD- (n=55)	FLT3/ITD+ (n=6)	FLT3/ITD- (n=77)
<i>Sex:</i>				
Males	10	29	3	50
Females	4	26	3	27
M:F ratio	2.5:1	1.1:1	1:1	1.8:1
<i>Age (in years):</i>				
Mean	11.3±5	7.5±4.6	9±5.7	7.9±5
Median	13	7	9.5	6.5
Range	3-18	0.3-18	1.5-16	0.5-17
< 2 yrs	0	6	1	4
2-10 yrs	6	29	2	41
≥ 10 yrs	8	20	3	32
<i>TLC x10⁹/L:</i>				
Mean ± SD	91.3±102.2	86±78.9	121.6±161.5	140.5±168.7
Median	41	63	72.4	66.6
Range	3.8-301	1.6-310	5.7-428	4.3-716
< 50x10 ⁹ /L	8	25	3	36
≥ 50x10 ⁹ /L	6	30	3	41
<i>BM blasts %:</i>				
Mean ± SD	73±0.2	59±0.2	88.8±12	86.7±15
Median	82	62	92	92
Range	22-92	5-98	65-97	30-99

Table (3): Clinical and laboratory characteristics of FLT3/ITD+ AML patients.

No.	Age	Sex	TLC x10 ⁹ /L	BM Blast	FAB	Induction Response	Status
1	15	M	57	62%	M2	RF	Resistant
2	15	M	13	85%	M1	CR	Died in CR
3	6	F	236	83%	M1	RF	Relapse
4	9	M	14	80%	M1	RF	Resistant
5	15	M	51	92%	M5b	RF	Resistant
6	3	M	3.8	75%	M3	RF	Relapse
7	12	M	16	22%	M3	CR	Relapse
8	16	F	31	90%	M1	Died	Died
9	8	F	11	--	M3	NE	NE
10	14	F	7.8	34%	M3	NE	NE
11	5	M	250	90%	M1	NE	NE
12	5	M	142	70%	M1	NE	NE
13	14	M	301	90%	ND	NE	NE
14	17	M	47	--	M1	NE	NE

RF: Remission Failure CR : Complete Remission
NE: Non-evaluable ND: Not done

Table (4): Clinical and laboratory characteristics of FLT3/ITD+ ALL patients.

No.	Age	Sex	TLC x10 ⁹ /L	BM Blast	Phenotype	Induction Response	Status
1	6	F	21.8	96%	Pre-B	CR	Relapse
2	5	M	428	95%	T-early	CR	CR
3	16	F	142.6	65%	T-early	Died	Died
4	13	M	123	90%	T-early	CR	CR
5	13	M	8.89	97%	T-inter	CR	CR
6	1.5	F	5.7	90%	Pre-B	NE	NE

CR: Complete Remission NE: Non-evaluable

Table (5): Status post induction of evaluable AML and ALL patients.

Status Post Induction	AML		ALL	
	FLT3/ITD+ (n=8)	FLT3/ITD- (n=41)	FLT3/ITD+ (n=5)	FLT3/ITD- (n=50)
Complete remission	2/8 (25%)	20/41(48.7%)	4/5 (80%)	37/50 (74%)
Remission failure	5/8 (62%)	13/41(31.7%)	0	6/50 (12%)
Died	1/8 (12.5%)	8/41(19.5%)	1/5 (20%)	7/50 (14%)

DISCUSSION

In this study we investigated tandem duplication of FLT3 gene by genomic PCR method in 152 newly diagnosed pediatric acute leukemia cases including 69 AML and 83 ALL cases. FLT3/ITD was found in 20.3% (14/69) of our childhood AML cases which is higher than most literature where figures of 5.3%, 11.3% and 13.8% were reported [4,6,8]. One study reported a relatively higher incidence of 16.5% [7], which is still lower than ours. It is comparable to the frequency reported in adult AML patients being 19.6% and 23% [12,8].

