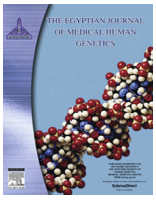
The Egyptian Journal of Medical Human Genetics 19 (2018) 345–351

HOSTED BY

Contents lists available at ScienceDirect

The Egyptian Journal of Medical Human Genetics

journal homepage: www.sciencedirect.com



Original article

FLT3 receptor/CD135 expression by flow cytometry in acute myeloid leukemia: Relation to *FLT3* gene mutations and mRNA transcripts

Mohamed Amin Mekawy, Deena Samir Eissa *, Mohamed Tarif Hamza, Gehan Mostafa Hamed, Mariam Karam Youssef

Department of Clinical Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

ARTICLE INFO

Article history:
Received 11 April 2018
Accepted 24 April 2018
Available online 3 May 2018

Keywords:
Acute myeloid leukemia
CD135
FLT3 receptor
FLT3 mRNA
FLT3-ITD
FLT3-TKD

ABSTRACT

Background: Alterations of the *FLT3* gene are the most frequent molecular aberrations seen at diagnosis of acute myeloid leukemia (AML). Two main types of *FLT3* mutations have been the most commonly detected; internal tandem duplication (ITD) in the juxtamembrane domain and point mutation D835Y in the tyrosine kinase domain (TKD). Both classes of mutations result in constitutive activation of FLT3 receptor/CD135.

Aim: To assess the frequency of *FLT3* gene mutations (ITD and TKD D835Y) and the flow cytometric expression of FLT3 receptor/CD135 among AML patients to define the role for FLT3 receptor expression in predicting *FLT3* gene mutational status and mRNA transcript level.

Subjects and methods: Eighty AML patients at diagnosis and 20 control subjects were enrolled. FLT3 receptor/CD135 expression, *FLT3* gene mutations, and *FLT3* transcript level were evaluated by flow cytometry, conventional polymerase chain reaction (PCR), and quantitative real-time reverse-transcription PCR, respectively. Fluorescence in situ hybridization was done to stratify patients into favorable, intermediate, and poor cytogenetic risk groups.

Results: *FLT3*-ITD was detected in 22.5% AML patients, while none had *FLT3*-TKD D835Y mutation. A cut-off value of >17% was assigned to define FLT3 receptor/CD135^{+} cases. FLT3 receptor/CD135 and *FLT3* transcripts were overexpressed in 100% AML patients; higher levels were found among AML-M5 subtype and poor cytogenetic group. AML patients harboring *FLT3*-ITD showed a trend for higher FLT3 receptor/CD135 expression and *FLT3* transcript level than those with wild-type *FLT3*. FLT3 receptor/CD135 >49% was predictive for *FLT3*-ITD. A positive correlation was found between FLT3 receptor/CD135 expression and *FLT3* transcript level.

Conclusion: Evaluation of FLT3 receptor/CD135 expression by flow cytometry at diagnosis of AML could constitute a predictor for the *FLT3*-ITD mutational status and *FLT3* transcript level.

1. Introduction

Acute myeloid leukemia (AML) is a remarkably complex neoplasm with considerable genetic, epigenetic, and phenotypic heterogeneity [1]. Gene expression profiling has improved the molecular classification and prognosis of AML, where molecular testing has become mandatory for AML with normal cytogenetics to further classify into prognostic groups [2]. Among the genetic aberrations, alterations of the FMS-like tyrosine kinase 3 (*FLT3*) gene have been shown to play a substantial role in AML pathogenesis and prognosis [3].

The *FLT3* gene maps to chromosome 13q12.2, consists of 24 exons, spans ~96 kb, and encodes a tyrosine kinase receptor that plays a key role in controlling survival, proliferation, and

Abbreviations: AML, acute myeloid leukemia; ANOVA, analysis of variance; BM, bone marrow; CT, cycle threshold; DNA, deoxyribonucleic acid; FAB, French-American-British; FISH, fluorescence in situ hybridization; FLK2, fetal liver tyrosine kinase 2; FLT3, FMS-like tyrosine kinase 3; FLT3L, FLT3 ligand; Hb, hemoglobin; IQR, interquartile range; ITD, internal tandem duplication; JMD, juxtamembrane domain; K-EDTA, potassium ethylene diamine tetra-acetic acid; MPO, myeloperoxidase; mRNA, messenger ribonucleic acid; NPV, negative predictive value; PB, peripheral blood; PCR, polymerase chain reaction; PPV, positive predictive value; qRT-PCR, real-time reverse-transcription polymerase chain reaction; ROC, receiver operating characteristic; SD, standard deviation; SPSS, statistical program for social science; SSC, side scatter; STK1, stem cell tyrosine kinase 1; TdT, terminal deoxynucleotidyl transferase; TKD, tyrosine kinase domain; TLC, total leucocytic count; TMD, transmembrane domain; WHO, World Health Organization.

Peer review under responsibility of Ain Shams University.

* Corresponding author at: Department of Clinical Pathology, Ain Shams University Hospitals, Ramses St., Abbassia, Cairo 11566, Egypt.

E-mail address: dr.deena_samir@yahoo.com (D.S. Eissa).

https://doi.org/10.1016/j.ejmhg.2018.04.006
1110-8630/

differentiation of hematopoietic cells [4]. In AML patients, two main types of activating mutations in the *FLT3* gene have been identified and represent the most frequently observed molecular aberrations at diagnosis of AML; the internal tandem duplications (ITDs) in exon 14 and 15 encoding the juxtamembrane domain (JMD), generated by the in-frame insertion of 18 to more than 100 bp, and the missense point mutations in exon 20, most commonly involving the amino acid substitution aspartic acid to tyrosine in codon 835 (D835Y) of the activation loop of the tyrosine kinase domain (TKD). These mutations lead to loss of the autoinhibition exerted by the JMD over the TKD resulting in overexpression or constitutive ligand-independent phosphorylation/activation of the FLT3 receptor, disturbing the intracellular signaling networks and deregulating the delicate balance between cell proliferation and differentiation. Less commonly in AML, ITDs can occur in the TKD, and point mutations in the JMD; although these mutations lead to constitutive activation of the receptor, their biological and clinical role remains to be clarified [5]. While there are diverging data concerning the prognostic impact for missense TKD mutations, many studies have supported that ITDs represent a major independent adverse prognostic indicator associated with increased risk for relapse and worse overall survival in AML patients [6–8].

