Prognostic Significance of FLT3 Internal Tandem Duplication in Egyptian patients with Acute Myeloid Leukemia with Normal or Favorable Risk Cytogenetics

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Internal tandem duplication (ITD) of the FLT3 gene (FLT3/ITD) has been linked to poor outcome in acute myeloid leukemia (AML). However, the prognostic value of FLT3/ ITD in various cytogenetic risk groups is still a matter of debate. The aim of this study was to evaluate the prognostic significance of FLT3/ITD in patients with de novo AML and normal or favorable risk cytogenetics (NFC-AML). Blood samples from 39 patients with AML were subjected to PCR of exons 14 and 15 of the FLT3 gene. Patients included 25 with normal cytogenetics, 8 with t(15;17), 4 with t(8;21) and 2 with inv(16). FLT3/ITD was found in 6/39 (15.4%) patients, 4 of them showed normal cytogenetic, 1 positive for t(15;17) and 1 positive for t(8;21). Patients were M1 3/13, M2 2/12, M3 1/9, M4 0/4 and M5 0/1. The patients were followed up for a mean of 34.5 ± 2.3 months. The complete remission (CR) rates for the FLT3/ITD+ and FLT/ITD- groups were 50% vs 63.6%, while the relapse rates were 50% vs 28.6% respectively. Interestingly, disease free survival (DFS) at 3 years was significantly different in studied patients: DFS was 5% in patients with FLT3/ITD+ vs 30% of patients with FLT3/ITD- (*P*= 0.001). Our data suggest a possible high prognostic value of FLT3/ITD in patients with normal/favorable cytogenetics.

Acute myelogenous leukemia (AML) is a clinically and molecularly heterogeneous disease (Bacher *et al.,* 2006), its diagnosis has been historically based on cytomorphology and cytochemistry using the French–American–British (FAB) classification (Bao *et al.,* 2006). The WHO classification subdivides AML predominantly according to karyotype since recurrent chromosomal abnormalities identify distinct leukemia entities and have a major impact on prognosis, favorable risk cytogenetics include t(15:17), t(8:21) and Inv(16), while normal karyotype is considered as intermediate risk. Moreover molecular analyses indicate that many AML patients have cytogenetically undetectable mutations that may affect prognosis (Bacher *et al.,* 2006). In the past 25 years, the majority of patients still succumb to the disease; prognosis is influenced by a number of factors, including white blood cell (WBCs) count on presentation, patient's age and the presence of chromosomal translocations in bone marrow aspirates (Bloomfield *et al.,* 1992). The most powerful prognostic factor in AML has been the karyotype of the leukemic cells, which predicts for both the CR rate and the relapse rate (RR) in those patients achieving a CR. Three cytogenetic risk groups, favorable, intermediate, and adverse are widely accepted, although there are minor differences in the precise definitions of these risk groupings among investigators (Bloomfield *et al.,* 1997; Grimwad *et al.,* 1998). A recent study has shown that an internal tandem duplication (ITD) in the FLT3 gene is a frequent mutation in AML (Pazhakh *et al.,* 2011). AML patients who carry the FLT3-ITD mutation appear to have poorer clinical outcomes. FLT3 mutations have been strongly associated with poor prognosis and high WBCs count in AML patients (Kottaridis *et al.,* 2001; Meshinchi *et al.,* 2001; Meshinchi

et al., 2006). Adult patients usually have a higher prevalence of FLT3/ITD than pediatric AML patients. This observation may partially explain why adult AML has a poorer clinical outcome than pediatric AML (Gregory *et al.,* 2009). Many clinical studies have shown that patients with an ITD at diagnosis have frequent disease relapse and a short duration of survival when compared to patients without an ITD (Schnittger *et al.,* 2002; Kim *et al.,* 2004).

FLT3, a member of the receptor tyrosine kinase class III family, is preferentially expressed on the surface of a high proportion of AML and B-lineage acute lymphoblastic leukemia (ALL) cells as well as hematopoietic stem cells (Stirewalt & Radich, 2003). An interaction of FLT3 and its ligand has been shown to play an important role in the survival, proliferation and differentiation of not only normal hematopoietic cells but also leukemia cells. Mutations of the FLT3 gene were first reported as an internal tandem duplication (ITD) of the juxtamembrane (JM) domain-coding sequence, and subsequently as a missense mutation of D835 within the activation loop (Yamamoto *et al.,* 2001). In addition, point mutations, deletions and insertions have been found in JM domain and in other codons of the kinase domain, although these are less common (Stirewalt *et al.,* 2004).

