Runx1 Gene in Acute Myeloid Leukemia: Expression Level Significance and Impact on Clinical Features

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Abstract

Background Acute myeloid leukemia represents the highest percentage of all adult acute leukemia variants. Runt-related transcription factor1 (RUNX1), a transcription factor with a known tumor suppressor function was recently reported as a tumor promotor in AML. We investigated the role of RUNX1 geneexpression levelin Egyptian AML patients and delineateits clinical significance. Methods This study recruited 91 AML patients that were recently diagnosed at our hospital with 14 healthy age- and sex-matched donors of bone marrow transplantation unit. We measured RUNX1 gene expression level using reverse transcription–quantitative polymerase chain reaction. Results We found that RUNX1 gene expression level was significantly higher compared to the control group ($p < 0.001$). Patients with FLT3 mutations had the higher expression level of RUNX1 ($p=0.023$). The male patients expressed significantly higher level of RUNX1 ($p=0.046$). Conclusion The RUNX1 gene expression may serve as a diagnostic marker in Egyptian AML patients. Its relation to FLT3 may give clue that patients carrying this mutation may benefit fromnew treatments that target RUNX1 in the future. Further studies on a larger number of patients and different ethnic groupsmay give a clearer vision about this new molecular therapeutic target.

Background

Each year, three to four new cases of acute myeloid leukemia (AML) are reported per 100,000 individuals. The prognosis of AML is highly variable despite intensive research for new markers and therapies [1]. Relapse is the most frequent cause of therapeutic failure [2, 3], less than 50% of patients have a5 yearoverall survival rate (OS) andonly 20% of elderly survive 2 years [1].

Genetic and epigenetic alterations in the hematopoietic stem and progenitor cells causing their aberrant proliferation and block of differentiation (HSPCs) leading to different clinical types of AML [4]. Several studies have recognized various genes affected by the somatic mutations due to different AML subtypes [5,6]. Mutations in transcriptional regulator, additional sex combs like 1, tumor protein 53, and FMsrelated tyrosine kinase 3 (FLT3) genes and certain chromosomal translocations (Breakpoint cluster regionAbelson murine leukemia viral oncogene homolog 1) were reported to predispose to AML [7]. The data from previous studies has helped in elucidation of AML biology, which could facilitate better risk assessment while determining novel drug targets and therapeutic strategies [5, 6].

The RUNX1 gene has emerged as anovel therapeutic target for AML [8].It belongs to atranscriptional regulator family called Runx that comprise three members: RUNX1,RUNX2 and RUNX3 [9]. It is located on chromosome 21. As shown in a previous study in a mouse model, RUNX1 gene is vital for the process of hematopoiesis [8]. It consists of a “Runt homology domain” (RHD) that facilitates the formation of the heterodimer of RUNX1 and PEBP2β that acts as a DNA binding and transcription factor [10].

RUNX1 gene activity is tightly regulated via several mechanisms, such as translational regulation, posttranslational modifications (PTM), and alternative splicing [11, 12]. The PTMs, such as methylation,

acetylation, and phosphorylation promote RUNX1 transcription [13]. Perturbation in the activity of RUNX1 leads to development of several hematopoietic neoplasms [9].

Several studies have shown the tumor suppressor activity of RUNX1 against myeloid neoplasms. AML patients often exhibit chromosomal translocations involving RUNX1 and its cofactor CBFB. The most common AML subtype, also referred to as CBF-AML, is characterized by the chromosomal aberrations inv(16) and t(8;21) that lead to the formation of CBFB-MYH11 and RUNX1-RUNX1T1 (AML1-ETO) fusion genes, respectively. Around 15 to 20% of adult de novo AML cases suffer from CBF-AML [14]. Another aberration t(3;21) leads to the formation RUNX1-MECOM (also called AML1-EVI1) fusion gene that retains the N-terminal of RUNX1 [15]. This fusion gene is found in chronic myeloid leukemia with blastic or accelerated phase, therapy-related myeloid neoplasms, and, rarely, in de novo AML. The formation of the abovementioned fusion genes disrupts the normal function of RUNX1-CBFB. In addition, RUNX1 is itself mutated in several myeloid neoplasms [16]. Germline RUNX1 mutations lead to familial platelet disorder with predisposition to AML [17, 18]. Around 15% of cytogenetically normal AML [19–21] and 6–11% of myelodysplastic syndromes [22–24], 10% of chronic myelomonocytic leukemia [25] and 20% of systemic mastocytosis cases exhibit somatic mutations in RUNX1 gene [26]. In general, such mutations affect the transcription activation domain or the Runt domain. They are usually frameshift or nonsense mutations that adversely affect the transcriptional activity of RUNX1 [27, 28]. Due to these mutations, RUNX1 is unable to participate in various crucial events like early hematopoietic development and myeloid maturation [29]. Several previous studies have shown the tumor suppressor role of RUNX1 via various mouse models [30–33].