FLT3 receptor/CD135, also known as FLK2 (fetal liver tyrosine kinase 2) or STK1 (stem cell tyrosine kinase 1), is a transmembrane tyrosine kinase receptor composed of 933 amino acids, with a molecular weight of 155–160 kDa, belonging to class III family of receptor tyrosine kinases. FLT3 receptor is composed of five extracellular immunoglobulin-like domains, transmembrane domain (TMD), JMD, and TKD consisting of two lobes (TK1 and TK2) interconnected by a tyrosine kinase insert [5]. FLT3 receptor is normally expressed on the surface of hematopoietic stem/progenitor cells and is lost upon hematopoietic cell differentiation [6]. The FLT3 ligand (FLT3L) is expressed both as a soluble form and a membrane-bound form by cells of the bone marrow (BM) microenvironment, including BM stromal cells and hematopoietic progenitor cells [3]. FLT3/FLT3L interaction induces receptor dimerization that promotes phosphorylation of tyrosine residues in the TKD and JMD, resulting in activation of the receptor and its downstream effectors, stimulating survival and proliferation, and inhibiting apoptosis of progenitor cells [5]. Overexpression of the FLT3 receptor has been reported to be associated with high risk for relapse in AML patients [9–11].

This study aimed to assess the frequency of *FLT3* gene mutations (ITD and TKD D835Y), and the flow cytometric expression of FLT3 receptor/CD135 among a cohort of de novo AML patients to define the role for FLT3 receptor expression in predicting *FLT3* gene mutational status and mRNA transcript level.

2. Subjects and Methods

This study was conducted on 80 newly-diagnosed de novo AML patients admitted to the Hematology/Oncology Unit, Ain Shams University Hospitals during the period March 2015 to January 2017. They were 46 males and 34 females (male to female ratio, 1.4:1), with mean age 43.7 ± 19.9 years (range, 23–65 years). Twenty age- and sex-matched healthy individuals recruited from BM donors candidate for allogenic BM transplantation were enrolled as a control group. They were 12 males and 8 females (male to female ratio, 1.5:1), with mean age 45.3 ± 16.7 years (range, 21–55 years). Informed written consents were obtained from patients and controls prior to enrollment. All procedures were in accordance with the standards of the Ethical Committee for Human Experimentation of Ain Shams University and the Helsinki Declaration of 1964, as revised in 2008. The patients were diagnosed and classified according to the World Health Organization (WHO) proposed criteria of myeloid neoplasms and acute leukemia [12]. The clinical and laboratory characteristics of AML patients are summarized in Table 1.

2.1. Sampling

Peripheral blood (PB) and BM specimens were collected on potassium ethylene diamine tetra-acetic acid (K-EDTA) (1.5 mg/mL) for morphologic, immunophenotypic, and molecular analyses,

Table 1
Clinical and laboratory characteristics of AML patients.

Parameters	AML patients (n = 80)	*FLT3*-ITD (n = 18)	*FLT3*-WT (n = 62)	P-value*
Age (years), mean ± SD	43.7 ± 19.9	44.1 ± 20.6	43.5 ± 20.1	0.942
Gender, n (%)				
Male	46 (57.5)	8 (44.4)	38 (61.3)	0.456
Female	34 (42.5)	10 (55.6)	24 (38.7)	
Hepatosplenomegaly, n (%)	24 (30)	6 (33.3)	18 (29)	1.000
Lymphadenopathy, n (%)	20 (25)	4 (22.2)	16 (25.8)	1.000
TLC ($\times 10^9$/L), median (IQR)	29.9 (10.9–66.1)	31.8 (3.9–50)	28 (11–68.4)	0.373
Hemoglobin (g/dL), mean ± SD	7.9 ± 1.7	7.9 ± 2.5	7.9 ± 1.5	0.944
Platelets ($\times 10^9$/L), median (IQR)	49 (21.5–102)	48 (38–112)	50 (18–94)	0.560
BM blasts (%), mean ± SD	79.6 ± 18.6	76.2 ± 22.5	80.6 ± 17.5	0.542
FAB subtypes, n (%)				
M0	12 (15)	4 (22.2)	8 (12.9)	0.413
M1	10 (12.5)	2 (11.1)	8 (12.9)	
M2	26 (32.5)	6 (33.3)	20 (32.3)	
M3	16 (20)	6 (33.3)	10 (16.1)	
M5	14 (17.5)	0 (0)	14 (22.6)	
M7	2 (2.5)	0 (0)	2 (3.2)	
Cytogenetic risk groups, n (%)				
Favorable	28 (35)	8 (44.4)	20 (35.7)	0.184
Intermediate	30 (37.5)	10 (55.6)	20 (35.7)	
Poor	16 (20)	0 (0)	16 (28.6)	
Not available	6 (7.5)	0 (0)	6 (9.7)	
FLT3 mRNA expression level, median (IQR)	35 (19.5–83)	47 (39–92)	30 (18–82)	0.060
FLT3 receptor/CD135%, mean ± SD	53.2 ± 22.4	64.2 ± 17.2	50 ± 23	0.070

AML, acute myeloid leukemia; BM, bone marrow; FAB, French-American-British; *FLT3*, FMS-like tyrosine kinase 3; IQR, interquartile range; ITD, internal tandem duplication; n, number; SD, standard deviation; TLC, total leucocytic count; WT, wild-type; *, P-value for comparison between *FLT3*-ITD and *FLT3*-WT groups. P-value <0.05 is significant, and P-value <0.01 is highly-significant.

and on lithium heparin for fluorescence in situ hybridization (FISH).