ITD of JM domain-coding sequence of the FLT3 gene (FLT3/ITD) on chromosome 13 has been identified in a group of patients with AML (Nakao *et al.,* 1996). In FLT3/ITD, a fragment of JM domain-coding sequence (Exons 14 and 15) is duplicated in direct head-to-tail orientation; length of ITD varies, but duplicated sequence is always in-frame (Yokota *et al.,* 1997). *in vitro* studies have shown that mutant FLT3/ITD receptors are dimerized in a ligand-independent manner, leading to autophosphorylation of the receptor through constitutive activation of the tyrosine kinase moieties (Kiyoi *et al.,* 1998). Such constitutive activation leads to autonomous, cytokine-independent growth in the mutant cells (Fenski *et al.,* 2000). The mechanism by which this mutation leads to tyrosine kinase activation is unknown (Thiede *et al.,* 2002).

We undertook this study to determine the frequency of FLT3/ITD and its prognostic significance in Egyptian AML with normal/favorable cytogenetics and its impact on the therapy of AML.

Patients and Methods

Newly diagnosed AML patients were included in this study; cases were recruited from and the Hematology-Oncology Centre, Internal Medicine Department (Unit 3), Mansoura School of Medicine, Egypt.

Exclusion criteria included: previous anticancer treatment, associated advanced medical comorbidity or patients diagnosed with poor cytogenetics.

After approval of the study by The Medical Ethical Committee of Mansoura School of Medicine, blood samples were obtained from 39 AML cases at initial diagnosis. Patients were followed by CBC done day after day during induction chemotherapy then at each clinical visit; bone marrow aspirate was done after induction chemotherapy to assure remission thereafter, on clinical suspension of relapse. After obtaining an informed consent from patients or their guardians; they were 19 males and 20 females, mean age was 47 years. The median percentage of blasts in the fresh bone marrow samples was 68% (range, 18%-89%).

Patients were managed according to our institutional protocols. Patients presenting with excessive leukocytosis at presentation are managed by emergency leukapheresis before commencing chemotherapy. Induction chemotherapy includes an anthracycline and cytosine arabinoside, with the well-known '3+7' regimen. Acute promyelocytic leukemia induction chemotherapy consists of all-trans retinoic acid (ATRA) and an anthracycline. During intensive chemotherapy, bone marrow was examined to monitor response. The accepted criteria of response were blast clearance in the bone marrow to <5% of all nucleated cells, morphologically normal haematopoiesis and return of peripheral blood cell counts to normal levels. Consolidation therapy was initiated once patients have reached clinical and hematological remission. In intermediate-risk patients, with an HLA-identical sibling are referred for allogeneicbone marrow

transplantation. In good risk patients and patients who are unsuitable for allogeneic stem cell transplantation, intensive consolidation chemotherapy, incorporating high-dose cytarabine is administered. Maintenance therapy has been used for first-remission acute promyelocytic leukemia only, where the combination of long-term chemotherapy and ATRA is used. The average duration of follow up was mean ± SD (34.5 ± 2.3 months).

Patients were classified according to the standard methods; morphological according FAB classification, cytochemical and immunological evaluation (Illmer *et al.,* 2005). Twenty subjects with matched age and sex were selected as a control group, recuirted from subjects referred to the oncology center for investigations who were proved to be free. Samples from patients and control were analyzed for mutation in Exons 14, 15 of the FLT3 gene using genomic PCR method. Genomic DNA was extracted from diagnostic blood samples of patients and control using the QIAamp DNA blood mini kit for DNA extraction provided by QIAGEN (Inc Chasworthy, CA). The concentration of extracted DNA was then measured by UV spectrophotometer at 260 & 280 nm and electrophoresed on agarose gel 2% stained with ethidium bromide.

PCR of FLT3 Gene

High molecular weight DNA was prepared using a standard procedure. Size marker ØX 174 Hae was used as a ladder. 328-bp fragment including exons14 and 15 of FLT3 gene were amplified using genomic Polymerase chain reaction (Meshinchi *et al.,* 2001). PCR amplification was performed in 50 µl reaction using primers

11F (5'-GCAATT TAG GTA TGAAAG CCAGC-3') and 12R (5'-CTT TCAGCATTT TGACGG CAA CC-3'). (Nakao *et al.,* 1996) - Applied Biosystem, USA.