Other studies have shown that, in CBF-MLL fusion leukemia, RUNX activity is needed to maintain the phenotype of leukemogenic cell [9]. RUNX1 down regulation led to apoptosis and cell-cycle arrest in human cord blood cells that expressed the RUNX1-RUNX1T1 and MLL-AF9 fusion genes [9]. In another study, a rapid development of leukemia was observed in a knock-in mice model that expressed a mutant CBFB-MYH11 fusion product that lacked the RUNX1 high-affinity binding domain and caused an inadequate RUNX1 suppression [34]. Similarly, in a mouse bone marrow transplant model, a truncated C-terminal version of RUNX1-RUNX1T1, RUNX1-RUNX1T1-9a, exhibited weak RUNX1suppression but high leukemogenic potential [35]. In addition, RUNX1 knockdown in ME-1 and Kasumi-1 cell (expressing CBFB-MYH11 and RUNX1-RUNX1T1, respectively) led to abnormal cell cycle and apoptosis [36] (Fig. 1).

In addition, phosphorylated RUNX1 was reported to conjugate with FLT3-ITD and induce AML [4]. Internal tandem duplication of the FLT3 gene (FLT3-ITD) is one of the commonest AML mutations that cause constitutive activation of FLT3 receptor tyrosine (Tyr) kinase [4]. Studies on mouse model have revealed that FLT3-ITD cannot induce AML on its own [36]. A previous study has reported an association between expression of RUNX1 and FLT1-ITD signaling in AML cells; both entities synergistically participate in AML development [4]. AML patients with FLT3-ITD mutations exhibit overexpression of RUNX1 RNA and its downstream target, HHEX. In addition, downregulation of RUNX1 adversely affects the leukemogenic activity of AML cells [4].

RUNX1 could act as both tumor promoter as well as suppressor depending on several factors, including its expression levels. Thus, regulation of RUNX1 expression could act as a potential therapeutic strategy against cancer [9].

It has been previously observed that RUNX1 downregulation promotes the differentiation of AML cells expressing FLT3-ITD. It indicates that RUNX1 downregulation did not induce any potential mutations that could adversely affect the differentiation potential of these cells [4]. Therefore, introduction of such mutations could act as a potential therapeutic strategy for AML patients with FLT3-ITD mutations [4].

Our study is aimed at assessing RUNX1 gene expression level and its association with other genetic markers and clinical outcome in Egyptian De-novo AML patients.

Methods

Study group

This study recruited 91 AML patients that were recently diagnosed at the Hematology clinic, National Cancer Institute, Cairo, Egypt, from June 2015 till December 2018. For control group, 14 healthy age- and sex-matched donors of bone marrow transplantation unit were recruited. The patients included 50 males and 41 females between the ages of 18 and 65 years (mean age: 35.5 years). We excluded all patients with ageless than 18 years or who just started treatment.The peripheral blood of all remaining patients was analyzed for measuring different blood parameters, such as hemoglobin (Hb), platelets count, andtotal leukocyte count (TLC). Blasts were counted inbone marrow samples and peripheral blood. Based on the FrenchAmericanBritish (FAB) classification of AML, the patients were divided into subgroups [37]. Cytogenetic and gene mutation analyses for detection of chromosomal abnormalities, FLT3-ITD, and NPM mutations were done for all patients (Table 1). Cytogenetic and gene mutation analyses for detection of chromosomal abnormalities, NPM1 and FLT3-ITD mutations respectively were done for all included patients (Table 1).

The institutional review board of National Cancer Institute, Cairo University, approved the protocol of the study and we followed the Helsinki guidelines for the protection of human subjects. Written informed consents were obtained from all participants.

Quantitative Reverse Transcriptase-PCR

RNA extraction and cDNA formation

QIAamp RNA extraction blood Mini kit (QIAGEN® Austin, Texas, USA catalogue no.52304) was used to extract total RNA from 1mLculture of BM cells preserved on K-EDTA. A spectrophotometer nano-drop (Quawell, Q-500, Scribner, USA) was used to assess the concentration and purity of RNA, which was then kept at–80˚C until further use.

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, USA; catalogue no. 4368814) was used to reverse transcribe the extracted RNA. Again, the spectrophotometer nano-drop was used to qualify and quantify the complementary DNA (cDNA), which was then kept at 20˚C.

