ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

OVEREXPRESSION OF LYMPHOID ENHANCER BINDING FACTOR-1 (LEF1) PREDICTS FAVORABLE OUTCOME IN CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA (CN-AML).

Shereen Mohamed Elhoseiny MD[1] and Raafat Mohamed Abdelfattah MD[2].

1. Department of Clinical and Chemical Pathology, Beni Suef Teaching Hospital, Beni Suef University, Egypt.
2. Department of Clinical Oncology, National Cancer Institute, Cairo University, Egypt.

Manuscript Info

Manuscript History:

Received: 14 January 2016
Final Accepted: 26 February 2016
Published Online: March 2016

Key words:
CN-AML, LEF-1, Wnt signaling pathway, RTQ-PCR .

Corresponding Author

Shereen Mohamed Elhoseiny MD.

Abstract

Background:- Lymphoid enhancer-binding factor 1 (LEF1) is a downstream effector of the Wnt/β-catenin signaling pathway, which controls cell growth and differentiation. Dysregulation of LEF1 expression may result in several disease patterns and hematological malignancies, as the Wnt signaling plays a pivotal role in development and cancerogenesis.

Patients and methods:- We investigated the influence of LEF1 expression in 72 adults with cytogenetically normal acute myeloid leukemia (CN-AML) patients together with 30 healthy controls using a Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction (RTQ-PCR) to assess the possible relation, association or correlation between LEF1 expression and CN-AML clinical and laboratory features at diagnosis and follow up.

Results:- We found that both the expression rate and expression level of LEF-1 gene were significantly higher in AML patients than in controls **(p=<0.001).** LEF-1 ^{high} expressors had significantly lower WBCs and BM blasts compared to LEF-1 ^{Low} expressors, the difference was statistically significant **(p=0.03, 0.02)** respectively. Hepatosplenomegaly was more encountered in LEF-1 ^{Low} expressors compared to LEF-1 ^{high} expressors **(p=0.05)**. LEF-1 ^{high} expressors were associated with favorable treatment outcomes as they were associated with significantly better overall survival (**p=0.001),** disease-free survival (**p=0.04)**, and event-free survival (**p=0.008).** Complete remission (CR) were more achieved in LEF-1 ^{high} expressors than LEF-1 ^{Low} expressors; yet the difference was not statistically significant (p=0.1).

Conclusion:- Our study has shown that LEF1 is a favorable prognostic factor in CN-AML. It could therefore be useful to improve risk stratification and to develop better treatment strategies and suggest the need of alternative regimens.

Introduction:-

The karyotype of acute myeloid leukemia (AML) assessed at diagnosis is generally recognized as the single most valuable prognostic factor in AML. However, using conventional cytogenetic techniques, karyotype abnormalities are detected in only half of all AML cases. The other half is commonly described as normal-karyotype AML (Cytogenetically normal AML (CN-AML**) (Bienz et al., 2005).** Such patients usually fall into intermediate-risk cytogenetics, but show obviously heterogeneous prognosis clinically. Mutations and gene expression signatures

have been used to distinguish and identify different prognostic subgroups **(Mrozek et al., 2007).** Mutations of NPM1 **(Falini et al., 2005)** and CEBPA **(Frohling et al., 2004)** are associated with favorable outcome, whereas mutations of FLT3-ITD **(Kiyoi et al., 1999)** and WT1 **(Becker et al., 2010**) supply indication for adverse prognosis. Also, low expression of BAALC and ERG **(Schwind et al., 2010),** have shown to be favorable prognostic factors. Because of the sizeable proportion of patients that failed to existing therapies and high relapse rate, identification of new diagnostic and prognostic biomarkers is valuable for finding novel targets and developing novel therapies **(Jinlong et al., 2015).**

In recent years, a number of researches have indicated that the pathogenesis of AML involves the abnormal activation of Wingless-type (Wnt) signaling pathway that has crucial roles in extensive cellular processes in differentiation and proliferation as well as hematopoietic cell growth and fate **(Widelitz 2005 and Kirstetter et al., 2006).** Aberrant Wnt signaling can lead to expansion of leukemic stem cells in myeloid and lymphoid neoplasias (**Qiang et al., 2003 and Ysebaert et al., 2006).** Wnts regulate multiple signaling pathways through both canonical mechanism (β-catenin dependent) and non-canonical mechanism (β-catenin indepedent) (**Anastas & Moon 2013).**

Lymphoid enhancer-binding factor 1 (LEF1) is a member of the LEF1/T-cell factor (TCF) family of transcription factors **(Spaulding et al., 2007).** LEF-1 is a downstream effector of the Wnt/ β -catenin signaling pathway and was originally identified as a lymphoid-specific DNA binding protein **(Reya & Clevers 2005)**.It mediates Wnt signals through recruiting β -catenin and its co-activators to Wnt response elements of target genes, and plays crucial roles during development, including normal hematopoiesis **(Arce et al., 2006).** During canonical Wnt signaling, LEF1/TCF proteins directly interact with β-catenin to induce expression of target genes, including the cell-cycle regulators cyclin D1 and c-myc.1 In addition, LEF1 has roles in normal hematopoiesis and leukemogenesis that are independent of its involvement in Wnt signaling **(Skokowa et al., 2006 and Spaulding et al., 2007).**

Dysregulation of LEF1 expression may result in a number of diseases such as cancer, where overactive Wnt signaling drives LEF1/the T-cell factor (TCF) family of transcription factors to transform cells **(Arce et al., 2006).**Increased expression of LEF1 affects normal expression of cell cycle and growth-promoting genes, such as Cyclin D1 and c-MYC and disturbs differentiation in hematopoiesis **(Petropoulos et al., 2008).**Aberrant expression of LEF1 has been reported to be involved in solid cancers and leukemia as CLL **(Liu et al., 2012),** ALL **(Jia et al., 2015 & Guo et al., 2015)** and AML **(Metzeler et al., 2012);** and LEF1 is required for the growth of leukemia cells **(Zhou et al., 2011).** The increase of LEF1 mRNA and Wnt target gene c-MYC was also shown in the blast phase (BP) of chronic myeloid leukemia (CML) **(Jamieson et al., 2004).**.These reports indicate that LEF1 has an oncogenic effect by promoting cell proliferation through regulation of target gene expression **(Guo et al., 2015).**

To our knowledge, the LEF-1 gene has never been studied in Egyptian AML patients. Thus, this study was done to investigate the expression level of Lymphoid Enhancer-Binding Factor 1 (LEF-1) gene in cytogenetically normal acute myeloid leukemia (CN-AML) patients with a specific aim of determining gene relation to clinical features and laboratory findings at diagnosis and its impact on the response to therapy.