The PCR mixture contained 50-100 ng of genomic DNA, PCR buffer (10 mM Tris HCl , pH 8.3, 50 mM KCl), 1.5 mM Mg Cl2, 200 mM of each deoxyribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, 40 pmol of each primer. PCR amplification was performed on Perkin-Elmer thermal cycler GenAmp9700. Denaturation, annealing and extension steps were performed at 94 ˚C for 30 seconds, 56˚C for 1 minute, and 72˚C for 2 minutes, respectively, for 35 cycles. There was initial 3 minutes denaturation step at 94˚C and a final extension at 72˚C for 7 minutes. PCR products were resolved on a 2% agarose gel stained with ethidium bromide. Source of internal control is normal peripheral lymphocyte. Size marker ØX 174 Hae was used as a ladder. Wild band is 133-bp and any extra PCR band is the mutant band

Cytogenetic Analysis

G banded metaphases were analyzed. Culture medium [Prepared by mixing of RPMI (Gibco laboratories) 100mL, Penicillin 10.000 u/mL and streptomycin 10mg/mL (Gibco laboratories] was prepared by placing 5mL of growth medium. The sample was added (5 drops of the blood sample) in each tube. Mix gently and then incubated for 24 hour at 37ºC in slanting position. For Harvesting different solutions was used including Colcemid solution (Gibco laboratories) 10mg/ml, hypotonic solution (0.56% KCL) and fixative solution made of 75mL absolute methanol+25mL glacial acetic acid. Two drops of Colcemid (0.02mL) were added to each culture tube with gentle shaking and were then incubated 45-60 minutes at 37ºC. Tubes were then centrifuged at 1000 rpm for 10 minutes. The supernatant fluid was discarded leaving as little medium as possible over the cell pellet. Five ml of the hypotonic solution were added drop by drop to each culture tube with shaking. The cultures were then incubated at 37ºC for 15 minutes, centrifuged for 10 minute and the supernatant discarded. Few drops of freshly prepared fixative were added to each tube. Tubes were then centrifuged at 1000rpm for 10 minutes and the supernatant discarded. The cells were re-suspended in a small volume of fixative. Few drops were dropped on a cold wet slide. The slide was then dried and G-Banding was done by putting the slides for one hour in a 90ºC oven, cooled to room temperature. Trypsinize the cells with 0.3 mL trypsin for 30 seconds to 3 minutes and then immersed in a jar filled with saline. Slides were then stained in Giemsa stain solution for 1-4 minutes. They were then rinsed in diluted water, air-dried and were examined using binocular high microscope (Olympus BX microscope and Cytovision data processing processing through Applied Biosystems). For each 20 metaphases, spreads were analyzed to detect any chromosomal aberrations. (Kohler, 1999) Vysis, USA.

The karyotypes were interpreted according to the International System for Human Cytogenetic Nomenclature (Mitelman *et al.,* 1991).

Statistical Analysis

Data were analyzed on a personal computer running SPSS© for windows (Statistical Package for Social Scientists) Release 15. All tests are considered significant if ($P \leq 0.05$), all statistical tests were two-sided. Qualitative data were expressed as frequency and percentage and quantitative data were expressed as median. Mann-Whitney U test and chi-square test

(fishers exact test if the assumptions of chi square were violated) were used for comparative analysis. Kaplan-Meier analysis was used for survival of patients. Multivariate analysis was performed using the Cox proportional hazards model.

Results

Genomic DNA was obtained from 39 patients with AML and exones 14,15 of the FLT3 gene were amplified by PCR. Mutant band was found in 15.4% (6/39) of AML cases compared to 0% in our controls (Fig. 1).

The frequency of FLT3/ITD+ gene mutation in relation to FAB classification is presented in Table (1).The highest frequency was associated with M1 and M2 FAB subtypes and not present in M4 and M5.

The frequency of FLT3/ITD+ gene mutation in relation to cytogenetics is shown in Table (2). Four (63.6%) FLT3/ITD+ gene mutations were found in patients with normal cytogenetic and one (16.7%) in patients with t(8;21). Among the patients with t(15;17) only one patient was positive for FLT3/ITD mutation (16.7%). None of the 2 patients with inv (16) was positive for FLT3/ITD (0%).