2.2. Flow cytometric immunophenotyping for standard acute leukemia panel and surface FLT3 receptor/CD135 expression

Immunophenotyping was performed on BM/PB specimens using the standard panel of FITC/PE/PC5-labeled monoclonal antibodies for acute leukemia (CD45, CD34, CD38, HLA-DR, CD117, CD13, CD33, CD14, myeloperoxidase [MPO], CD19, CD10, CD20, CD79a, CD22, terminal deoxynucleotidyl transferase [TdT], CD2, CD3, CD5, CD7) (Beckman Coulter, Miami, USA) on Epics XL flow cytometer (Beckman Coulter, Inc., Hialeah, FL, USA). Antigens were scored positive using a cut-off value of 20% or more leukemic blasts staining brighter than an isotype-matched negative control (anti-mouse IgG1, Beckman Coulter). A cut-off value of 10% was used for CD34, MPO, and TdT [13].

The FLT3 receptor/CD135-PE-labeled monoclonal antibody (R&D Systems, Minneapolis, MN, USA) was used for evaluation of the surface expression of FLT3 receptor/CD135. Fresh specimens were counted using Coulter LH 750 Hematology Analyzer (Beckman Coulter Inc., Fullerton, California, USA), and the total leucocytic count (TLC) was adjusted to 5.0×10^9/L using phosphate-buffered saline (8.5 g NaCL, 1.07 g Na_2HPO_4, 0.39 g NaH_2PO_4, in 1 L deionized water, adjusted to pH 7.4; Sigma-Aldrich, Saint Louis, MO, USA). Fifty μL of the adjusted specimens were added to the control and test tubes, as well as 5 μL of the isotype-matched negative control and FLT3 receptor/CD135-PE monoclonal antibody added to the control and test tubes, respectively. The control and test tubes were incubated at room temperature, protected from light, for 15 min, followed by the addition of 1–2 mL of ammonium chloride-based erythrocyte lysing solution (8.29 g [0.15 M] NH_4Cl, 1 g [10 mM] $KHCO_3$, 0.037 g [0.1 mM] EDTA, in 1 L distilled water, adjusted to pH 7.3; Sigma-Aldrich, Saint Louis, MO, USA). Tubes were vortexed and analyzed on Coulter Epics XL flow cytometer. FLT3 receptor/CD135 expression was assessed on the gated CD45dim/side scatter (SSC) low blast cell region. The percentage of FLT3 receptor/CD135$^+$ blast cells was determined as a percentage of the gated blast cell population.

2.3. Conventional polymerase chain reaction (PCR) for FLT3 gene mutations

The LeukoStrat™ *FLT3* Mutation Assay-Gel Detection (Invivoscribe Technologies, Inc., CA, USA) was used for PCR-based detection of *FLT3* activating ITD and TKD mutations. *FLT3*-ITD master mix contained dNTPs, the forward (5'-CAATTTAGGTATGAAAGCC-3') and reverse (5′-GTACCTTTCAGCATTTTGAC-3′) primers, whereas *FLT3*-TKD D835Y master mix contained dNTPs, the forward (5′-CCGCCAGGAACGTGCTTG-3′) and reverse (5′-GCAGCCTCA CATTGCCCC-3′) primers (Invivoscribe Technologies, Inc., USA). Positive and negative control DNAs (Invivoscribe Technologies, Inc., USA), AmpliTaq Gold DNA polymerase (Applied Biosystems, Life Technologies™, USA), 100 bp DNA ladder (Invitrogen, Life Technologies™, USA), and EcoRV restriction endonuclease for TKD mutation (Invitrogen, Life Technologies™, USA) were used. The DNA was extracted from fresh BM/PB specimens using QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer's protocol. The eluted DNA was buffered and stored at −30 °C till assay.

Veriti™ thermal cycler (Applied Biosystems, Life Technologies™, USA) was employed for DNA amplification with conditions of initial denaturation at 95 °C for 7 min followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, then a final extension step at 72 °C for 10 min. EcoRV restriction endonuclease digest was performed post PCR for the *FLT3*-TKD D835Y master mix. Amplified PCR products together with DNA molecular weight marker were run on 2% agarose gel stained with ethidium bromide.

FLT3-ITD alleles yield a product that exceeds 330 bp, whereas *FLT3*-wild-type alleles yield a 330 bp product. *FLT3*-TKD D835Y mutant alleles yield a 130 bp digestion product, whereas *FLT3*-wild-type alleles yield an 80 bp digestion product from the original undigested amplicon product of 150 bp.

2.4. Real-time reverse-transcription polymerase chain reaction (qRT-PCR) for quantification of FLT3 mRNA transcript level

qRT-PCR for the *FLT3* mRNA transcript level was performed using the QuantiFast Probe RT-PCR Plus kit designated for quantitative, probe-based, real-time, one-step RT-PCR with integrated genomic DNA removal (Applied Biosystems, Foster City, CA, USA). QuantiFast Probe Assay for *FLT3* target gene included forward primer (5′-TTTCACAGGACTTGGACAGAGATTT-3′), reverse primer (5′-GAGTCCGGGTGTATCTGAACTTCT-3′), and FAM-labeled TaqMan probe (5′-TCCAAATTCCAGCATGCCTGGTTCAAG- 3′), whereas QuantiFast Probe Assay for *β-actin* reference gene included forward primer (5′-TGACGGGGTCACCCACAC-3′), reverse primer (5′-CTAGA AGCATTTGCGGTGGA-3′), and VIC-labeled TaqMan probe (5′-TTC ACCACCACGGCCGAGC- 3′). Both QuantiFast Probe Assays were labeled with a non-fluorescent quencher. The total RNA was extracted from fresh BM/PB specimens using QIAamp® RNA Blood Mini kit (Qiagen, Germany) according to the manufacturer's protocol. The eluted RNA was immediately flash frozen in liquid nitrogen and stored at −70 °C till assay.