Patients and methods:-

Patients:- The current study was conducted on 72 de novo adult patients with cytogenetically normal Acute Myeloid Leukemia **(CN-AML).**Thirty age and sex matched healthy unrelated individuals were served as a control group. Both patients and controls were recruited from the National Cancer Institute (NCI), Cairo University and Beni Suef University Hospital. Data confidentiality was preserved according to the **Revised Helsinki Declaration of Bioethics (2008).** Informed consent was obtained from all the patients who agreed and participated in this study. Follow up of patients was carried out for three years.

Patients were subjected to full clinical examination , CT chest, CT abdomen and pelvis to assess lung, liver, spleen, lymph nodes and kidneys for possible pathological alterations, echo cardiology to assess cardiac condition, in addition to routine laboratory investigations as complete blood picture (CBC) , liver and kidney functions, serum uric acid, LDH and coagulation profile.

Diagnosis of AML was made based on (1) morphologic findings from Giemsa-stained smears of bone marrow (BM) aspirates, (2) cytochemical stains criteria such as positivity of myeloperoxidase (MPO) and Sudan black B (SBB), and (3) immunophenotyping (IPT) criteria as positivity of CD13 and CD33 by flowcytometry.

Cytogenetic analysis of BM samples of AML patients was performed using the conventional cytogenetic analysis. To be considered cytogenetically normal, at least 20 metaphase cells were analyzed and the karyotype found to be normal in each case.

AML patients were 33 males (45.8%) and 39 (54.2%) females with a mean age of 38.2±12.8 (18-64) years. As for the controls; they were 16 (53.3%) males and 14 females (46.7%), with mean age of 36.0±14.6 (18-60) years.

AML patients were classified according to the French-American-British [FAB] classification into: 8 (11.1%) M0, 21 (29.2%) M1, 26 (36.1%) M2, 11 (15.3%) M4, 4 (5.6%) M5 and 2 cases (2.8%) M6.

Therapy:-Patients were treated according to the standard AML protocol of the NCI ***(El-Zawahry et al., 2007).*** All patients received the 3 and 7 protocols which consisted of adriamycin 30 mg/m^2 for 3 days and ARAC 100 mg/m^2 by continuous infusion for 7 days.

Methods:- Lymphoid enhancer Binding Factor-1 **(LEF-1)** gene expression was analyzed using a real time quantitative reverse transcription polymerase chain reaction (RTQ-PCR) in all patient samples in comparison with normal healthy controls to investigate a possible relation, association, or correlation with the clinical features of AML patients at diagnosis, such as age, gender, hemoglobin(HB), total leucocytic count(TLC), platelets count, bone marrow blast, cell infiltration and FAB classification. Patients' samples were taken prior to treatment. Follow up of patients was carried out for three years to study any possible association between (LEF-1) gene expression and the response of patients to therapy.

RNA isolation and real-time quantitative RT-PCR:-
Mononuclear cells (MNCs) were isolated from 2ml BM aspirate or peripheral blood at diagnosis by Ficoll density gradient centrifugation. Total RNA was extracted from MNCs using a **QIAamp® RNA Blood Mini Kits (Catalog No. 52304) (Qiagen, Germany).**

Complementary DNA (cDNA) was synthesized using (dt) 15-mer primer by superscript III Reverse transcriptase using **High-Capacity cDNA Archive Kit. Applied Biosystems (Part Number: 4322171)** and stored at -20°C till use.

The mRNA expression levels of the LEF-1 gene and its housekeeping gene glyceraldehyde -3-phosphate dehydrogenase **(GAPDH)** were measured by quantitative RT-PCR using an ABI PRISM 7000 Sequence Detector System (Applied Biosystems, Foster City, CA). The quantitative RT-PCR amplification was performed using the pre-developed Assays-on –demand Gene Expression Set for LEF-1 (Assay ID: Hs01547250_m1, Catalog no. 4331182,Applied Biosystems) and TaqMan GAPDH control reagents (Applied Biosystems, TaqMan GAPDH control reagents (human) part no.402869) for the GAPDH gene in combination with the TaqMan Universal PCR Master Mix (Catalog no. 4440043, Applied Biosystems).

Comparative real-time RT-PCR assays were performed for each sample in duplicate in a final reaction volume of 25 ul. GAPDH and LEF-1 were amplified in the same tube using 5ul cDNA, 12.5 ul universal master mix, 1.25 ul LEF-1 gene readymade primer and probe , 1.25 ul GAPDH gene readymade primer and probe , together with 5uL distilled water (DW).

Amplification was carried out at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 PCR cycles at 95°C for 15 seconds, and 60°C for 1 minute. All reactions were done using an ABI PRISM 7000 Sequence Detection Software (Applied Biosystems) and **Applied Biosystem Step One ^{TM} Instrument (USA).**
The expression levels of LEF-1 gene in tested samples were expressed in the form of CT (cycle threshold) level then Normalized copy number (Relative quantitation) was calculated using the Δ Δ CT equation, **Relative Quantification(RQ) =2 $^{-\Delta\Delta CT}$**. A negative control without template was included in each experiment.

Statistical Analysis:-

LEF-1 gene expression was dichotomized at its median (27.0) with patients classified as low LEF-1 if they had expression values within the lower 50% and as high LEF-1 if they had LEF-1 expression values within the upper 50%.

Remission status was assessed after completion of induction chemotherapy. **Complete remission (CR)** was defined as follows: granulocyte count of at least $1.5x10^9$/L, platelet count of at least $100x10^9$/L, no PB blasts, BM cellularity of at least 20% with maturation of all cell lines and less than 5% blasts, and no extramedullary leukemia. **Relapse** was defined as reappearance of PB blasts, more than 5% blasts in BM, or appearance of extramedullary manifestations after CR was achieved.

Overall survival (OS) was measured from the protocol on-study date until the date of death regardless of cause, censoring for patients alive at last follow-up. **Event-free survival (EFS)** was defined for those achieving CR as the time from on-study until relapse or death regardless of cause, censoring for those alive at last follow-up. If a patient did not achieve CR but expired within 2 months of the on-study date, then EFS was defined as the time from on-study until death, regardless of cause. **Disease-free survival (DFS)** was defined only for those patients achieving a CR. It was measured from the CR date until date of relapse or death, regardless of cause, censoring for patients alive at last follow-up **(Kühnl et al., 2011).**

Statistical Methods:-
Data were analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Pearson product-moment was used to estimate correlation between numerical variables. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Multivariate analysis was done using Cox-regression method for the significant factors affecting survival on univariate analysis. Odds ratio (OR) and hazard ratio (HR) with it 95% confidence interval (CI) were used for risk estimation. All tests were two-tailed. A p-value < 0.05 was considered significant **(Dawson & Trapp 2001).**

Results:-

The present study included 72 patients with cytogenetically normal Acute Myeloid Leukemia **(CN-AML),** together with 30 healthy unrelated controls.