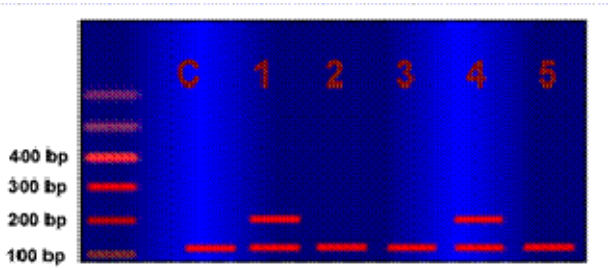

Figure 1. Genomic PCR for FLT3 .C for normal control, 1-5, samples from 5 different cases. Lane 1 and 4 represent FLT3/ITD+ cases. Lane 2, 3 and 5 represent FLT3/ITD- cases. *The 133 bp fragment indicates the size of the wild-type FLT3 gene in the absence of ITD (lane 2,3,5), **whereas additional upper bands of variable size (in the figure, it was 200 bp) were detectable in cases with the FLT3/ITD mutation (lane 1,4).

Table 1. Frequency of FLT3/ITD in AML Patients according to FAB Typing.

FAB typing	FLT3/ITD –ve (n=33)	FLT3/ITD +ve (n=6)
M1	10 (30.3%)	3 (50.0%)
M2	10 (30.3%)	2 (33.3%)
M3	8 (24.2%)	1 (16.7%)
M4	4 (12.1%)	0 (0%)
M5	1 (2.6%)	0 (0%)

FAB= French–American–British classification; FLT3/ITD= Internal tandem duplication of the FLT3 gene.

Table 2. Frequency of FLT3/ITD in AML Patients according to the Cytogenetics Classification

Cytogenetics		FLT3/ITD –ve (n=33)	FLT3/ITD +ve (n=6)
Normal		21 (63.6%)	4 (66.7%)
Favorable Risk	t(15:17)	07 (21.2%)	1 (16.7%)
	t(8:21)	03 (9%)	1 (16.7%)
	Inv(16)	02 (6%)	0 (0%)

The relations of FLT3/ITD status to patients and disease related variables in cases with normal cytogenetics are presented in Table (3). FLT3/ITD+ cases have higher median WBCs values and B.M blast. In addition, cases with FLT3/ITD+ with normal cytogenetic were associated with a lower complete remission (CR) rate (50% vs. 67%), and higher relapse rate 100% vs. 57%), however the difference was not statistically significant.

Table 3. Clinical Features of FLT3/ITD+ve versus FLT3/ITD-ve AML Patients with Normal Cytogenetics

		FLT3/ITD + N =4	FLT3/ITD – N=21	*P*=
Sex	Male	2	13	NS
	Female	2	7	
Age (Median)		52	45	NS
TLCx10^9 (Median)		97.4	62.6	0.037
BM blast cells (%)		83	52	0.041
Remission	CR	2(50%)	14 (67%)	NS
	No CR	2 (50%)	11 (33%)	
Relapse (cases in CR)	Relapse	2 (100%)	8 (57.1%)	NS
	Disease Free	0 (0%)	6 (42.9%)	

CR, complete remission; TLC, total leukocyte count; B.M, bone marrow. *P*<0.05 is significant. NS= not significant.

Table (4) shows status post induction and outcome in cases that tested positive for FLT3/ITD. Two cases achieved complete remission (33.3%); one of them relapsed early during consolidation, the other one (AML-M3) suffered relapse during maintenance. Remission failure was found in 66.7% of cases. Also follow up of the patients showed that 5/6 (83.3%) died.

Table 4. Clinical and Laboratory Characteristics of FLT3/ITD+ve AML Patients with Normal/Favorable Cytogenetics.

Induction Response	Outcome
CR	Relapsed during maintenance
RF	Death
Death	Death during the aplastic period of induction chemotherapy
RF	Death
RF (underwent leukapheresis and received only low dose cytarabine)	Death while on supportive treatment
CR	Relapsed During Consolidation and died after salvage chemotherapy

CR, complete remission; RF, remission failure.

The probability of disease free survival (DFS) was 5% in patients with FLT3/ITD+ vs 30% of patients with FLT3/ITD-, the difference was statistically significant (*P*= 0.001) (Fig.2). Multivariate modeling in patients with normal/favorable cytogenetics, including age,

WBCs, cytogenetic, and FLT3/ITD showed that FLT3/ITD+ was the sole independent adverse prognostic factor for DFS (P = 0.001, HR=1.52) (Table 5).

