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

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Abstract: Background: FLT3 plays an important role in stem cell proliferation, differentiation, and survival. The most common mutation in the *FLT3* gene is internal tandem duplication (*FLT3*/ITD). Several studies have demonstrated that FLT3 mutations are a strong detrimental prognostic factor in AML and ALL. Aim: Our study was designed to evaluate the relative frequency of *FLT3*/ITD status in adult AML and ALL patients and its possible impact on response to treatment and overall survival. Patients and Methods: This study was carried out on 110 newly diagnosed adult acute leukemia cases including 61 AML and 49 ALL. Bone marrow or peripheral blood samples were collected from patients at diagnosis. All samples were analyzed for mutation in exon 11 of the *FLT3* gene using genomic PCR method. *FLT3*/ITD appears as an extra PCR band (mutant band) in addition to the 133-bp wild band. ALL cases were treated according to the risk adapted chemotherapy protocol while mature B phenotype cases were excluded from the study. AML cases received induction with 3&7 regimen combing Daunorubicin, 45m/m2 IV day 1-3 and cytosine arabinoside100 mg /m2 by continuous infusion from day 1 to 7 as an induction regimen. Evaluation of response was carried out at the end of induction. Results: *FLT3*/ITD mutant band was found in 21.3 % (13 / 61) of AML cases. The highest frequency of *FLT3*/ITD was associated with M3 (40%) followed by M5 (37.5%) and M4 (33.3%), less frequent in M2 (13.6%), M1 (13.3%) and none in M0 and M7. In ALL cases *FLT3*/ITD was detected in (5/44) 10.2%. The highest frequency was associated with precursor B phenotype (11.4%) and less in T- ALL patients (7.1%). No association was detected between the *FLT3*/ITD on one side and age, gender, high leucocytic count, BM blasts, DNA index or CD34 expression on the other side in either AML or ALL. We could not find statistically significant difference in response to treatment between *FLT3*/ITD positive and negative cases in either AML or ALL patients. Conclusion: *FLT3*/ITD in our AML patients was 21.3 % which is comparable to the literature. However in adult ALL it was much higher than that reported in literature. In contrast to the literature we failed to demonstrate a detrimental effect of *FLT3*/ITD on treatment outcome in adult AML patients. Further study on a large scale is recommended to identify the prognostic impact of *FLT3*/ITD in adult ALL. [Journal of American Science 2010;6(9):344-352]. (ISSN: 1545-1003).

Key Words: Prognostic factors – Adult AML and ALL – FLT3

1. Introduction

The fms-like tyrosine kinase 3 (FLT3) is a member of the class III receptor tyrosine kinase, with a structure that resembles KIT, FMS, and the platelet-derived growth factor receptor [1, 2]. It is predominantly expressed on hematopoietic progenitor cells, but it is also found in a variety of hematological malignancies including acute myeloid leukemia (AML), B-precursor cell acute lymphoblastic leukemia (ALL), a fraction of T cell ALL, myelodysplastic syndrome in leukemic transformation, and chronic myelogenous leukemia in blast crisis [3, 4]. *FLT3* plays an important role in stem cell proliferation, differentiation, and survival. In normal hematopoesis, *FLT3* ligand binding to the FLT3 receptor causes dimerization of the receptor, autophosphorylation, activation of tyrosine kinase, and induction of multiple intracellular signaling pathways, which are involved in cell proliferation and leukemogenesis [5, 6]. The most common mutation in the *FLT3* gene is internal tandem duplication (*FLT3*/ITD) of the region coding for the juxtamembrane (JM) domain of the FLT3 receptor [7]. It can be found in approximately 20–30% of adult AML patients [8]. The frequency among children is much lower, approximately 5–22% [9]. The presence of a *FLT3*/ITD mutation is clearly associated with poor prognosis in both groups of patients.