The **LEF-1** gene was expressed in all 72/72 (100%) CN-AML patients while it was expressed in only 20/30 (66.7%) of controls. The mean expression level of LEF-**1** in the AML patients was 45.87±64.69 (range 2.62 -365.19), while it was 0.001±0.002 (range 0.000-0.009) in controls. Both expression rates and expression levels of LEF-1 gene were significantly higher in AML patients than in normal controls **(p<0.001).**
Characteristics of AML patients are shown in **Table 1.**

Table (2) represents follow up of AML patients

LEF-1 expression in relation to clinical and laboratory parameters (Table 3):
According to LEF-1 median expression (27.0); patients with values above this median were classified as high LEF-1 (36 patients), while those with values below this median were classified as low LEF-1 (36 patients). Patients with LEF-1 ^{high} were 16 (48.5%) male and 20 (51.3%) female with a mean age of 37.6±13.8 (18-64) years, while they were 17 (51.5%) male and 19 (48.7%) female with a mean age of 38.8±11.9 (18-62) years in LEF-1 ^{Low}. No statistically significant differences was found between LEF-1 ^{high} and LEF-1 ^{Low} expression patients as regards gender and age (p= 0.8, 0.5) respectively.
There was a statistically significant differences between LEF-1 ^{high} and LEF-1 ^{Low} patients as regards white blood cells (WBCs) **(p=0.03),** patients with LEF-1 ^{high} expression had significantly lower TLC (43.2±46.1) compared to LEF-1 ^{Low} (98.0±108.4) patients.

Patients with LEF-1 ^{high} expression showed lower BM blasts (60.2±24.4) than LEF-1 ^{Low} patients (72.7±24.3), the difference was statistically significant **(p=0.02)**. Hepatosplenomegaly was more encountered in LEF-1 ^{Low} patients, it was17 (65.4%) in LEF-1 ^{Low} expressors compared to 9 (34.6%) in LEF-1 ^{high} expressors, the difference was statistically significant **(p=0.05)**.

There was no statistically significant differences between LEF-1 ^{high} and LEF-1 ^{Low} patients as regards hemoglobin, platelets count or LNs enlargement (p >0.05).

Impact of LEF-1 Gene Expression on Follow up of AML patients (High versus Low LEF-1) (Table 4):
Although patients who achieved complete remission (CR) were more encountered in LEF-1 ^{high} expression patients (54.5% vs 45.5% in LEF-1 ^{Low} patients) and patients who did not achieve CR were more in LEF-1 ^{Low} expressors (64.7% vs 35.3% in LEF-1 ^{high} expressors), yet the difference did not reach statistical significance (p=0.1).

LEF1 ^{high} expression associates with favorable OS, DFS, and EFS (Table 4), (Fig 2,3,4):
LEF-1 ^{high} expression levels were associated with favorable treatment outcomes as they had better overall survival (OS),cumulative survival at 3 years was 87.5% in LEF-1 ^{high} compared to only 47.5% in LEF-1 ^{Low} patients, the difference was statistically significant **(p=0.001).** Hazards ratio was 0.19 (95% CI:0.06-0.57) which means that high level of LEF-1 gene is 81% protective against the hazard of death.

Also disease free survival (DFS) were more achieved in LEF-1 ^{high} than LEF-1 ^{Low} patients, cumulative survival at 3 years for DFS was 68.3% in LEF-1 ^{high} vs 50.2% in LEF-1 ^{Low} patients, the difference was statistically significant **(p=0.04).** Hazards ratio was **0.41** (95% CI:0.16-1.02) which means that high level of LEF-1 gene is 59% protective against the hazard of relapse and/or death.

As for event free survival (EFS), it was longer in LEF-1 ^{high} patients; cumulative survival at 3 years was 64.3% in LEF-1 ^{high} vs only 35% in LEF-1 ^{Low} patients, the difference was statistically significant **(p=0.008).** Hazards ratio was 0.41 (95% CI:0.21-0.81) which means that high level of LEF-1 gene is 59% protective against the hazard of not achieving CR, relapse and/or death.

Multivariable analyses shows that LEF1 expression associates with favorable OS, DFS, and EFS:-
We performed multivariable analyses to determine the prognostic significance of LEF1 expression after adjusting for the impact of other known risk factors. LEF1 gene was the only independent factor affecting significantly the overall survival **(p=0.005)** HR 0.20 (95% CI:0.068-0.620). LEF-1 is protective since HR<1, high LEF-1 gene is 80% protective against hazards of death. As for DFS; LEF1 gene was again the only independent factor affecting significantly DFS **(p=0.04)** HR 0.28 (95% CI:0.080-0.977). High LEF-1 gene is 72% protective against hazards of relapse and/or death. Again, in EFS; LEF1 gene was the only independent factor affecting significantly EFS **(p=0.002)** HR 0.33 (95% CI:0.169-0.682). High **LEF-1** gene is 67% protective against hazards of not achieving CR, relapse and/or death.

Impact of LEF-1 Gene Expression on Follow up of all 72 AML patients (Table 5):-
We used Cox Regression to determine Hazard Ratio. Age was an important risk factor for follow up of patients; as patients< 50 years had longer OS **(p=0.02)** HR 2.65 (95% CI:1.11-6.31), longer DFS **(p=0.01)** HR 3.13 (95% CI:1.27-7.69) and longer EFS **(p=0.01)** HR 2.39 (95% CI:1.22-4.68) than older patients >50 years. Hepatosplenomegaly was also an important risk factor for follow up of patients; patients without HSM had longer OS **(p<0.001)** HR 8.50 (95% CI:3.05-23.64), longer DFS **(p=0.001)** HR 4.37 (95% CI:1.80-10.57) and longer EFS **(p<0.001)** HR 5.0 (95% CI:2.59-9.90) than patients with HSM.

Patients with lower WBCs had longer OS **(p=0.004)** HR 1.0 (95% CI:1.002-1.009), longer DFS **(p<0.001)** HR 1.0 (95% CI:1.00-1.01) and longer EFS **(p<0.001)** HR 1.0 (95% CI:1.0-1.0) than higher WBCs patients. Also patients with lower BM blasts had longer OS **(p=0.02)** HR 1.04 (95% CI:1.00-1.08), longer DFS **(p=0.04)** HR 1.02(95% CI:1.00-1.04) and longer EFS **(p=0.002)** HR 1.04 (95% CI:1.01-1.07) than higher BM blasts patients. So when using cox regression age, HSM, WBCs & BM blasts were important risk factors for follow up of patients.