Molecular Detection of RUNX1 gene expression

RUNX1 mRNA expression was quantified using TaqMan® Universal PCR Master Mix; 2X conc (Cat No.: 4440040, Thermo Fisher scientific, Applied Biosystems, USA), Taqman readymade gene expression assay, for RUNX1 mRNA (Hs 02558380_S1; Thermo Fisher Scientific, USA, Cat No.: 4331182) and β-actin [38]. The total reaction volume for PCR mix was20μL, and the PCR protocol was as follows: 95˚C for 10 min (polymerase activation), followed by 40 cycles of 95˚C for 30 s (denaturation), 60˚C for 60 s (annealing and extension).The StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to detect the fluorescence. The following primers were used for quantitative PCR: RUNX15'-AGTGGAAGAGGGAAAAGC-3'(sense) and 5'-ATCCACTGTGATTTTGATGG-3'(antisense) andβ-Actin (control) 5'-GTGGGCCGCTCTAGGCACCAA-3'(sense) and 5'-CTCTTTGATGTCACGCACGATTTC-3' (antisense). Comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to assess the relative RUNX1 expression level that was expressed as fold change normalized against β-Actin expression levels [39]. The median follow-up period was 7 months (range: 0.03–40.2 months).

Treatment and follow up

All patients were treated by standard induction regimen, which comprised ofadministration of cytarabine (100–200 mg/m^2/d) for 7 days along with administration of anthracycline and either idarubicin (12 mg/m^2/d) or daunorubicin (45–90 mg/m^2/d) for 3 days. The individuals, who underwent complete remission (CR) after one or two courses of induction chemotherapy, further received two to six cycles of high-dose cytarabine-based consolidation chemotherapy. Follow up was done by complete blood count, bone marrow aspirate, flow cytometry, and molecular analysis.

Statistical analysis

IBM SPSS® Statistics version 22 (IBM® Corp., Armonk, NY, USA) was used for statistical analysis. Numerical data was respresented as either median and range or mean and standard deviation. The qualitative data was represented as either percentage or frequency. The correlation between qualitative variables was evaluated by Fisher's exact test or Pearson's Chi-square test. In case of quantitative data, Student's t-test was used to compare two groups belonging to normally distributed data, and Mann-Whitney test (non-parametric t-test) was used for groups belonging to data not normally distributed. The correlation among numerical variables was analyzed using Spearman-rho method. Median value of the markers in the study group was used as the cutoff point with values above considered as overexpression and values below it as low expression. Markers were evaluated via calculation of specificity, sensitivity, negative predictive value (NPV), positive predictive values (PPV), and overall accuracy. Kaplan-Meier

method was used forsurvival analysis. Log rank test was used for comparison between two survival curves. All the tests were two-tailed. Statistical significance was defined at $p < 0.05$.

Results

RUNX1 expression is significantly higher in AML cases

RUNX1 gene expression were measured in BM samples of De novo AML patients and 14 control subjects of same age and sex. We found that the AML group showed significantly high RUNX1 gene expression level than control cases ($p < 0.001$, Table 2) (Fig. 2). For AML patients, the fold change in RUNX1 expression ranged between 0.02 and 1382.78 (median: 16.81). Patients with values below 16.81 were considered low expressors while those with values above were considered high expressers. None of the control group was high RUNX1 expressor (Table 2). Using this median value, specificity, sensitivity, negative predictive value, positive predictive value, and overall accuracy for diagnosis of AML were 100%, 50.5%, 23.7%, 100%, and 57.1%, respectively. The risk of AML had an odds ratio of 1.31 (95% CI: 1.14–1.51).

Characteristics of the AML patients by high and low expression levels of RUNX1

As shown in Table 3, the male patients and the patients with mutant FLT3 exhibited significantly higher RUNX1 expression ($p = 0.046$ and $p = 0.023$, respectively). In males, the median RUNX1 level was 30.0 (range: 0.2-878.2) compared to 16.0 (range: 0.8-1382.8) in females. RUNX1 expression was not significantly associated with other clinical, laboratory and genetic characteristics of the studied group with RUNX1 expression status (Table 4) (Fig. 3).

Relation of Patients survival to the level of RUNX1 gene expression

Overall survival (OS) is referred to as the period from diagnosis of AML till the patient's death due to any cause. The patients that were alive on last follow-up date were censored for that date. Progression free survival (PFS) refers to the period of start of therapy till death or documented progression. The patients that did not exhibit any disease progression during the analysis period were censored on last follow-up date [40].

During the period of the study, there were 54 patients died. Median follow-up period was 7 months (range: 0.03–40.2 months). Median OS was 11.3 months, while the cumulative overall survival after 12 months was 46.6% (Table 5). Overall and event-free survival were not affected by RUNX1 expression ($p = 0.804$ and $p = 0.314$, respectively) (Fig. 4).

Discussion

In last decade, the RUNX family suggested as a tumor biomarker that play dual role in acute myeloid leukemia, that it is why it is important to continue with the molecular studies that discuss their importance in diagnosis and prognosis of AML [41–43].