The reaction protocol started with reverse transcription at 50 °C for 20 min, followed by 40 cycles of heating at 95 °C for 5 min (hot start PCR), heating at 95 °C for 15 s (denaturation), and finally heating at 60 °C for 30 s (annealing/extension). PCR and data analysis were carried out on Applied Biosystems StepOne™ (Applied Biosystems, Life Technologies™, USA).

FLT3 expression levels in unknown specimens were calculated by relative quantification using the ΔΔCT method which relies on comparison of cycle threshold (CT) values of *FLT3* (target gene) to *β-actin* (reference gene) in unknown and normal calibrator specimens. The results were presented as the fold change in gene expression normalized to the endogenous reference gene and relative to the normal calibrator [14].

2.5. Fluorescence in situ hybridization (FISH)

Probes for t(8;21)(q22;q22), t(15;17)(q22;q12), t(9;22)(q34;q11), trisomy 8, and 11q23 rearrangement (Vysis, Downers Grove, USA) were used. The patients were stratified into cytogenetic-based risk groups: *favorable*; t(8;21)(q22;q22) and t(15;17)(q22;q12), *intermediate*; normal karyotype and trisomy 8, and *poor*; t(9;22)(q34;q11) and 11q23 rearrangement [12].

2.6. Statistical analysis

Statistical Program for Social Science, version 20 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Categorical data were expressed in the form of frequency and percentage. Continuous data were expressed in the form of mean ± standard deviation (SD), or median and interquartile range (IQR) for parametric and non-parametric variables, respectively. Fisher's exact test was used for comparison of categorical variables between two independent groups. Student *t*-test and Mann-Whitney test were used for comparing continuous parametric and non-parametric variables between two independent groups, respectively. ANOVA and Kruskal-Wallis tests were used for comparing continuous parametric and non-parametric variables between more than two study

groups, respectively. Pearson (r) and Spearman (rs) correlations were used to assess the strength of association between parametric and non-parametric variables, respectively. The receiver operating characteristic (ROC) curve was employed to assign the best cut-off value for FLT3 receptor/CD135% positivity and predictability for *FLT3*-ITD. P-value of <0.05 was considered significant and of <0.01 was considered highly-significant in all analyses.

3. Results

3.1. FLT3 receptor/CD135 expression in AML patients

FLT3 receptor/CD135 expression was higher in AML patients (mean ± SD, 53.2 ± 22.4%) compared with control subjects (mean ± SD, 8.4 ± 4.7%) ($p < 0.001$). Representative example for flow cytometric expression of surface FLT3 receptor/CD135 in BM specimen of control subject and AML patient is shown in Fig. 1. ROC curve was constructed to determine the best cut-off value for FLT3 receptor/CD135% positivity in AML patients; a cut-off of >17% was assigned to define FLT3 receptor/CD135$^+$ cases with 100% sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and efficacy (area under curve = 1; $p < 0.001$). Accordingly, 80 of 80 (100%) AML patients were FLT3 receptor/CD135$^+$, and 20 of 20 (100%) control subjects were FLT3 receptor/CD135$^-$.

FLT3 receptor/CD135 expression was not equally distributed among the different FAB subtypes, recording the highest expression in M5 followed by M3, M0, M2 and M1 ($p = 0.001$). Similarly, FLT3 receptor/CD135 expression was higher in the poor cytogenetic risk group compared with the intermediate and favorable risk groups ($p = 0.007$). In contrast, FLT3 receptor/CD135 expression was consistent in relation to gender, hepatosplenomegaly, and lymphadenopathy ($p > 0.05$) (Table 2).

FLT3 receptor/CD135 expression was positively correlated with the percentage of BM blasts ($r = 0.659$; $p = 0.023$), while no significant correlations were found with age ($r = 0.124$), TLC ($rs = 0.127$), hemoglobin (Hb) level ($r = 0.021$), and platelets count ($rs = -0.113$) ($p > 0.05$).

3.2. FLT3 gene mutations in AML patients

FLT3-ITD was detected in 18 of 80 (22.5%) AML patients, while none of the patients had *FLT3*-TKD D835Y mutation. Control subjects showed neither *FLT3*-ITD nor *FLT3*-TKD D835Y mutation. Although AML patients harboring *FLT3*-ITD mutation had higher FLT3 receptor/CD135 expression and *FLT3* mRNA transcripts than those with wild-type *FLT3*, the difference showed a trend with failure to reach statistical significance ($p = 0.07$; $p = 0.06$, respectively) (Table 1, Fig. 2). *FLT3*-ITD mutational status was comparable in relation to other clinical and laboratory characteristics of AML patients ($p > 0.05$) (Table 1).