Several studies have demonstrated that *FLT3* mutations are a strong prognostic factor in AML [8-17]. To date several large-scale analyses have revealed that *FLT3* mutations are essentially found in myeloid-lineage leukemia.[18,19,20]. However, *FLT3* mutations within an activation-loop were found in few cases of acute lymphoblastic leukemia (ALL) with mixed-lineage leukemia *(MLL)* gene-rearranged ALL [21]. It is notable that FLT3 is highly expressed in *MLL* gene-rearranged ALL, leading to the constitutive activation of wild-type FLT3 kinase, and that primary ALL cells and an ALL cell line SEMK2-M1, which strongly expressed FLT3 but did not carry

FLT3 mutations, had the same sensitivity to a potent FLT3 inhibitor as leukemia cells and a cell line with *FLT3* mutations [21, 22]

FLT3 is not only of utmost interest regarding physiological processes of hematopoietic cells but also with regard to pathological aspects, namely leukemogenesis, prognosis and therapy of leukemia. AML patients with *FLT3* mutations tend to have a poor prognosis; thus *FLT3* is an attractive target of therapy, for instance using kinase inhibitors [23].

2. Patients and Methods:

This study was carried out on 110 newly diagnosed adult acute leukemia cases including 61 AML and 49 ALL. All cases presented to the Medical Oncology Department of the National Cancer Institute, Cairo University.

AML patients included 37 male and 24 female with age ranging from 19-72 years with a median of 34 years while ALL patients included 33 male and 16 female with age ranging from 18-75 years with a median of 23.5 years. Cases were diagnosed according to standard methods including morphological, cytochemical and immunological evaluation. FAB subtype was determined in AML cases. ALL cases were classified according to immunophenotypes.

Bone marrow or peripheral blood cells were collected from patients at diagnosis. Mononuclear cells were obtained by Ficoll-Hypaque density-gradient centrifugation method and stored at –80^{o}C until use. DNA extraction was performed using salting out technique [24].

Immunophenotypic analysis: Immunophenotypic analysis was performed using whole blood staining method. Fresh samples were obtained from peripheral blood or bone marrow at the time of diagnosis. A wide panel of monoclonal antibodies (Mo Abs) was used. Myeloid Markers included CD13, CD33, CD14, CD41, CD61, Glycophorin A and Myeloperoxidase Lymphoid markers included CD19, CD22 for B Lineage and CD1, CD2, CD3, CD4, CD5 and CD7 for T- lineage and other markers used included CD45, HLA-DR, CD10, and CD34. All the monoclonal antibodies were obtained from Coulter Hialeah, FL.

Double and Triple marker labeling was performed, including proper isotype controls. Samples were analyzed on Flow Cytometer (Coulter Epics, XL, Hialeh).

Detection of surface markers by direct staining:-

The whole blood staining method was performed as previously described [25]. In short, 10 µl labeled Mo Ab was added to 100 µl whole blood (or BM), incubated in the dark for 20 minutes, hemolyzed (No-wash Lysing solutions, A11894, Beckman Coulter) and washed by PBS then analyzed.

DNA index (DI): was done for 49 ALL cases. Bone marrow or blood samples were processed with the DNA-Prep coulter Reagents Kit. The sample was shacked on an automatic shaker then 100µl of the suspension was taken. Hundred µl of the DNA-Prep LPR for lysing and permeabilizing cells was added to the suspension with continuous shacking for 8 to 12 seconds and 2 ml of the DNA-Prep Stain (propodium iodide) was added to the previous suspension with continuous shacking for another 30 seconds [26].The DNA-Prep stains DNA and double stranded RNA with Propidium iodide; ribonuclease is included to digest RNA. Samples were analyzed on the Coulter Epics XL-MCL flow cytometer.

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 located.

High molecular weight DNA was prepared using a standard procedure. Fifty to 100 ng of genomic DNA was amplified in a 50 µl reaction containing 10mM tris-HCL (pH8.3), 50 mMKCL and 1.5 mM MgCl2, 200 µM 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 ^{o}C for denaturation, 45s at 50 ^{o}C for annealing and 1 min at 72 ^{o}C for the final extension. The sequence of the primers used is:

-11F (Sense) 5’ CAATTTAGGTATGAAAGCC-3’

-11R (Antisense) 5’ CAAACTCTAAATTTTCTCT-3’

Eight to 10 µl of the PCR product were electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed. Size marker ϕX 174 Hae was used as a ladder.