Translocationsand mutations of RUNX1 gene locus were reported tocauseincrease or decrease in its functionand to induce leukemia [43–45].

Several studies have shown that RUNX1 could trigger the development of AML by promoting proliferation of leukemic cells [2629]. Goyama *et al*. reported that RUNX1 overexpression inhibited cord blood cell growth by triggering myeloid differentiation[9, 35, 46].

To the best of our knowledge, our study was the first to present different expression levels of RUNX1 gene in Egyptian population, as the previous human studies was conducted on Chinese and polish populations [47, 49].

We found that, compared to control group, RUNX1 expression was significantly higher in AML patients ($p < 0.001$). Fu *et al*. estimated RUNX1 expression using microarrays on the bone marrow samples of 157 cytogenetic normal AML (CN-AML) Chinese patients and normal bone marrow samples [48]. They reported that, compared to normal bone marrow samples, CN-AML samples exhibited significantly higher RUNX1 expression ($P < 0.001$). In our study, the patients with higher RUNX1 expression were more likely to harbor FLT3-ITD mutation compared to patients with lower RUNX1 expression ($p = 0.023$). Our results indicated that, under high expression, RUNX1 could act as an oncogene that induces leukemogenesis and act as a surrogate marker for other mutations. In agreement with the previous results, Behrens *et al*. reported that, after upregulation and phosphorylation, RUNX1 could trigger AML, in conjugation with FLT3-ITD [49]. Thus, suppression of RUNX1 could hold high potential as a therapeutic strategy that could markedly enhance the current therapeutic approaches, involving FLT3 inhibitors, by reversing the differentiation block and making therapy more effective [49].

Furthermore, we observed a significantly higher RUNX1 expression in male patients ($p = 0.046$). This result was in contrast to the findings of KRYGIER A et al who reported in his study on 43 polish De Novo AML cases using RT qPCR analysis technique that RUNX1 significantly expressed in females [47]. This disagreement may be due the difference in sample size or could be due to racial difference. In his study, Fu et al reported that among the 157 CN-AML patients enrolled by them, the group of patients with higher RUNX1 gene expression comprised of a significantly higher proportion of patients with FAB M1 and M2 subtypes, compared to the group of patients with lower RUNX1 expression [48]. These results differed from our present study, where we found no association between the FAB subtypes and RUNX1 expression ($P = 0.348$). Furthermore, they reported that higher RUNX1 expression in CN-CML patients is associated with poorer PFS and OS ($p = 0.011$ and $p = 0.009$, respectively). However, in this study, we did not find any correlation between RUNX1 expression and FAB classification, OS, and PFS ($P = 0.348$, $P = 0.804$, $P = 0.314$, respectively). This is similar to KRYGIER A et al, who concluded that high expression of RUNX1 has

no relation to FAB classification of AML and mortality [47]. Our study found no associations between age, TLC, Hb, PLT, peripheral and bone marrow blasts and different ELN genetic groups and the level of RUNX1 expression.

No detection of correlation between RUNX1 expression and the clinicopathological parameters could be considered as a limitation of this study that could be owed to recruitment of small number of patients and short follow-up period. In future, researchers could consider enrolling a higher number of patients, a longer follow-up period, and acquisition of more detailed clinical information to obtain higher quality results.

Conclusion

In conclusion, RUNX1 could serve as a potential diagnostic target and modulation of its expression could prove to be a better therapeutic strategy than the current approaches. We also suggested that RUNX1 gene expression level and AML development might be associated with gender of the patient. Males may have higher levels of RUNX1 and more prone to AML development However, we must confirm the obtained results in a larger cohort study.

Declarations

Ethics approval and consent to participate

The institutional review board of National Cancer Institute, Cairo University, approved the protocol of the study and we followed the Helsinki guidelines for the protection of human subjects. Written informed consents were obtained from all participants.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

FSAM, RES & NMH collected, analyzed and interpreted the patient data. FSAM, RES & NMH performed the examination and contributed majorly in writing the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Laboratory characteristics of the AML group (n=91).