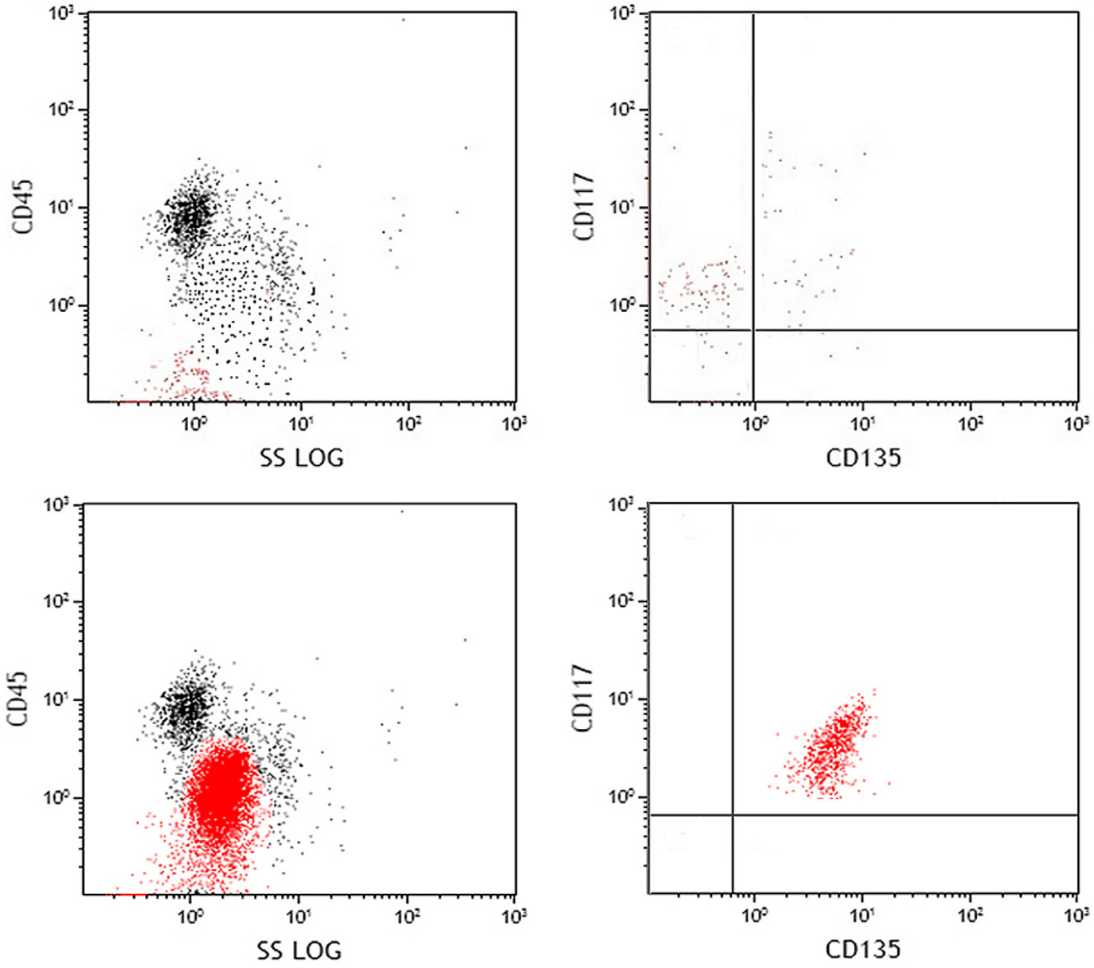


Fig. 1. Flow cytometric expression of surface FLT3 receptor/CD135 in bone marrow specimen of control subject and AML patient. Upper Panel; control subject showing negative CD135 expression (13%), Lower Panel; AML patient showing positive CD135 expression (86%). SS LOG, side scatter log.

Table 2
FLT3 receptor/CD135 expression and *FLT3* mRNA transcript level in relation to clinical and laboratory characteristics of AML patients.

Parameters	CD135% Mean ± SD	P-value	*FLT3* mRNA Median (IQR)	P-value
Gender				
Male	49.7 ± 21.5	0.255	30 (18–82)	0.331
Female	57.9 ± 23.3		42 (22–115)	
Hepatosplenomegaly				
Yes	63.3 ± 21.5	0.060	77 (30–92)	0.042*
No	48.9 ± 21.7		29.5 (18–48.5)	
Lymphadenopathy				
Yes	60.1 ± 22.8	0.232	38.5 (27–92)	0.444
No	50.7 ± 22.1		35 (19–82)	
FAB subtypes				
M0	50.3 ± 17.7	0.001**	18 (16–55)	0.007**
M1	42.6 ± 18.7		19 (14–50)	
M2	43.1 ± 17.9		30 (18–43)	
M3	60.6 ± 19.6		32.5 (28–103.5)	
M5	77.6 ± 18.3		115 (72–147)	
Cytogenetic risk groups				
Favorable	49.5 ± 21.6	0.007**	29.5 (21–42)	0.023*
Intermediate	46.3 ± 17.1		30 (18–47)	
Poor	73.7 ± 21.9		83 (82–125)	

AML, acute myeloid leukemia; FAB, French-American-British; *FLT3*, FMS-like tyrosine kinase 3; IQR, interquartile range; SD, standard deviation; *, P-value <0.05 is significant; **, P-value <0.01 is highly-significant.