Table 1: Characteristics of AML patients

Characteristic	Value
LEF-1 expression	45.87±64.69 (2.62 -365.19)*
Gender: No (%) **Male** **Female**	 33(45.8%) 39(54.2%)
Age at diagnosis: (years) Median	38.2±12.8 (18-64) 37.5
White blood cells x10^9/L Median	70.6 ± 87.2 (0.9-365.7) 42.5
Hemoglobin gm/dl Median	8.1 ± 1.9(2.7-12) 8.3
Platelets x10^9/L Median	66.9 ± 65.2 (8-360) 46.5
Bone Marrow blasts Median	66.5 ± 24.2(13-100) 74.5
Hepatosplenomegaly (%)	26 cases (36.1%)
LN enlargement (%)	9 cases(12.5%)
FAB classification: **M0: No (%)** **M1: No (%)** **M2: No (%)** **M4: No (%)** **M5: No (%)** **M6: No (%)**	 8 (11.1%) 21(29.2 %) 26 (36.1 %) 11 (15.3 %) 4 (5.6 %) 2 (2.8 %)

***Mean ± SD (range) FAB classification: French American British classification**

Table 2: Follow up of all AML patients

Characteristic	Value
Follow up time(months) Median	26.2±14(2-47.3) ***** 31.2
Disease Free Survival time (DFS)(months) Median	21.3±15.6(0-46.4) 22.6
Complete Remission (CR): No (%) **No CR: No (%)**	55 (76.4%) 17 (23.6%)
Overall Survival (OS): **Alive: No (%)** **Dead: No (%)** **Cumulative survival at 3 years: %**	 51 (70.8%) 21 (29.2%) **68.1%**
Disease Free Survival (DFS): of the 55 CR patients: **CR: No (%)** **Relapse or Death: No (%)** **Cumulative survival at 3 years: %**	 35 (63.6%) 20 (36.4%) **59.2%**
Event Free Survival (EFS): **CR: No (%)** **No CR, Relapse or Death: No (%)** **Cumulative survival at 3 years: %**	 35 (48.6%) 37 (51.4%) **49.1%**

***Mean ± SD (range)**

Table 3: Comparison between AML patients with High and Low LEF-1 expression.

Characteristic	High LEF-1	Low LEF-1	P value
Number (%)	36(50%)	36(50%)	
LEF-1 expression	77.3±80.1(27-365.2)*	14.5±6.6 (2.6-26.5)	
Median	46.3	13.7	**<0.001**
Gender: No (%)			
Male	16 (48.5%)	17(51.5%)	0.8
Female	20 (51.3%)	19 (48.7%)	
Age(years)	37.6±13.8(18-64)	38.8±11.9 (18-62)	
Median	36.5	38.0	0.5
White blood cells x10^9/L	43.2±46.1(0.9-210)	98.0±108.4(2.4-365.7)	
Median	24.4	54.7	**0.03**
Hemoglobin gm/dl	8.3±1.8 (3.6-11)	7.9±2.0 (2.7-12)	
Median	8.2	8.4	0.5
Platelets x 10^9/L	73.1±73.9 (9-360)	60.7±55.4 (8-250)	
Median	53.0	42.5	0.5
Bone Marrow blasts	60.2±24.4 (13-100)	72.7±24.3(21`-100)	**0.02**
Median	61.0	80.5	
Hepatosplenomegaly (%) 26 cases	9 (34.6%)	17 (65.4%)	**0.05**
LN enlargement (%) 9 cases	4 (44.4%)	5 (55.6%)	1.00
FAB classification:			
M0: No (%) 8 cases	4 (50%)	4 (50%)	
M1: No (%) 21 cases	12(57.1 %)	9(42.9 %)	
M2: No (%) 26 cases	13 (50 %)	13 (50 %)	
M4: No (%) 11 cases	4 (36.4 %)	7 (63.6 %)	
M5: No (%) 4 cases	2 (50 %)	2 (50 %)	
M6: No (%) 2 cases	1 (50 %)	1 (50 %)	

***Mean ± SD (range)**

FAB classification: French American British classification

Bold values are statistically significant

P<0.05 Sig; P<0.001 HS; P>0.05 NS.

Table (4): Impact of LEF-1 Gene Expression on Follow up of AML patients (High versus Low LEF-1)

Characteristic	High LEF-1 36 cases (50%)	Low LEF-1 36 cases (50%)	P value	Hazard Ratio (95% CI)
Complete Remission (CR): No (%) **No Complete Remission: No (%)**	30 (54.5%) 6 (35.3%)	25 (45.5%) 11 (64.7%)	0.1	0.45(0.14-1.40)
Overall Survival (OS) **Alive: (51 cases)No (%)** **Dead: (21 cases) No (%)**	 31 (86.1%) 5 (13.9%)	 20 (55.6%) 16 (44.4%)	**0.001**	**0.19(0.06-0.57)**
OS: Cumulative survival at 3 years: %	87.5%	47.5%		
Disease Free Survival (DFS): of the 55 CR patients **CR: 35 cases: No (%)** **Relapse or Death: 20 cases: No (%)**	 22 (73.3%) 8 (26.7%)	 13(52%) 12(48%)	**0.04**	**0.41 (0.16-1.02)**
DFS: Cumulative survival at 3 years: %	68.3%	50.2%		
Event Free Survival (EFS): **CR: 35 cases: No (%)** **No CR, Relapse or Death: 37 cases: No (%)**	 22(61.1%) 14(38.9%)	 13(36.1%) 23(63.9%)	**0.008**	**0.41(0.21-0.81)**
EFS: Cumulative survival at 3 years: %	64.3%	35%		

CI: Confidence interval
Bold values are statistically significant
P<0.05 Sig; P<0.001 HS; P>0.05 NS.

Table (5): Impact of LEF-1 Gene Expression on Follow up of all 72 AML patients

Parameters	Overall Survival (OS)			Disease Free Survival (DFS)			Event Free Survival (EFS)		
	P-value	Hazard Ratio (95% CI)		P-value	Hazard Ratio (95% CI)		P-value	Hazard Ratio (95% CI)	
Age: **<50 years versus >50 years:**	**0.02**	**2.65**	**1.11-6.31**	**0.01**	**3.13**	**1.27-7.69**	**0.01**	**2.39**	**1.22-4.68**
Gender: **Male versus Female:**	0.6	0.81	0.33-1.96	0.3	0.66	0.26-1.67	0.8	1.08	0.56-2.06
HSM: **Yes versus No:**	**<0.001**	**8.50**	**3.05-23.64**	**0.001**	**4.37**	**1.80-10.57**	**<0.001**	**5.0**	**2.59-9.90**
White blood cells	**0.004**	**1.00**	**1.002-1.009**	**<0.001**	**1.00**	**1.00-1.01**	**<0.001**	**1.00**	**1.00-1.00**
Haemoglobin	0.1	0.86	0.70-1.06	0.9	0.99	0.79-1.23	0.6	0.96	0.81-1.13
Platelets count	0.2	0.99	0.98-1.00	0.3	0.99	0.98-1.00	0.1	0.99	0.98-1.00
BM blasts	**0.02**	**1.04**	**1.00-1.08**	**0.04**	**1.02**	**1.00-1.04**	**0.002**	**1.04**	**1.01-1.07**

CI: Confidence interval
Bold values are statistically significant
P<0.05 Sig; P<0.001 HS; P>0.05 NS.