	Value
Total leukocytic count ($x10^3/mm^3$)	38.9 (1.0-440.0)
Hemoglobin concentration (gm/dL)	8.0±1.8
Platelet count ($x10^3/mm^3$)	35.0 (5.0-297.0)
Peripheral blood blasts (%)	51.0 (0.0-98.0)
Bone marrow blasts (%)	70.0 (0.0-97.0)
FAB classification	
M1	11 (12.1%)
M2	38 (41.8%)
M4	31 (34.1%)
M5	10 (11.0%)
M7	1 (1.1%)
BM cellularity	
Hypercellular	68 (74.7%)
Normocellular	12 (13.2%)
Hypocellular	5 (5.5%)
FLT3 Mutation	14 (15.4%)
NPM Mutation	9 (39.1%)
Molecular markers	
-ve	77 (84.6%)
+ve	14 (15.4%)
inv (16)	8 (8.8%)
t(8,21)	1 (1.1%)
t(8,21),t(9,22)	1 (1.1%)
t(8:21)	4 (4.4%)
Genetics	
Abnormal	24 (26.4%)
Normal	67 (73.6%)
Genetic risk	

Favorable	18 (19.8%)
Intermediate	51 (56.0%)
Adverse	22 (24.2%)

Table 2 Comparison of RUNX1 gene expression value in the AML group and the Control group of same age and sex.

	AML Group (n=91)	Control Group (n-14)	p value
Age (years)	35.5±11.6	31.1±8.7	0.177
Sex (male/female)	50/41	7/7	0.730
RUNX1 gene expression level	16.81 (0.20-1382.78)	1.00 (0.72-10.17)	< 0.001

Table 3 RUNX1 expression levels in AML and Control groups.

	AML Group (n=91)	Control Group (n-14)	p value	Odds Ratio (95%CI)
RUNX1				
Over expression	46 (50.5%)	0 (0.0%)	< 0.001	1.31 (1.14-1.51)
Low expression	45 (49.5%)	14 (100.0%)		

Table 4 Characteristics of the AML patients by high and low expression levels of RUNX1.

	RUNX1 over-expressors (n=46)	RUNX1 low-expressors (n=45)	p value
Age (years)	36.0±10.9	34.9±12.3	0.434
Sex			
Male	30 (60.0%)	20 (40.0%)	**0.046**
Female	16 (39.0%)	25 (61.0%)	
TLC ($x10^3/mm^3$)	50.7(1.0-440.0)	25.1 (1.7-281.7)	0.191
Hb (gm/dL)	7.8±1.8	8.3±1.7	0.267
PLT ($x10^3/mm^3$)	32.0 (6.0-208.0)	35.0 (5.0-297.0)	0.923
PB blasts (%)	59.0 (10.0-95.0)	48.5 (0.0-98.0)	0.229
BM blasts (%)	72.0 (30.0-97.0)	70.0 (0.0-95.0)	0.282
FAB1			
M1 M2	27 (55.1%)	22 (44.9%)	0.348
M4, M5, M7	19 (45.2%)	23 (54.8%)	
FLT3			
Mutant	11 (78.6%)	3 (21.4%)	**0.023**
Wild	35 (45.5%)	42 (54.5%)	
NPM			
Mutant	5 (55.6%)	4 (44.4%)	1.000
Wild	7 (50.0%)	7 (50.0%)	
Molecular markers			
-ve	39 (50.6%)	38 (49.4%)	0.964
+ve	7 (50.0%)	7 (50.0%)	
Genetics			
Abnormal	11 (45.8%)	13 (54.2%)	0.590
Normal	35 (52.2%)	32 (47.8%)	
Genetic risk			
Favorable	8 (44.4%)	10 (55.6%)	0.363
Intermediate	24 (47.1%)	27 (52.9%)	
Adverse	14 (63.6%)	8 (36.4%)	

*TLC: Total leukocytic count, Hb: hemoglobin concentration, PLT: Platelet count, PB: Peripheral blood, BM: Bone marrow

Table 5 Overall and event-free survival and their relation to RUNX1 expression in the patients.

Overall Survival	n	No of events	Cumulative survival at 12 months	Median survival (months)	p-value
Whole Group	91	54	46.60%	1.3 (7.1-15.4)	
RUNX1					
Over-expressors	46	27	46.30%	9.5 (3.7-15.4)	0.804
Low-expressors	45	27	47.00%	11.5 (5.2-17.8)	
Event-free Survival					
Whole Group	91	60	40.70%	8.6 (6.1-11.1)	
RUNX1					
Over-expressors	46	32	36.80%	7.4 (2.6-12.1)	0.314
Low-expressors	45	28	44.40%	9.5 (7.0-12.1)	