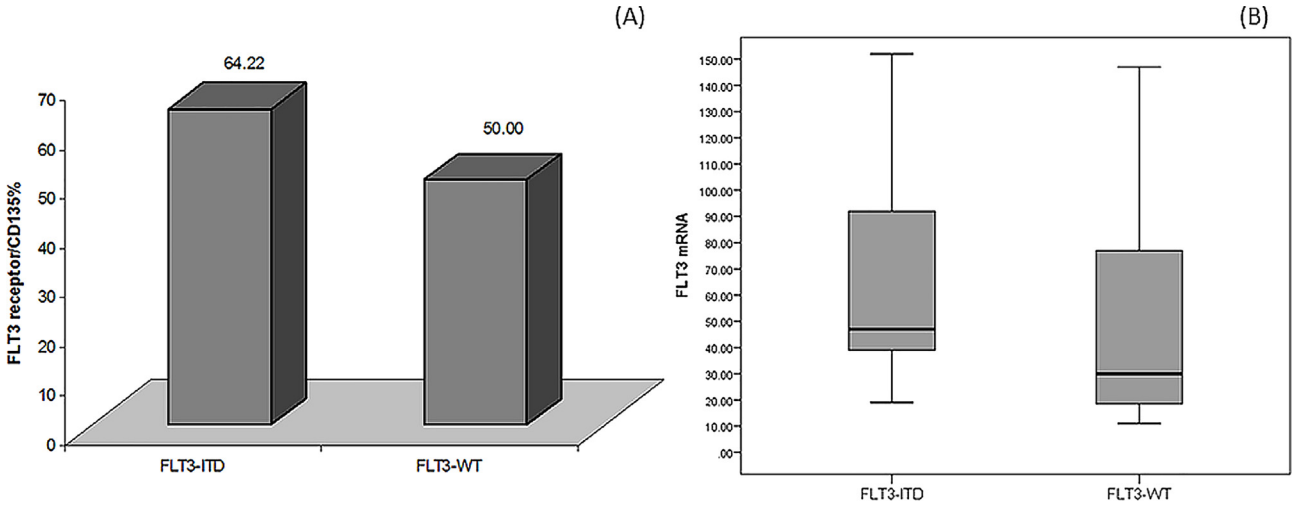


Fig. 2. FLT3 receptor/CD135 expression (A) and *FLT3* mRNA transcript levels (B) in *FLT3*-ITD and *FLT3*-WT patient groups. WT, wild-type.

3.3. *FLT3* mRNA transcript level in AML patients

FLT3 mRNA transcripts were detected in 80 of 80 (100%) AML patients, with higher levels found in AML patients; median (IQR), 35 (19.5–83), compared with control subjects; median (IQR), 1 (0.7–1.4) ($p < 0.001$).

FLT3 mRNA transcript level was higher in AML patients presented with hepatosplenomegaly ($p = 0.042$). Moreover, FLT3 mRNA transcript level was not equally distributed among the different FAB subtypes, recording the highest expression in M5 followed by M3, M2, M1 and M0 ($p = 0.007$). Similarly, *FLT3* mRNA transcript level was higher in the poor cytogenetic risk group compared with the intermediate and favorable risk groups ($p = 0.023$). In contrast, *FLT3* mRNA transcript level was comparable in relation to gender and lymphadenopathy ($p > 0.05$) (Table 2).

No significant correlations were found between *FLT3* mRNA transcript level and age ($rs = 0.084$), TLC ($rs = -0.017$), Hb level ($rs = 0.047$), platelets count ($rs = -0.092$), and percentage of BM blasts ($rs = 0.188$) ($p > 0.05$).

3.4. Role for FLT3 receptor/CD135 expression in predicting *FLT3*-ITD and FLT3 mRNA transcript level in AML patients

ROC curve was constructed to define the best cut-off value for FLT3 receptor/CD135% that could predict the presence of *FLT3*-ITD at diagnosis of AML. FLT3 receptor/CD135 >49% predicted *FLT3*-ITD with 89% sensitivity, 61.3% specificity, 40% PPV, 95% NPV, and 67.5% efficacy (area under curve = 0.708; $p = 0.01$). Moreover, a positive correlation was found between FLT3 receptor/CD135% and *FLT3* mRNA transcript level ($rs = 0.965$; $p < 0.001$) (Fig. 3).

4. Discussion

In this study, we detected *FLT3*-ITD in 18 of 80 (22.5%) AML patients, while none of our patients showed *FLT3*-TKD D835Y mutation. Control subjects showed neither *FLT3*-ITD nor *FLT3*-TKD D835Y mutation. *FLT3*-ITD was first detected by Nakao and

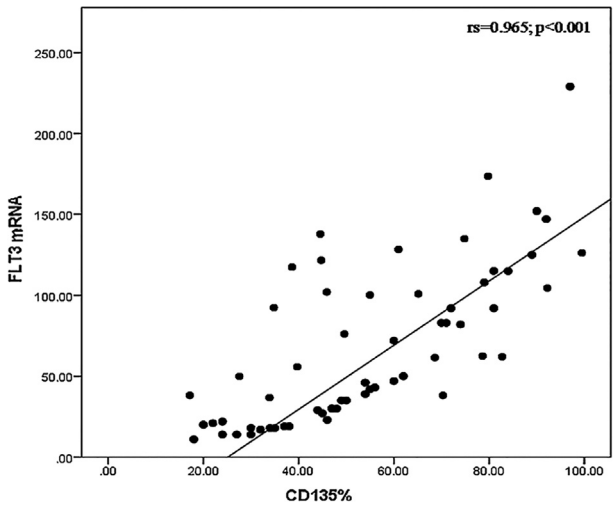


Fig. 3. Positive correlation between FLT3 receptor/CD135% and *FLT3* mRNA transcript level in AML patients.

coworkers [15] who reported approximately 23% incidence in AML, followed by many study groups reporting the incidence of *FLT3*-ITD to range from 10 to 38% [6,11,16–20]. Previous studies [6,11,21] have also denoted the low incidence for *FLT3*-TKD D835Y mutation in AML, ranging from 4 to 7%.

We assigned a cut-off value of >17% to define FLT3 receptor/CD135$^+$ AML patients. FLT3 receptor/CD135 and *FLT3* mRNA were overexpressed in all studied AML patients compared with controls. Other studies have reported FLT3 receptor/CD135 overexpression as well as increased median expression levels of *FLT3* transcripts in 70–100% AML patients [10,11].

In the present study, we found comparable differences between each of FLT3 receptor/CD135, *FLT3*-ITD, and *FLT3* mRNA, and the patients' age and gender, complying with the results of other studies [11,22,23]. We also did not find significant association between each of FLT3 receptor/CD135 and *FLT3*-ITD and the clinical parameters of AML patients, however, *FLT3* mRNA was significantly elevated in AML patients presented with hepatosplenomegaly. Moreover, no significant correlations were found between each of FLT3 receptor/CD135, *FLT3*-ITD, and *FLT3* mRNA, and the studied hematological parameters except for a significant positive correlation between FLT3 receptor/CD135 expression and percentage of BM blasts.