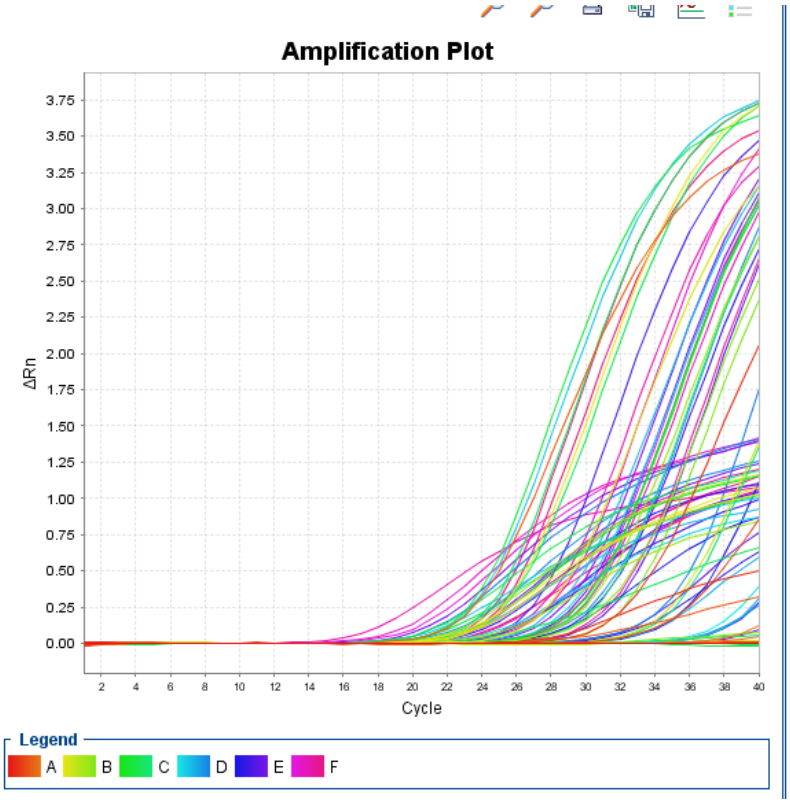

Fig 1: Curves showing LEF-1 gene and GAPDH expressions in AML cases and controls by Realtime Relative Quantitative PCR (RT-PCR).

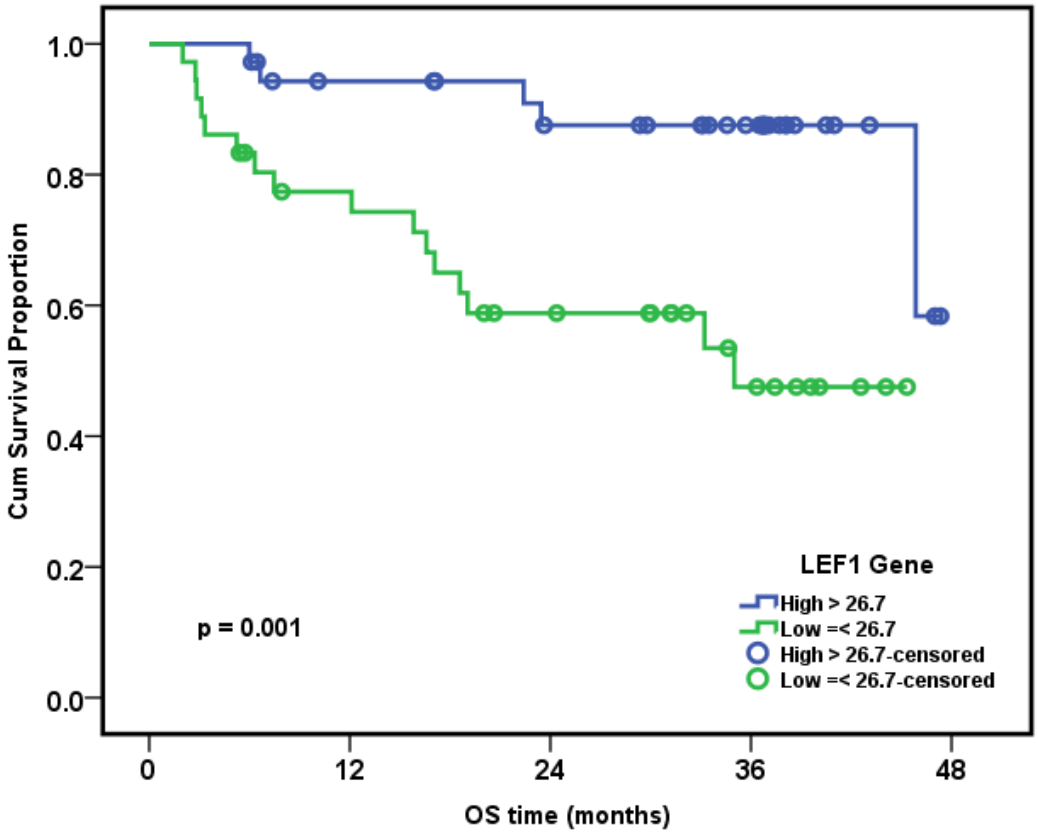

Fig 2: Impact of LEF-1 gene on Overall Survival (OS) (High vs Low).