Clinical Evaluation and Follow up:

Treatment protocol: AML cases received induction with 3 and 7 regimen combining Daunorubicin, 45m/m2 IV day 1-3 and cytosine arabinoside 100 mg /m2 by continuous infusion from day 1 to 7 as an induction regimen .Patients who attained complete remission were subjected to consolidation chemotherapy with high dose cytosine arabinoside and mitoxantrone (HAM regimen) or referred for stem cell transplantation according to their risk stratification. For the M3 cases induction

regimen included ATRA 45 mg/m^2 P.O.daily till CR or for a maximum of 90 days and Daunorubicin 60 mg/m^2 I.V. day 1-3 for 3 courses (one course every month). ALL cases were treated according to our risk adapted chemotherapy protocol: The patients were stratified according to their prognostic factors into standard, high, and very high risk groups. Mature B phenotype were excluded. The treatment plan included: **Prephase** for patients with high TLC and/or organomegaly. **Induction phase I**: Four drugs: Vincristine, Doxorubicin, L-Asparaginase and prednisone with intrathecal MTX. Patients that attained CR were subjected to cranial irradiation with 24 Gy and intrathecal MTX for four injections. **Phase II induction** with Cyclophosphamide and Cytarabine. **Consolidation phase I:** Vincristine, Doxorubicine and prednisone with Triple intrathecal. **Phase II consolidation**: Cyclophosphamide, Cytarabine and Etoposide with triple intrathecal. **Maintenance therapy**: two years with 6 mercaptopurine and methotrexate. For patients with high and very high risks who were not planned to stem cell transplantation, one cycle of high dose cytarabine and mitoxantrone (HAM regimen) was added between induction and consolidation while those with HLA identical donor ready for stem cell transplantation were referred for transplantation as post remission therapy after induction chemotherapy. Mature B phenotype cases were excluded from the study

Bone marrow aspirate was done to evaluate response to chemotherapy (status post induction). Cases who died before treatment or who didn't receive treatment due to poor performance status or elderly cases kept on supportive treatment 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.

Statistical Analysis:

SPSS was used for data analysis. Mean and standard deviation described quantitative data. Non parametric test compared two independent groups. Kaplen Meier method estimated survival. Log rank test compared survival curves; p-value is significant at 0.05 levels.

3. Results:

This study included newly diagnosed 61 AML and 49 ALL patients. AML patients included 37 male and 24 female with age ranging from 19-72 years with a median of 34 years. ALL patients included 33 male and 16 female with age ranging from 18-75 years with a median of 23.5 years.

An extra PCR band (mutant band) in addition to the 133-bp wild band was found in 21.3 % (13 / 61) of AML cases. The size of the mutant bands was variable. Representative samples of FLT3/ITD+ AML and ALL cases are shown in Fig (1).

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 (40%) followed by M4 (33.3%) and M5 (37.5%), lesser in M1 (13.3%), M2 (13.6%) and none in M0 and M7.

In ALL cases, the frequency was 11.4% in precursor B phenotype and only one T- ALL case (7.1%).

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

No statistically significant relation was observed in AML cases between *FLT3*-ITD and sex, age (p=0.53), TLC (p=0.46), or BM blasts (p=0.42), or in ALL cases between *FLT3*-ITD and sex, age (p=0.36), TLC (P=0.11), or BM blasts counts (P=0.29).

No statistically significant relation was observed in AML cases between *FLT3*/ITD and CD34 (P=0.61). In ALL cases there was, also no statistically significant association between FLT3+/ITD and CD34 (P=0.12) or DNA index (P=0.51).

The clinical and laboratory findings of *FLT3*/ITD AML cases are detailed in table (3). The clinical and laboratory findings of *FLT3*/ITD ALL cases are detailed in table (4).

Clinical outcome and follow up:

AML: Eight out of 13 patients with *FLT3*/ITD and 22/48 FLT3/WT cases were evaluable. Complete remission was achieved in 5/8 (62.4%) of FLT3/ITD+ patients versus12/22 % (37.52%) of *FLT3*/ITD- patients, no statistical significance was encountered between the two groups (p=0.95).

ALL:Four out of 5 *FLT3*/ITD and 27/44 of F*LT3*/WT cases were evaluable.

Complete remission was achieved in 2/4 (50%) of *FLT3*/ITD patients versus 17/22 (77.3%) of *FLT3*/WT patients; no statistical significance was encountered between the two groups (p=0.37).