Vora and coworkers [23] found no association between FLT3 receptor/CD135 expression and TLC or Hb level, yet found significant association with the percentage of BM blasts; the latter could be expected due to the impact of overexpressed FLT3 receptor on the uncontrolled proliferation and survival of BM leukemic blast cells. Abd El-Ghaffar et al. [24] and Kuchenbauer et al. [10] also reported significant association of FLT3 receptor/CD135 with high TLC and BM blast count.

Peng and colleagues [22] found *FLT3*-ITD was associated with higher TLC and percentage of BM blasts, yet their acute leukemia study group comprised a mixture of AML, B-acute lymphoblastic leukemia (ALL), and T-ALL. Mehta et al. [7] reported a trend for *FLT3*-ITD with low Hb level and low platelets count, while detected similar incidence for *FLT3*-ITD among the TLC subgroups. They also showed that *FLT3* mRNA was not related to TLC, although they could find significant associations between *FLT3* mRNA and low Hb level, low platelets count and high percentage of BM blasts; they alternatively employed absolute quantification of *FLT3* mRNA transcript level and sub-grouped their patients considering the median value as the cut-off level ($<16 \times 10^5$ and $\geq 16 \times 10^5$). Furthermore, Kuchenbauer et al. [10] reported significant association of *FLT3* mRNA with leucocyte and BM blast counts. This difference could possibly be related to their inclusion of AML with myelodysplasia-related changes and therapy-related AML among their de novo AML patients as well as to the use of SYBR Green instead of Taqman probes.

Interestingly, *FLT3* has been reported to be persistently expressed during the process of monocyte differentiation, where the ligand for FLT3 is required for complete differentiation of monocytes from CD34$^+$ cells. Thus, it has been proposed that FLT3 signaling is more likely to be associated with AML-M5 subtype [3,24,10]. In agreement, we found FLT3 receptor/CD135 expression and *FLT3* mRNA transcript level to be highest among AML-M5 patients, whereas none of our *FLT3*-ITD$^+$ patients were classified as AML-M5 or related to other FAB subtypes.

Although *FLT3* mutations have long been identified within the intermediate cytogenetic risk group of AML patients especially those having normal karyotype [25], we found the highest FLT3 receptor/CD135 expression and *FLT3* mRNA transcript level among the poor cytogenetic risk group. This may be because the highest level for both FLT3 receptor/CD135 expression and *FLT3* mRNA transcript was among AML-M5 patients with 11q23 (*MLL*) rearrangement which has been classified among the poor cytogenetic risk group. In contrast, Abd El-Ghaffar et al. [24] found comparable FLT3 receptor/CD135 expression among the cytogenetic risk groups; a finding possibly confounded by the inclusion of pediatric among adult AML patients, the former being known for its lower frequency of *FLT3*-ITD than the latter [26].

In our study, although there was a trend for higher expression of FLT3 receptor/CD135 and *FLT3* mRNA transcripts among *FLT3*-ITD than *FLT3*-wild-type AML patients, the results could not reach statistical significance. Receiver operating characteristics showed that FLT3 receptor/CD135 expression by >49% blast cells could predict *FLT3*-ITD$^+$ AML patients. These findings complied with the results reported by Peng and coworkers [22] who found that despite the upregulated FLT3 receptor/CD135 and *FLT3* mRNA transcripts in patients with *FLT3*-ITD, the difference was comparable. Hence, it could be indicated that quantification of FLT3 receptor and *FLT3* transcripts detects the total expression of both wild-type and mutant alleles [11]. However, according to Abd El-Ghaffar et al. [24], higher levels of FLT3 receptor/CD135 were found among CD135$^+$*FLT3*-wild-type than CD135$^+$*FLT3*-ITD patients, and a cut-off level of <62% for CD135 was determined to indicate *FLT3*-ITD mutation. Moreover, Riccioni et al. [3] reported that 10–15% AML patients display high expression of FLT3 receptor associated with wild-type *FLT3* which represents an unfavorable prognosis, the same as that with *FLT3*-ITD.

An explanation for the association of FLT3 receptor/CD135 overexpression with wild-type *FLT3* rather than *FLT3*-ITD was provided by Kuchenbauer et al. [10] who hypothesized that high *FLT3* mRNA expression may represent an alternative to the mutational activation of the FLT3 receptor. In the same context, they denoted that *FLT3* transcriptional upregulation is associated with a partial loss of the wild-type allele, whereas a total loss of the wild-type fragment (ITD/-) leads to low *FLT3* expression, proposing that *FLT3* expression in the presence of *FLT3*-ITD depends on the coexpression of wild-type and mutant alleles.

As the quantity of total cellular protein expression essentially reflects the gene transcript level, in congruence with others [10,22] we found a significant positive correlation between FLT3 receptor/CD135 expression and *FLT3* mRNA transcript level.

The identification of FLT3 receptor expression levels and its molecular mutations represent new opportunities in the treatment of AML. Over the past decades, the biology and function of wild-type and mutant FLT3 receptor have been well characterized [5]. Different compounds including tyrosine kinase inhibitors and immunotherapy using anti-FLT3 monoclonal antibody have been

investigated *in vitro* and *in vivo* as FLT3 inhibitors; however, clinical trials have produced only partial and transitory results. Therefore, additional data about FLT3 receptor expression levels and molecular mutations are still required to investigate the underlying causes for resistance to FLT3 inhibitors in a trial to optimize AML treatment protocols [5,27].