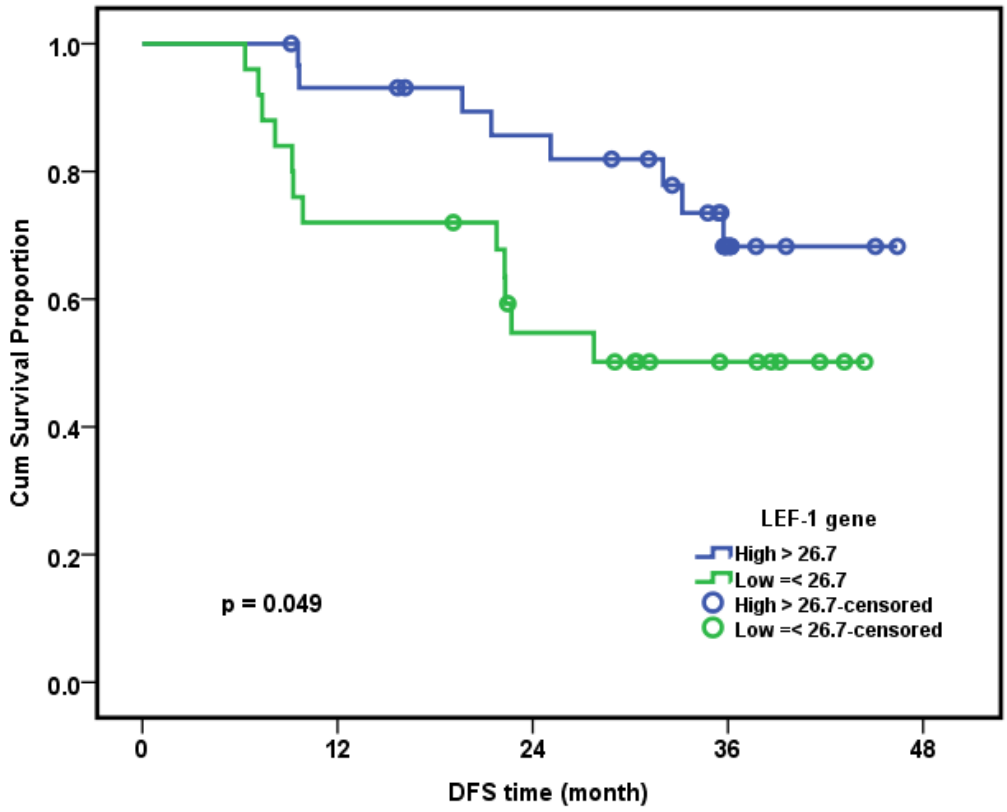

Fig 3: Impact of LEF-1 gene on Disease Free Survival (DFS) (High vs Low).

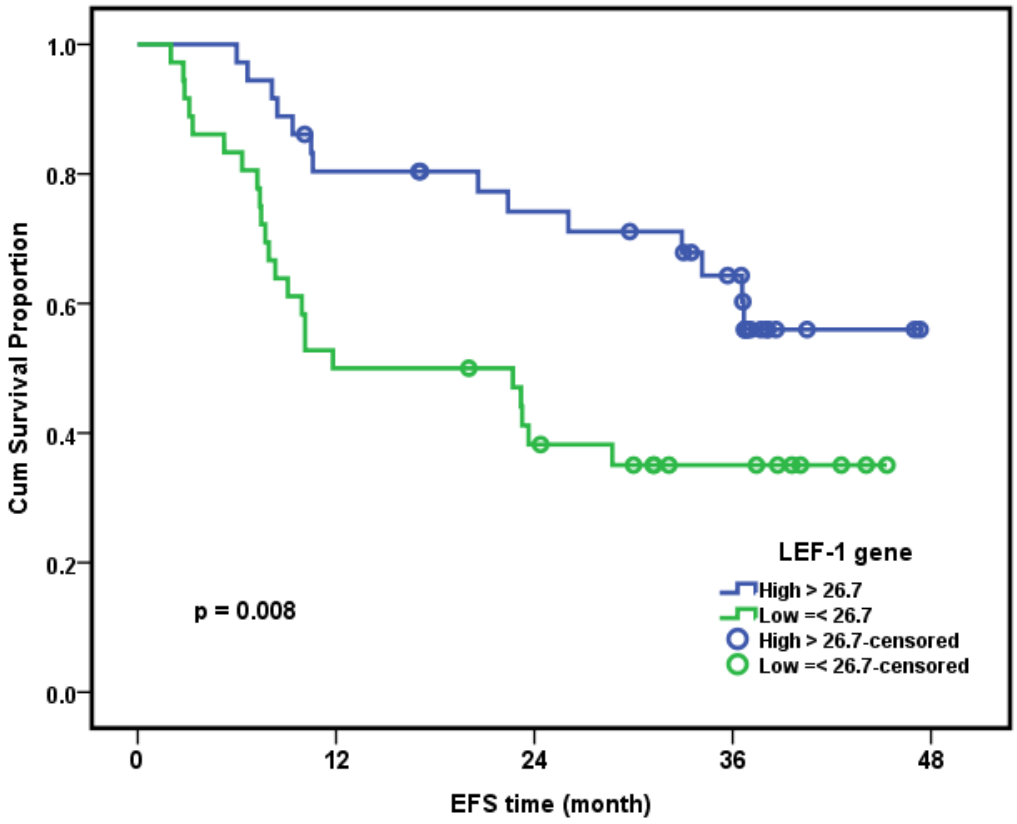

Fig 4: Impact of LEF-1 gene on Event Free Survival (EFS) (High vs Low).

Discussion:-

The identification of prognostic markers in AML, particularly in cytogenetically normal (CN) patients, is important for the development of new molecular therapies and might also allow to improve risk-adapted treatment stratification for these patients (**Kühnl et al., 2011).**

Lymphoid enhancer-binding factor 1 (LEF1) is a downstream effector of the Wnt/β-catenin signaling pathway, which controls cell growth and differentiation **(Reya & Clevers 2005)**. Dysregulation of LEF1 expression may result in several disease patterns, as the Wnt signaling plays a pivotal role in development and cancerogenesis and also controls self-renewal, proliferation and differentiation of many types of stem cells **(Holland et al., 2013).**

In normal hematopoiesis, LEF1 plays a crucial role in the development of B- and T-lymphocytes as well as neutrophilic granulocytes **(Skokowa et al., 2006)**.In different hematologic malignancies, including lymphomas, **(Spaulding et al., 2007 and Gelebart et al., 2008),** CLL **(Liu et al., 2012),** ALL **(Jia et al., 2015 & Guo et al., 2015)** and AML **(Metzeler et al., 2012);** LEF1 was found to be highly expressed**.**

From this context, this study was done to investigate the possible relationship between LEF-1 gene expression and CN-AML patients with a specific aim of determining gene relation to clinical features and laboratory findings at diagnosis and its impact on the response to therapy in a case–control study conducted on a cohort of Egyptian AML patients.

The expression of LEF-1 mRNA was analyzed in 72 CN-AML patients together with 30 healthy unrelated controls. We found that both the expression rates and expression levels of LEF-1 were significantly higher in AML patients than in controls **(p=<0.001).** These findings were in accordance with previous studies **(Metzeler et al., (2012) and Fu et al., 2014)**.

These findings can be explained by the fact that the Wnt signaling pathway is a critical regulator of stem cell function in healthy tissues and cancer, including acute myeloid leukemia (AML). Studies in humans and mice have demonstrated that the Wnt pathway is essential for maintenance, activation, and proliferation of normal hematopoietic stem cells **(Reya & Clevers 2005)**.

Given the pivotal role of LEF-1 in normal hematopoiesis, it is not surprising that its dysregulation is associated with certain kinds of hematologic malignancies. Animals transplanted with bone marrow cells expressing LEF1 presented a severe disturbance of normal hematopoietic differentiation and finally developed B lymphoblastic leukemia and AML, indicating the critical function of balanced LEF1 expression for an ordered hematopoietic development **(Petropoulos et al., 2008).**

Aberrant LEF1 signaling could lead to increased growth and proliferation of myeloid progenitor cells through up-regulation of its target genes such as c-myc and cyclin D1 **(M ü ller-Tidow et al., 2004).**

Therefore, constitutive expression of LEF-1 in early hematopoietic progenitor cells might result in a shift toward the myeloid lineage. This important role of **LEF-1** in myeloid development is supported by **Petropoulos et al., (2008)** findings showing expression in early and myeloid hematopoietic cells in mice and **Fu et al., (2014)** whose data indicates that dysregulation of LEF1 may contribute to the pathophysiology of AML.