Figures

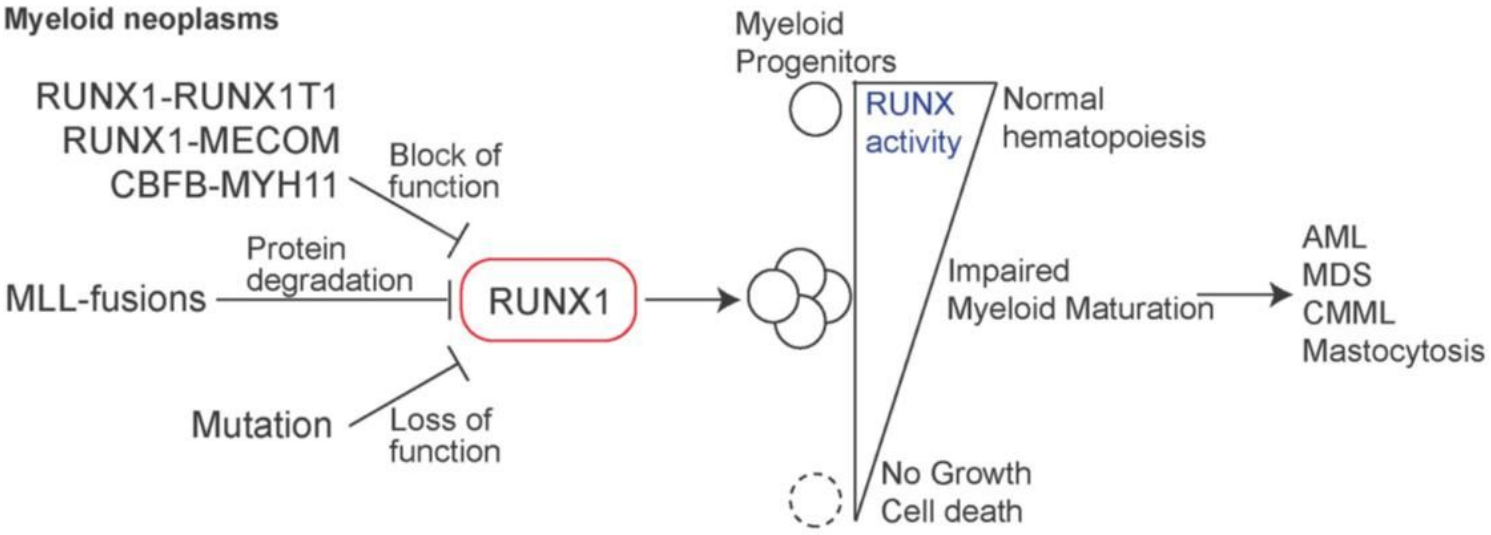

Figure 1

Regulation of RUNX1 function in hematopoietic neoplasms

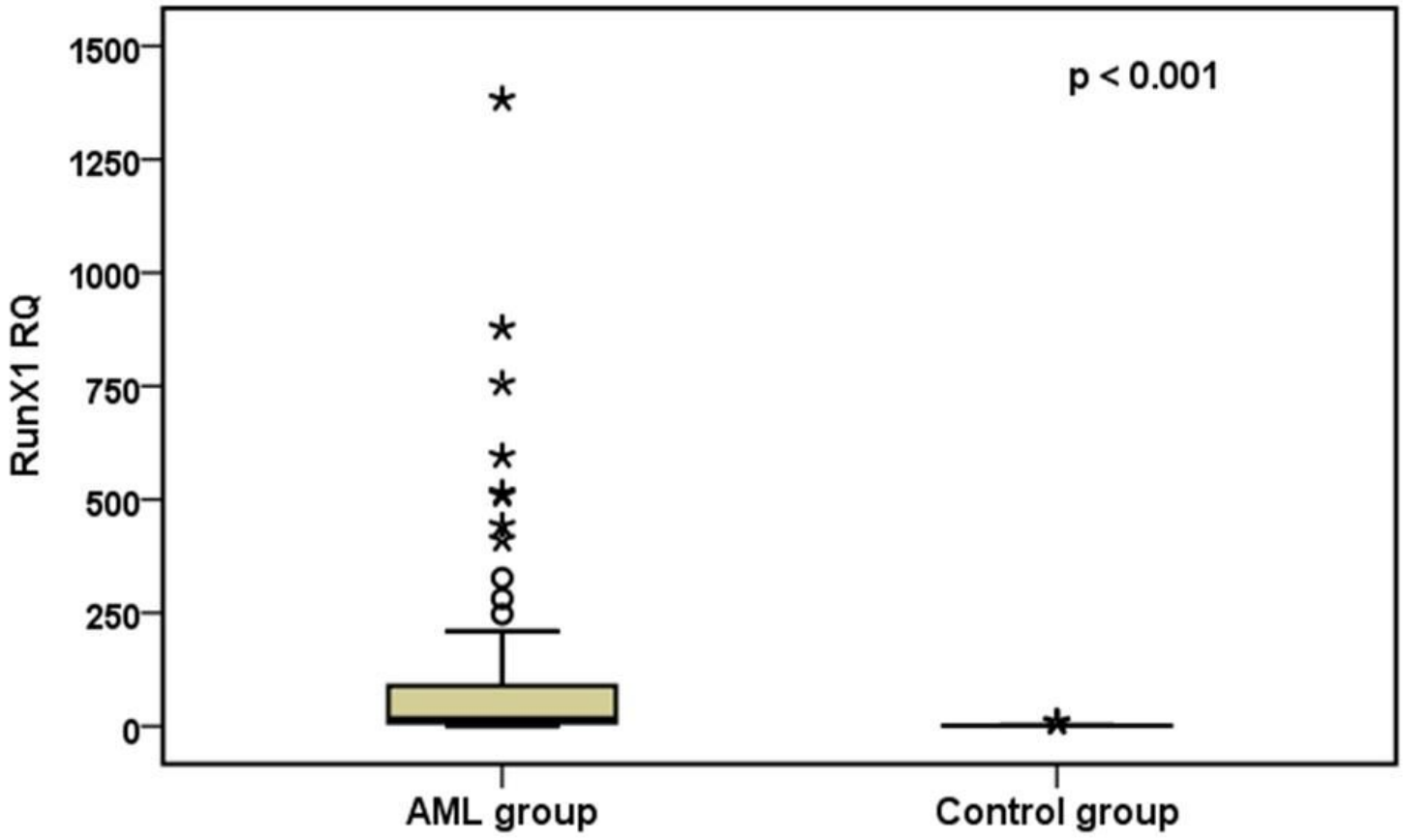