Overall Survival: The mean survival for FLT3/ITD+ AML cases was 5+_1.96 versus 11.2+_5.2 months in FLT3/ITD- cases(p=0.95) while the mean survival for FLT3/ITD+ALL cases was 5.3+_4.8 compared to 26.6+_9.1months in FLT3/ITD- cases (P=0.37).

Table (1): Frequency of *FLT3*/ITD in AML and ALL patients:

Diagnosis	Number of patients	Tandem duplication
<u>AML:**</u>	**61**	**13 (21.3%)**
M0	2	0
M1	15	2 (13.3%)
M2	22	3 (13.6%)
M3	5	2 (40%)
M4	9	3 (33.3%)
M5	8	3 (37.5%)
M7	1	0
<u>**ALL:**</u>	**49**	**5 (10.2%)**
Precursor B	35	**4 (11.4%)**
***T-ALL:**	14	**1 (7.1%)**
T-early	7	0
T-intermediate	5	1* (20%)
T-late	2	0

Only one T- ALL case (T-intermediate)*; FAB subtype was not available in 4 AML cases**.

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

	AML		ALL	
	FLT3/ITD+ (n=13)	*FLT3*/WT (n=48)	*FLT3*/ITD+ (n=5)	*FLT3*/WT (n=44)
Sex:				
Male	9	28	3	29
Female	4	20	2	15
M:F ratio	2.25:1	1.4:1	1.5:1	1.93:1
Age in years:				
Mean ± SD	34.8±14.6	36.8±14.6	32.5±11.9	28.1±13.9
Median	29	34.5	32.5	23
Range	19-64	19-72	20-42	18-75
$TLCX10^9/L$:				
Mean ± SD	70.5±98.2	71.5±106.9	21.4±8.9	116.6±262.7
Median	47	25	16.7	48.5
Range	33-98	20-95		
BM blast %:				
Mean ± SD	66±23.4	59.5±23.3	60.5±36	80±17.1
Median	72	60	60.5	86
Range	33-98	20-97	35-86	45-97

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

No	Age	Sex	$TLCX10^9/L$	BM blasts	FAB	Induction response	Status
1	42	F	182	86	M1	No CR	Died
2	28	M	30	39	M3	NE	NE
3	64	M	15	77	M4	NE	NE
4	19	M	1.5	36	M3	NE	NE
5	29	M	62.4	60	M5	CR	Died
6	20	M	47	50	M1	No CR	Died
7	22	F	180	80	M2	No CR	Died
8	32	M	115	60	M4	CR	Died
9	46	M	103	33	M2	NE	NE
10	51	M	26.4	45	M2	CR	Died
11	25	F	143	98	M1	CR	Died
12	48	F	5.2	51	M2	CR	alive
13	35	M	6	83	M5	NE	NE

CR= complete remission NE= non evaluable

Table (4): Clinical and laboratory Characteristics in FLT3/ITD+ ALL patients:

No	Age	Sex	TLCX10^9/L	BM blasts	Phenotype	Induction response	Status
1	19	F	16.7	69	Pro-B	CR	Died
2	26	M	27.1	73	CALL	CR	Died
3	23	F	6.7	35	Pre-B	No CR	Died
4	18	M	28	88	Pre-B	No CR	Died
5	36	M	26.6	100	T-intermediate	NE	Died