LEF-1 was classified according to its median expression (27.0) into high and low LEF-1 expressors; a similar approach was adapted by several studies **(Metzeler et al., 2012, Fu et al., 2014 Albano et al., 2014 and Jia et al., 2015)**.

In the current study, patients with high LEF1 expression had lower pretreatment WBCs than patients with low LEF-1 expression, the difference was statistically significant **(p=0.03).** Our results are in accordance with **Metzeler et al., (2012)** who also found lower WBCs count in LEF-1 ^{high} compared to LEF-1 ^{Low} patients when studied 210 CN-AML patients and with **Albano et al., (2014)** who studied LEF-1 expression in 78 adult acute promyelocytic leukemia (APL) patients.

In this study, patients with LEF-1 ^{high} expression showed lower BM blasts percentages than LEF-1 ^{Low} patients, the difference was statistically significant **(p=0.02)**. This finding are in accordance with **Metzeler et al., (2012)**.

These findings could be explained by previous studies that found that apart from its involvement in Wnt signaling, LEF1 is also involved in multiple other cellular pathways **(Spaulding et al., 2007 and Skokowa & Welte 2007).**LEF1 is a crucial transcription factor in neutrophilic granulopoiesis. LEF1 expression is low or absent in patients with severe congenital neutropenia, leading to downregulation of CEBPA and to a block of neutrophilic differentiation **(Skokowa & Welte 2007).** Thus, low LEF1 expression may also contribute to the differentiation block in MDS and AML blasts, as reflected by the higher WBC and blast percentages in LEF1low CN-AML and MDS **(Pellagatti et al., 2009)**.

In the current study, hepatosplenomegaly was more encountered in LEF-1 ^{Low} patients compared to LEF-1 ^{high} expressors, the difference was statistically significant **(p=0.05)**. This may be due to disease progression in LEF-1 low patients and lower treatment outcome compared to LEF-1 high patients. The only known previous two studies in AML **Metzeler et al. (2012) and Fu et al., (2014)** didn't report if there was a difference or not.

We did not find any statistically significant differences between patients with high and low LEF-1 expression as regards other patients parameters as age, gender, hemoglobin, platelets, or LN enlargement. This is in agreement with previous studies **(Metzeler et al. 2012 and Fu et al., 2014)** except for platelets count in which **Metzeler et al., (2012)** found higher platelet counts in LEF-1 ^{high} patients compared to LEF1low patients **(p=0.04)**.

When response to treatment was considered, high LEF1 expression levels were associated with favorable treatment outcomes. Complete remission (CR) were more achieved in LEF-1 ^{high} expressors compared to LEF-1 ^{Low} expressors; yet the difference was not statistically significant (p=0.1). These findings were in agreement with **Metzeler et al., (2012)** and **Fu et al., (2014)** of the association of LEF-1 ^{high} expressors with better achievement of complete remission, but contrary to our results, they found it statistically significant (p=0.03) in both studies. This may be due to our small sample size (72 AML patients) compared to 210 in **Metzeler et al., (2012)** and 101 in **Fu et al., (2014)**.

LEF-1 ^{high} expressors were associated with longer overall survival (OS) than LEF-1 ^{Low} expressors **(p=0.03),** HR<1 which means that LEF-1 ^{high} expressors are highly protective against hazards of death. Our results agree with the previous two studies in AML **(Metzeler et al. 2012 and Fu et al., 2014)** and that's of APL also **Albano et al., (2014).**

Also disease free survival (DFS) were more achieved in LEF-1 ^{high} than LEF-1 ^{Low} patients, the difference was statistically significant **(p=0.05)** and again HR<1. In **Metzeler et al., (2012)** and **Fu et al., (2014)** studies; LEF-1 ^{high} expressors had longer relapse free survival (RFS) than LEF-1 ^{Low} patients,

In the current study, LEF-1 ^{high} expressors had also longer event free survival (EFS) than LEF-1 ^{Low} expressors, the difference was statistically significant **(p=0.01)** and HR<1. Again, these findings were in agreement with **Metzeler et al., (2012)],** but **Fu et al., (2014)** didn't study EFS in their patients.

LEF1 gene was a strong independent favorable prognostic factor affecting significantly the OS **(p=0.005)**, DFS **(p=0.04)** and EFS **(p=0.002)** in multivariable analyses. Our findings were in agreement with **Metzeler et al., (2012)**.

The mechanisms underlying the association between high LEF1expression and favorable treatment outcomes are unclear. However, our results are of particular interest because LEF1 is an important downstream effector of Wnt signaling, a pathway that is required for self-renewal of normal hematopoietic and leukemic stem cells **(Wang et al., 2010).** Leukemogenic fusion genes and gene mutations can induce Wnt signaling in AML, aberrant activation of the Wnt effector β-catenin has been detected in primary AML samples, and small-molecule Wnt pathway inhibitors are cytotoxic for AML blasts **(Minke et al., 2009).** Previous studies showed that over expression of LEF-1 in murine BM leads to disturbed hematopoiesis and, ultimately, to the development of myeloid and lymphoid leukemias **(Petropoulos et al., 2008).** Interestingly, the myeloid leukemias arising in this model originated from a leukemic stem cell with lymphoid characteristics and showed coexpression of lymphoid markers. **Metzeler et al., (2012)** analyzed genome-wide gene expression profiles to identify biologic pathways that are associated with LEF1 expression in CN-AML. In line with previous observations in mice, LEF1high patients also showed up-regulation of gene sets related to T-lymphoid differentiation. On the other hand, gene sets related to cell proliferation, DNA replication, and DNA repair were down regulated in LEF1high patients, which might contribute to their favorable outcomes **(Metzeler et al. 2012)**.

However, there were some limitations to our study, as small patients sample size. Also due to financial limitations, we could not correlate LEF-1 gene with other prognostic genes in CN-AML as mutations of NPM1, and CEBPA**,** FLT3-ITD or WT1**.**Therefore, these results must be verified by further studies with larger patient populations . It will also be interesting to investigate its relation with other known prognostic genes in CN-AML

Conclusion:-

Our study has shown that LEF1 is a favorable prognostic factor in CN-AML. High LEF1 expression is associated with favorable prognosis and better treatment outcome. LEF1 might be involved in the process of disease progression, and possibly can serve as a molecular parameter for risk assessment and/or monitoring of treatment in AML patients. Thus, molecular assessment of LEF1 at diagnosis may be of value to add to the prognostic work of AML patients.

Conflict of interests:-

The authors have no conflicts of interests to declare.