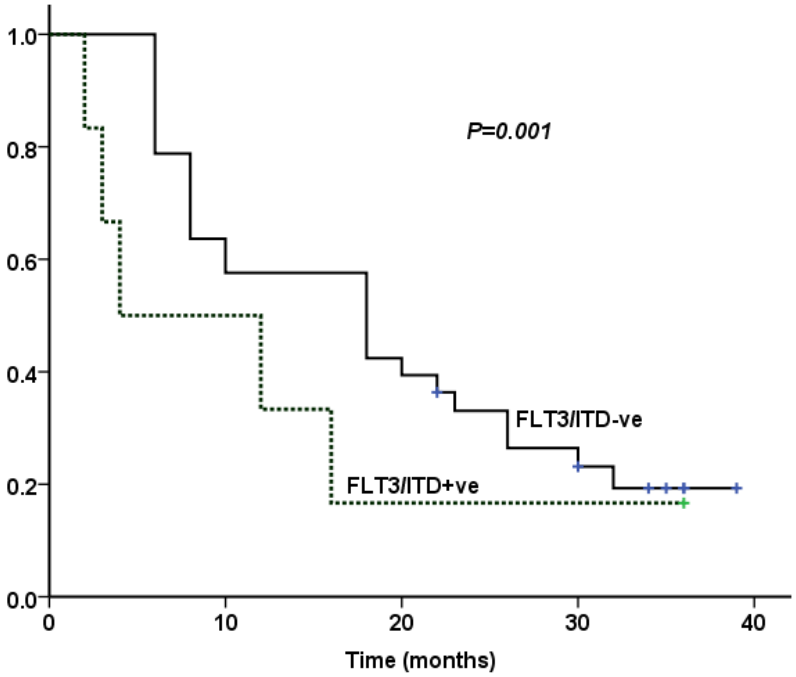

Figure 2. Disease free survival in AML cases withand without FLT3/ITD+ mutation (median 4 versus 18 months respectively)

Table 5. Multivariate Analysis for Disease Free Survival.

DFS	95% CI for HR	HR	*P*
FLT3/ITD positive vs FLT3/ITD negative	1.11-1.54	1.24	0.001

DFS, disease free survival; HR, hazard ratio. P<0.05 is significant.

Discussion

Despite significant efforts toward improving the clinical outcome of AML patients, much progress is still needed. The presence of FLT3/ITD had received significant attention for identification of high-risk patients. The clinical significance of FLT3/ITD has been suggested in previous studies (Kottaridis *et al.,* 2001; Meshinchi *et al.,* 2006; Thiede *et al.,* 2002; Ozeki *et al.,* 2004). Adult studies have shown a prevalence of 20-35% for the FLT3/ITD with an additional 7% for FLT3/ITD point mutation of the activation loop domain (Stirewalt *et al.,* 2004, Fenski *et al.,* 2000). In the current study, we evaluated the clinical and the prognostic significance of FLT3/ITD in a group of 39 AML patients with normal/favorable cytogenetics.

Despite growing data on the clinical significance of FLT3 mutations, difference in prognostic significance of FLT3/ITD as well as other factors within FLT3/ITD patient's group have precluded its use in risk-based therapy. Nakao *et al* first reported FLT3/ITD in AML and suggested its major role in the pathogenesis of AML (Nakao *et al.,* 1996).

The frequency of FLT3/ITD in pediatric AML appears to be somewhat lower than in adults with AML, occurring in about 10% to 15% of pediatric patients. In addition, the frequency of FLT3/ITD appears to be higher in elderly patients with AML (Gilliland & Griffin, 2002). In the current study, we demonstrated a low frequency of FLT3/ITD + mutation in Egyptian AML patients (6/39; 15.4%). This frequency was nearly similar to that recently reported from Iran (Pazhakh *et al.,* 2011) and was lower than reports from other studies (Kainz et al., 2002, Shih et al., 2002).Various studies have reported a high occurrence of ITD in 385 of 1595 of adult patients (24%) with AML and another study has shown that ITD mutations occur in 20 to 30% of AML cases (Nakao *et al.,* 1996; Yamamoto *et al.,* 2001). Overall, FLT3 ITD was found in 20-30% of patients with AML who have no cytogenetic abnormalities (Yanada *et al.,* 2005).