CR= complete remission NE= non evaluable

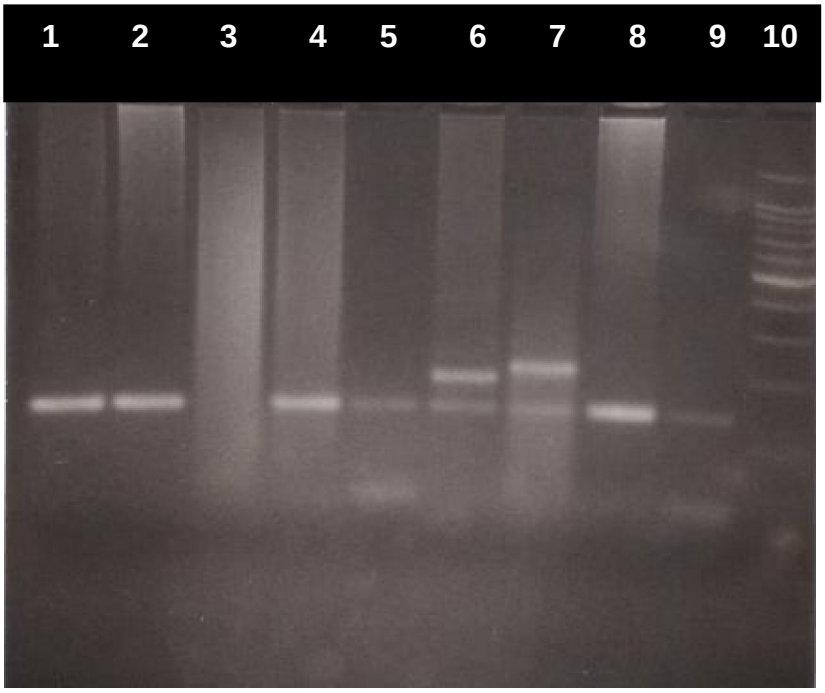

Figure 1: *FLT3* ITD:lane 6,7: wild & mutant bands. Lane1, 2, 4, 8 & 9: wild band. Lane 10: One Kb Molecular weight marker,Lane 3: degraded DNA.4.

4. Discussion:

In this study we investigated tandem duplication of *FLT3* gene by genomic PCR method in 110 newly diagnosed adult acute leukemia cases including 61 AML and 49 ALL. *FLT3*-ITD was found in 21.3 % (13 / 61) of our adult AML cases. These findings are comparative to several study groups on adult AML which reported a *FLT3*-ITD frequency ranging from 17.3% to 29% [27, 28, and 29]. In a large study on 854 AML patients from United Kingdom Medical Research Council, they found the incidence of *FLT3*-ITD mutation to be 27%, which is marginally higher than that found in the Japanese (23%), Dutch (22%), and German (23%) studies (13). It is possible that all studies based on DNA banks slightly overestimate the true incidence of *FLT3*-ITD mutations because there may be bias toward availability of DNA from patients with higher peripheral WBC count, which was shown to be associated with such mutations. In addition, 19% of their cases were FAB type M3, which has the highest incidence of *FLT3*-ITD mutations (36%).

FLT3-ITD was also found in 10.2% (5/49) of our adult ALL cases. In another Egyptian study done by EL Ghazaly et al (30) the incidence of this mutation in Egyptian adult ALL cases was 7.7% which is comparable to our findings. However a lower incidence was found by a Japanese study [18] who reported 2.8% (1/36). Up to our best knowledge, no much available data regarding the incidence of *FLT3*/ITD in adult ALL.

In our work no relationship was detected between the status of *FLT3*/ITD and age, gender, high leucocytes count, BM blasts or CD34 expression

in both adult AML and ALL or DNA index in ALL. These findings are in agreement with most of the studies except for a significant association noted by many authors between FLT3/ITD and high WBC [10, 23, 31]

According to FAB classification the highest frequency in our cases was in M3.

Abu-Duhier et al [12] reported that *FLT3*-ITD has been identified in all FAB subtypes and appears at the highest frequency in M3 and at the lowest frequency in M2 subtype. Kiyoi et al. [32] and Schnittger et al. [33] reported the highest frequency of *FLT3*/ITD among their M3 cases (28.6%) and (20.3%) respectively.

In our work we also encountered *FLT3*-ITD in 33.3% of M4 and 37.8% of M5, cases. This is in agreement with Colovic et al [35] who detected the highest frequency of *FLT3*/ITD mutations in FAB M5 [34, 17, 35].They reported that *FLT3* is persistently expressed during the process of monocyte differentiation, and that FLT3-L is required for complete differentiation of monocytes from CD34+ cells. According to these facts, they presumed that constitutive activation of *FLT3* might be associated with monocyte differentiation.

In this work, failure to achieve CR post induction was observed in 3/8 (37.5%) of evaluable AML patients with *FLT3* -ITD, as opposed to 10/22 (45.5%) with *FLT3*-WT. Although this difference was not statistically significant (P=0.37), our results may indicate that *FLT3* mutation had a bad impact on the overall outcome in adult AML as out of eight with *FLT3*-ITD positive patients no one remained alive in CR.

On the other hand, ALL cases with *FLT3* mutation were not statistically associated with any of the prognostic factors. The remission rate was comparable in *FLT3*-ITD and FLT3-WT patients. However we are dealing with only 4 precursor B *FLT3*/ITD cases and only one T- ALL case with *FLT3*-ITD+ which made it impossible to make fair comparison.