According to FAB classification the highest frequency of FLT3/ITD in our cases was in M3: 30.7% (4/13) and M1: 29.1% (7/24) subtypes. The frequency in M3 in the present study is comparable to the results of Kondo et al., 1999 and Liang et al., 2002 who reported the highest percentage of duplication among their M3 cases 66.6% (2/3) and 25% (3/12) respectively [6,13]. However, this is not in accordance with Xu et al. [7] who reported the highest percentage in M4 cases 27.3% (3/11) vs. none in our cases (0/8). The high frequency in our M1 cases 29.1% is much higher than that reported by Iwai et al., 1999, Kondo et al., 1999 as well as Xu et al., 1999 who reported the frequencies of 11% (1/9), 15% (2/13) and 20% (1/5) respectively [4,6,7].

On the other hand, 7.2% (6/83) of our ALL cases showed tandem duplication of FLT3 gene. Up to our best knowledge only two studies were reported in literature one by Nakao et al., 1996 who did not report this mutation in any of their 50 ALL cases [8]. The other study by Xu et al., 1999 [7] that reported the mutation in only two cases (2/60; 3.3%) both were biphenotypic expressing both lymphoid and myeloid antigens. Our 6 FLT3/ITD+ ALL cases included two Pre-B, three T-early and one T-intermediate. In our hands T-early fare worse than more mature T-ALL [12].

The only parameter that showed statistically significant difference between FLT3/ITD+ and

FLT3/ITD- AML cases was age. The mean age of FLT3/ITD+ cases was 11.3 vs. 7.5 years in FLT3/ITD- cases (p 0.01). All other prognostic factors including gender, TLC, BM blasts and FAB subtypes did not show any statistically significant difference.

Controversy exists as to the prognostic significance of these mutations. In the Japanese study by Kiyoi et al., 1999 and in the UK study by Kottaridis et al. 2001, presence of the FLT3 mutation did not appear to influence the CR rate but predicted for relapse rates (RR) and adversely predicted the overall survival (OS) [14,15]. A subsequent Dutch study; suggested that FLT3 mutations were associated with both lower CR rate and an increased RR [16]. A German study; has shown no prognostic significance to the presence of a FLT3 mutation [17]. All these studies were carried out on adult AML patients.

In our study, failure to achieve CR post induction was observed in 62.5% (5/8) of evaluable AML pediatric patients with FLT3 tandem duplication, as opposed to 31.7% (13/41) of patients without duplication. Furthermore, 37.5% (3/8) of patients with FLT3 duplication remained refractory to therapy compared to 14.6% (6/41) of patients without duplication. Although these differences did not attain statistical significance, our results may indicate that FLT3 mutation had an impact on clinical outcome in childhood AML, as there is an appreciable trend toward bad prognosis associated with FLT3/ITD+ cases. Indeed, of eight positive patients no one remained alive in CR. Similarly, other studies carried on childhood patients with AML demonstrated that the tandem duplication of FLT3 gene was associated with poor prognosis. Xu et al., 1999 reported that most of the patients with FLT3 tandem duplication were resistant to initial chemotherapy and could not be induced to enter CR [7]. Also, Kondo et al., 1999 demonstrated that FLT3/ITD was significantly associated with both high-induction failure rate and low event free survival (EFS) rate [6] and it was shown to be associated with significantly shorter disease

free survival in the study by Iwai et al., 1999 [4].

On the other hand, ALL cases with FLT3 mutation were not statistically associated with any of the prognostic factors even age. The remission rate and DFS were comparable in FLT3/ITD+ and FLT3/ITD- Patients. However we are dealing with only five evaluable FLT3/ITD+ cases, which made it impossible to make fair comparison.

The discovery of tandem duplication of FLT3 gene in AML raises 2 other issues besides prognosis. First, the presence of a mutation that activates a tyrosine kinase receptor introduces the possibility of using specific kinase inhibitors to treat this disease, analogous to the use of ST1571 in chronic myeloid leukemia. Second, a FLT3/ITD mutation might serve as a suitable marker for detection of minimal residual disease, although more extensive analysis of matched presentation and relapse samples would be needed before this could be applied clinically.

In conclusion we are reporting probably for the first time the presence of tandem duplication of FLT3 gene in classical pediatric ALL cases. The incidence reported in AML is higher than that reported in the literature. Yet we failed to report statistically significant impact on prognosis. These differences might be attributed to a biologically different disease. A larger number of cases with the mutation are needed to be studied.

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