Evaluation of the characteristics of patients with and without FLT3/ITD demonstrated no statistically significant difference between FLT3/ITD+ and FLT3/ITD- cases regarding age and gender, a finding which is in agreement with those reported in literatures (Zwaan et al., 2003; Bao *et al.,* 2006). Clinically, AML patients with FLT3/ITD+ tend to have higher WBC counts and an increased percentage of leukemic blasts (Fröhling *et al.,* 2002). In our study, positive association has been found between FLT3/ITD mutation versus WBCs and blast counts, in accordance with others (Gilliland & Griffin, 2002; Bao *et al.,* 2006).

FLT3/ITD has been shown to cause constitutive activation of receptor tyrosine kinase (Kim *et al.,* 2004), leading to autonomous, cytokine-independent cellular proliferation (Fenski *et al.,* 2000; Rombouts *et al.,* 2000) and consequently leukocytosis, however the mechanism by which this mutation leads to tyrosine kinase activation is still unknown (Meshinchi *et al.,* 2001).

According to FAB classification, the highest frequency of FLT3/ITD in our cases was in M1 and M2, similar to the findings of Zwaan et al (2003), however the highest frequency in the work of Pazhakh and collegues was in the M3 cases (33.3%) (Pazhakh *et al.,* 2011) and in the work of Xu *et al.* (Xu F *et al.,* 1999), was in M4 cases (27.3%) but M4 cases with FLT3/ITD in our study were not found (0%), this may be attributed to the low number of our cases. In the current work, M1 cases with FLT3/ITD + constituted 50% of the total positive case, which is much higher than that reported in literatures 11-15% (Meierhoff *et al.,* 1995; Kondo *et al.,* 1999). Although FLT3/ITD has been described to occur frequently in acute promyelocytic leukemia (Thiede *et al.,* 2002, Liang *et al.,* 2002, Arrigoni *et al.,* 2003; Beitinjaneh *et al.,* 2010), we could not assess this issue as the number of that FAB type was low among patients positive for FLT3/ITD (16.7%).

Although the clinical significance of this FLT3 mutation especially in NFC-AML is not yet clear, several studies indicate that it is also an adverse prognostic indicator (Zheng *et al.,* 2005; Kiyoi *et al.,* 2006). Several studies have demonstrated that FLT3/ITD+ in NFC-AML patients correlates with an adverse prognosis for both DFS and OS (Kainz *et al.,* 2002; Ciolli *et al.,* 2004; Bienz *et al.,* 2005). In our study, FLT3/ITD patients had significantly worse prognosis in terms of complete remission (CR) rate, than FLT3/ITD- patients. Some studies showed that patients harbored this mutation had an extremely poor outcome compared with patients without FLT3/ITD (Meshinchi *et al.,* 2001). In a Japanese study by Kiyoi et al and in UK study by Kottaridis *et al.,* the presence of the FLT3 mutation did not appear to influence the achievement of CR (Kottaridis *et al.,* 2001). Lower remission and

higher relapse has been associated with FLT3 mutations in a Dutch study (Rombouts *et al.,* 2000). All the above studies were done in adults, also many pediatric AML studies have confirmed the poor clinical outcome of patients with FLT3/ITD (Fröhling *et al.,* 2002, Gilliland & Griffin, 2002).

In the present study, failure to achieve post induction remission was observed in 50% (3/6) of evaluable patients with FLT3/ITD+, as opposed to 33.3% (11/33) of patients without duplication. Most of patients with FLT/ITD+ were found to be resistant to initial chemotherapy and failed to achieve complete remission (Xu *et al.,* 2000, Arrigoni *et al.,* 2003). The most significant impact of FLT3/ITD is its association with increased relapse rate (RR) which has been reported in most studies of adults less than 60 years of age (Kottaridis *et al.,* 2003); RR among patients with normal/favorable cytogenetic who had achieved CR were higher in FLT3/ITD+ patients (100%) than in those with FLT3/wild (57%), a result that is in accordance with Kumiko *et al.* (2005).

In conclusion, FLT3/ITD+ is a promising prognostic marker in patients with AML .It may play an important role for diagnostic and therapeutic strategies in patients with AML with normal/favorable cytogenetics as may it help in identifying a subclass of these patients that may be reclassified as a poor risk category. Since the detection of FLT3 mutations is fast, easy and inexpensive (Kiyoi *et al.,* 2006), mutations analysis should be performed as a routine test in AML patients. A larger number of cases with FLT3/ITD mutation are needed to be studied.

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