Figure 2

Comparison between relative RUNX1 gene expression in AML cases and control group

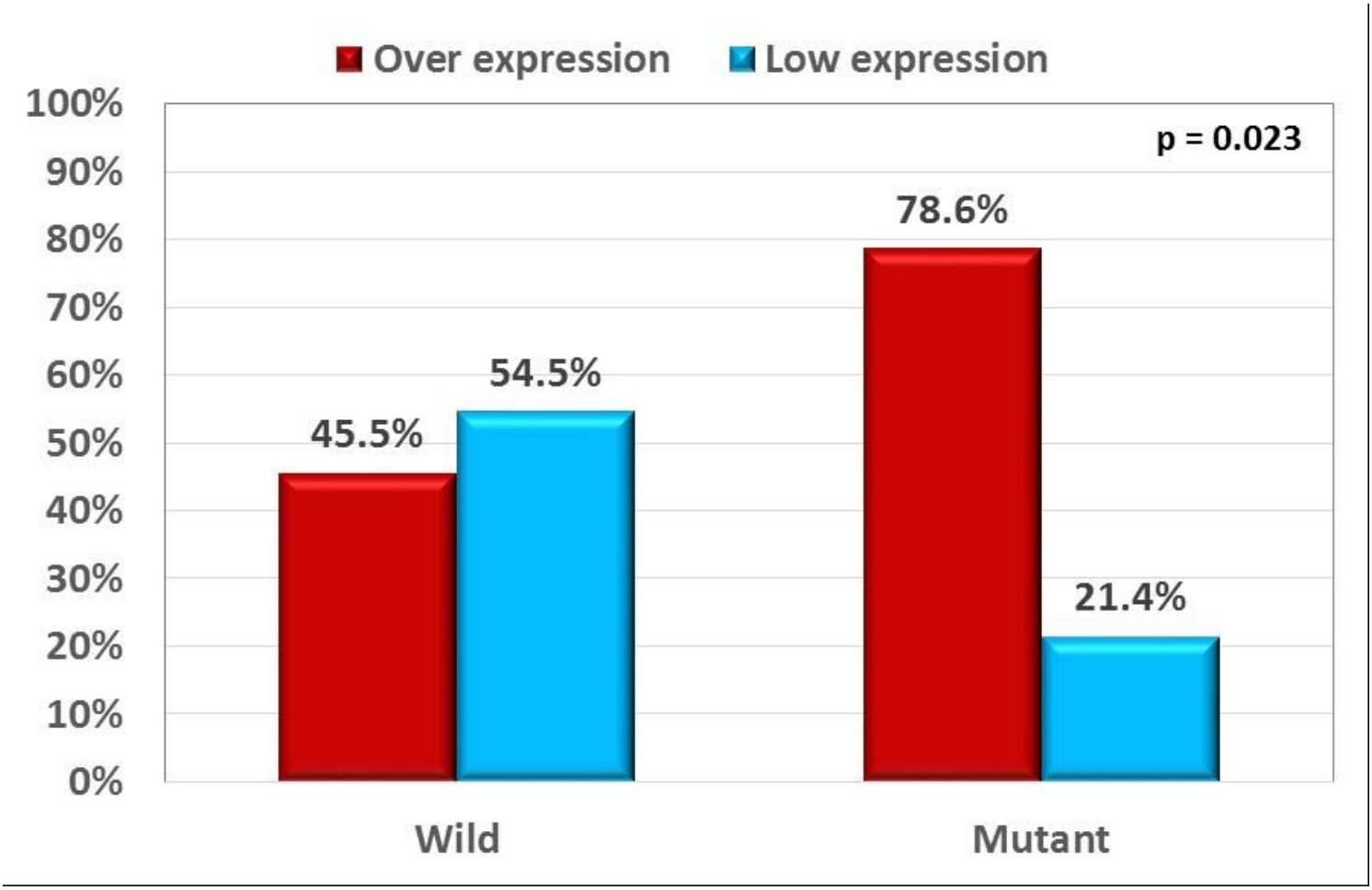

Figure 3

Comparison of expression (high, low) of RunX1 in wild and mutant FLT3

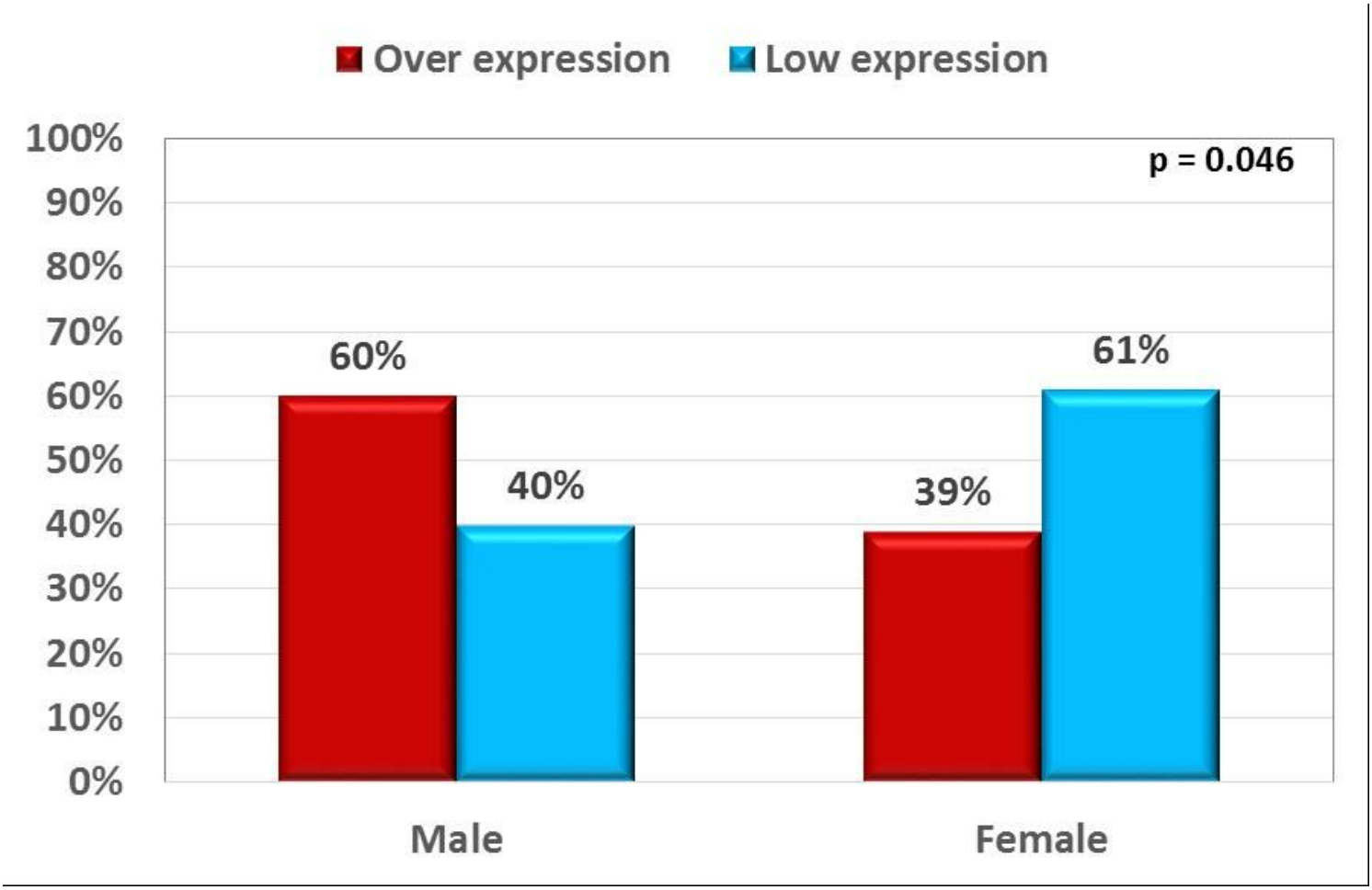

Figure 4

Comparison of expression (high, low) of RunX1 in males and females