5. Conclusions

Our study provides further insights and contributes to understanding the role for FLT3 receptor expression in predicting *FLT3*-ITD mutational status and *FLT3* gene expression. We propose that evaluation of FLT3 receptor/CD135 expression by flow cytometry in association with the standard acute leukemia panel at diagnosis of AML could constitute a predictor for *FLT3*-ITD mutational status and *FLT3* mRNA transcript level. The frequent overexpression of FLT3 receptor in AML, with or without *FLT3*-ITD, points to its importance in selecting patients who could likely benefit from the incorporation of FLT3 inhibitors along with conventional chemotherapy. The identification of *FLT3*-ITD using more advanced methodologies as the next generation sequencing or capillary electrophoresis-based fragment analysis, considered as the gold standard for *FLT3*-ITD detection since conventional electrophoresis of amplified *FLT3* sequences may not be sensitive enough for small ITD insertions, are warranted. Future studies to evaluate the potential role for FLT3 receptor expression level in predicating the ITD allelic burden and length are worthwhile.

Conflict of interests

The authors declare no conflict of interests with respect to the research, authorship, and/or publication of this article.

Sources of funding

The research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

References

[1] Löwenberg B, Rowe JM. Introduction to the review series on advances in acute myeloid leukemia (AML). Blood 2016;127:1.
[2] Seegmiller A, Jagasia M, Wheeler S, Vnencak-Jones C. Molecular profiling of acute myeloid leukemia. My Cancer Genome 2016; http://www.mycancergenome.org/content/disease/acute-myeloid-leukemia/.
[3] Riccioni R, Pelosi E, Riti V, Castelli G, Lo-Coco F, Testa U. Immunophenotypic features of acute myeloid leukaemia patients exhibiting high FLT3 expression not associated with mutations. Br J Haematol 2011;153:33–42.
[4] Markovic A, MacKenzie KL, Lock RB. FLT-3: a new focus in the understanding of acute leukemia. Int J Biochem Cell Biol 2005;37:1168–72.
[5] Grafone T, Palmisano M, Nicci C, Storti S. An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. Oncol Rev 2012;6:e8.
[6] Elyamany G, Awad M, Fadalla K, Albalawi M, Al Shahrani M, Al Abdulaaly A. Frequency and prognostic relevance of FLT3 mutations in Saudi acute myeloid leukemia patients. Adv Hematol 2014;2014:141360.
[7] Mehta SV, Shukla SN, Vora HH. Comprehensive FLT3 analysis in Indian acute myeloid leukaemia. J Blood Lymph 2012;2:102.
[8] Mrózek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD. Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? Blood 2007;109:431–48.
[9] Kang HJ, Lee JW, Kho SH, Kim MJ, Seo YJ, Kim H, et al. High transcript level of FLT3 associated with high risk of relapse in pediatric acute myeloid leukemia. J Korean Med Sci 2010;25:841–5.
[10] Kuchenbauer F, Kern W, Schoch C, Kohlmann A, Hiddemann W, Haferlach T, et al. Detailed analysis of FLT3 expression levels in acute myeloid leukemia. Haematologica 2005;90:1617–25.
[11] Ozeki K, Kiyoi H, Hirose Y, Iwai M, Ninomiya M, Kodera Y, et al. Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. Blood 2004;103:1901–8.
[12] Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 2016;127:2391–405.
[13] Bain BJ, Barnett D, Linch D, Matutes E, Reilly JT. General Haematology Task Force of the British Committee for Standards in Haematology (BCSH), British Society of Haematology. Revised guideline on immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders. Clin Lab Haematol 2002;24:1–13.
[14] VanGuilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 2008;44:619–26.
[15] Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia 1996;10:1911–8.
[16] Levis M. FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? Hematology Am Soc Hematol Educ Program 2013;2013:220–6.
[17] Levis M, Small D. FLT3: ITDoes matter in leukemia. Leukemia 2003;17:1738–52.
[18] Muñoz L, Aventín A, Villamor N, Juncà J, Acebedo G, Domingo A, et al. Immunophenotypic findings in acute myeloid leukemia with FLT3 internal tandem duplication. Haematologica 2003;88:637–45.
[19] Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood 2002;100:59–66.
[20] Abu-Duhier FM, Goodeve AC, Wilson GA, Gari MA, Peake IR, Rees DC, et al. FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. Br J Haematol 2000;111:190–5.
[21] Abu-Duhier FM, Goodeve AC, Wilson GA, Care RS, Peake IR, Reilly JT. Identification of novel FLT-3 Asp835 mutations in adult acute myeloid leukaemia. Br J Haematol 2001;113:983–8.
[22] Peng HL, Zhang GS, Gong FJ, Shen JK, Zhang Y, Xu YX, et al. Fms-like tyrosine kinase (FLT) 3 and FLT3 internal tandem duplication in different types of adult leukemia: analysis of 147 patients. Croat Med J 2008;49:650–69.
[23] Vora HH, Shukla SN, Brahambhatt BV, Mehta SH, Patel NA, Parikh SK, et al. Clinical relevance of FLT3 receptor protein expression in Indian patients with acute leukemia. Asia Pac J Clin Oncol 2010;6:306–19.
[24] Abd El-Ghaffar AA, El-Gamal RA, Mostafa NN, Abou Shady NM. FLT3 (CD135) and Interleukin-2 receptor alpha-chain (CD25) expression in acute myeloid leukemia: improving the correspondence to FLT3 - internal tandem duplication mutation. Int J Lab Hematol 2016;38:e69–72.
[25] Motyckova G, Stone RM. The role of molecular tests in acute myelogenous leukemia treatment decisions. Curr Hematol Malig Rep 2010;5:109–17.
[26] Zwaan CM, Meshinchi S, Radich JP, Veerman AJ, Huismans DR, Munske L, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. Blood 2003;102:2387–94.
[27] Reiter K, Polzer H, Krupka C, Maiser A, Vick B, Rothenberg-Thurley M, et al. Tyrosine kinase inhibition increases the cell surface localization of FLT3-ITD and enhances FLT3-directed immunotherapy of acute myeloid leukemia. Leukemia 2018;32:313–22.