Colovic et al [35] demonstrated that the presence of *FLT3*-ITD mutation at diagnosis predicts the poor outcome of induction chemotherapy and attainment of CR in adult AML. Moreover, it was the most significant factor predicting for OS in their patients (P= 0.0287). Decreased OS has been reported in numerous studies of AML patients harboring the *FLT3*/ITD mutation, including the meta-analysis of Yanada et al. [36]. However, in several studies, the presence of the *FLT3*/ITD mutation did not affect OS. According to the author's explanations, it could be due to a higher percentage of elderly patients or patients with therapy-related AML, myelodysplastic syndrome, or AML with preceding myelodysplasia included in the study, short follow-up, or the application of more intensive induction chemotherapy [8, 37].

Suzki et al [38] suggested that FLT3/ITD mutation may be a factor indicating a lower OS probability and may be used as a prognostic marker even in unselected AML patients. They also stated that the probability of relapse tended to be higher in *FLT3*-ITD cases than in *FLT3*-WT cases after allografting but not after autologous transplantation.

However, Schnittger et al. [33], who performed a multivariate analysis, reported that the *FLT3*-ITD is not an independent prognostic factor for DFS or OS in the intermediate risk group of AML patients.

Colovic et al [35] investigated whether FLT3-ITD can be used as a marker of minimal residual disease. Most of the patients (75%) maintained the same FLT3 status at presentation and relapse except one who acquired the *FLT3*-ITD mutation at relapse.

Two studies, Yamamoto et al [18] and Kottaridis et al [8] who examined a thousand patients four years after receiving comparable therapies demonstrated that *FLT3*-ITD expression correlated with decreased overall survival [13,18]. Two other studies, Schnittger et al [33] and Thiede et al [17] observed no effect of the *FLT3*-ITD mutation on overall survival, although these results may be explained by the relatively short follow-up of 11.1 and 12.2 months.

In a retrospective study Scholl et al [39] analyzed MRD status of 11 AML patients and its potential impact on the follow up of these patients; they encountered relapse in five out of six patients with a positive *FLT3*-ITD based MRD status

Inhibitors of FLT3 are being tested in adult AML patients, with promising preliminary results. Yee et al [23] suggested that the addition of potent FLT3 inhibitors such as SU11248 to AML chemotherapy regimens could result in improved treatment results.

They tested the effect of combining the FLT3 inhibitor SU11248 with cytarabine or daunorubicin on the proliferation and survival of cell lines expressing either wild-type (WT) *FLT3 or* mutant (*FLT3*- ITD or *FLT3*- D835V). They reported that combining SU11248 and cytarabine synergistically inhibited the proliferation of primary AML myeloblasts expressing *FLT3*- ITD but not WT *FLT3* protein. More recent trial by Zarrinkar et al 2009(40) reported the characterization of AC220, a second-generation FLT3 inhibitor, and a comparison ofAC220with the first-generation FLT3 inhibitors CEP-701, MLN-518, PKC-412, sorafenib, and sunitinib. AC220 exhibits low nanomolarpotency in

biochemical and cellular assays and exceptional kinase selectivity, and in animal models is efficacious at doses as low as 1 mg/kg given orally once daily making it a suitable drug for FLT3 inhibitor in clinical trial. A trial determined the antileukemicactivity of CEP-701, a potent and selective FLT3 inhibitor, in 8 ALL cell lines and39 bone marrow samples obtained at diagnosis from infants and children with various subtypes of ALL. CEP-701 induced pronounced apoptotic responses in a higher percentage of samples that expressed high levels of FLT3 suggesting CEP-701 as a novel molecularly targeted agent for clinical trial in the treatment of ALL with FLT3 expression (41).

5. Conclusion:

FLT3-ITD was encountered in 21.3 % as reported in the literature. On the other hand the frequency in ALL was higher than that reported in the literature. Further study on a large scale is recommended to identify the prognostic impact of *FLT3*-ITD in adult ALL. We recommended that early detection of *FLT3* mutation should become a standard diagnostic procedure especially for AML patients as it can be used for MRD detection as well as a target of FLT3 inhibitors to improve the treatment outcome.

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