References:-

- **Albano F, Zagaria A, Anelli L, Orsini P, Minervini CF, Impera L, et al. (2014)**: Lymphoid enhancer binding factor-1 (LEF1) expression as a prognostic factor in adult acute promyelocytic leukemia. Oncotarget Feb 15;5(3):649-58.
- **Anastas JN and Moon RT. (2013):** WNT signalling pathways as therapeutic targets in cancer. Nat Rev Cancer; 13: 11-26.
- **Arce L , Yokoyama NN , Waterman ML . (2006):** Diversity of LEF/TCF action in development and disease . Oncogene; 25 : 7492 – 7504 .
- **Becker H, Marcucci G, Maharry K, Radmacher MD, Mrozek K, et al. (2010):** Mutations of the Wilms tumor 1 gene (WT1) in older patients with primary cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. Blood 116: 788–792.
- **Bienz M, Ludwig M, Leibundgut EO, Mueller BU, Ratschiller D, Solenthaler M, Fey MF, Pabst T. et al (2005):** Risk assessment in patients with acute myeloid leukemia and a normal karyotype. Clin Cancer Res, 11:1416-1424.
- **Dawson B and Trapp RG (2001)** : Basic and clinical biostatistics; Mcgraw-Hill Inc.;Third edition.
- **El-Zawahry HM, Zeeneldin AA, Samra MA, Mattar MM, El-Gammal MM, Abd El-Samee A, et al.(2007):** Cost and outcome of treatment of adults with acute myeloid leukemia at the National Cancer Institute, Egypt**.** J Egypt Natl Canc Inst, 19 (2), pp. 106–113.
- **Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, et al. (2005)**: Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 352: 254–266.
- **Frohling S, Schlenk RF, Stolze I, Bihlmayr J, Benner A, et al. (2004)**: CEBPA mutations in younger adults with acute myeloid leukemia and normal cytogenetics: prognostic relevance and analysis of cooperating mutations. J Clin Oncol 22: 624–633.
- **Fu Y, Zhu H, Wu W, Xu J, Chen T, Xu B, Qian S, Li J, Liu P.(2014):** Clinical significance of lymphoid enhancer-binding factor 1 expression in acute myeloid leukemia. Leuk Lymphoma. Feb;55(2):371-7.
- **Gelebart P, Anand M, Armanious H, et al. (2008):** Constitutive activation of the Wnt canonical pathway in mantle cell lymphoma. Blood;112(13):5171-5179.
- **Guo X, Zhang R, Liu J, Li M, Song C, Dovat S, Li J, Ge Z.(2015):** Characterization of LEF1 High Expression and Novel Mutations in Adult Acute Lymphoblastic Leukemia. PLoS One. May 5;10(5):e0125429.
- **Holland JD, Klaus A, Garratt AN, Birchmeier W. (2013):** Wnt signaling in stem and cancer stem cells. Curr Opin Cell Biol. 2013; 25: 254-264.
- **Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, et al. (2004)**: Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med 351: 657–667.
- **Jia M, Zhao HZ, Shen HP, Cheng YP, Luo ZB, Li SS, Zhang JY, Tang YM. (2015):** Overexpression of lymphoid enhancer-binding factor-1 (LEF1) is a novel favorable prognostic factor in childhood acute lymphoblastic leukemia. Int J Lab Hematol. May 8. doi: 10.1111/ijlh.12375. [Epub ahead of print].

- **Jinlong S, Lin F, Yonghui L, Li Y, Weidong W (2015):** Identification of let-7a-2-3p or/and miR-188- 5p as Prognostic Biomarkers in Cytogenetically Normal Acute Myeloid Leukemia**.** PLoS One. 2015 Feb 3;10(2):e0118099.
- **Kirstetter P, Anderson K, Porse BT, Jacobsen SE, Nerlov C (2006):** Activation of the canonial Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol; 7: 1048-56.
- **Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, et al. (1999)**: Prognostic implication of FLT3 and NRAS gene mutations in acute myeloid leukemia. Blood 93: 3074–3080.
- **Kühnl A, Gökbuget N, Kaiser M, Schlee C, Stroux A, Burmeister T, Mochmann LH, Hoelzer D, Hofmann WK, Thiel E, Baldus CD.(2011):** Overexpression of LEF1 predicts unfavorable outcome in adult patients with B-precursor acute lymphoblastic leukemia. Blood. 2011 Dec 8;118(24):6362-7.
- **Liu P, Xu B, Shen W, Zhu H, Wu W, Fu Y, Chen H, Dong H, Zhu Y, Miao K, Xu W, Li J.(2012):** Dysregulation of TNFα-induced necroptotic signaling in chronic lymphocytic leukemia: suppression of CYLD gene by LEF1. Leukemia. Jun;26(6):1293-300.
- **Metzeler KH, Heilmeier B, Edmaier KE, Rawat VP, Dufour A, Döhner K, Feuring-Buske M, Braess J, Spiekermann K, Büchner T, Sauerland MC, Döhner H, Hiddemann W, Bohlander SK, Schlenk RF, Bullinger L, Buske C. (2012):** High expression of lymphoid enhancer-binding factor-1 (LEF1) is a novel favorable prognostic factor in cytogenetically normal acute myeloid leukemia. Blood. Sep 6;120(10):2118-26.
- **Minke KS, Staib P, Puetter A, et al. (2009):** Small molecule inhibitors of WNT signaling effectively induce apoptosis in acute myeloid leukemia cells. Eur J Haematol. 82(3):165-175.
- **Mrozek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD (2007):** 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 109: 431–448.
- **M ü ller-Tidow C , Steff en B , Cauvet T , et al . (2004):** Translocation products in acute myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells . Mol Cell Biol ; 24 : 2890 – 2904 .
- **Pellagatti A, Marafioti T, Paterson JC, Malcovati L, Della Porta MG,et al.(2009):** Marked downregulation of the granulopoiesis regulator LEF1 is associated with disease progression in the myelodysplastic syndromes. Br J Haematol. 146(1):86-90.
- **Petropoulos K, Arseni N, Schessl C, Stadler CR, Rawat VP, Deshpande AJ, et al. (2008)**: A novel role for Lef-1, a central transcription mediator of Wnt signaling, in leukemogenesis. J Exp Med 205: 515–522.
- **Qiang YW, Endo Y, Rubin JS, Rudikoff S. (2003):** Wnt signaling in B-cell neoplasia. Oncogene. 22(10):1536-1545.
- **Reya T and Clevers H .(2005):** Wnt signalling in stem cells and cancer. Nature; 434 : 843 – 850 .
- **Schwind S, Marcucci G, Maharry K, Radmacher MD, Mrozek K, et al. (2010):** BAALC and ERG expression levels are associated with outcome and distinct gene and microRNA expression profiles in older patients with de novo cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. Blood 116: 5660–5669.
- **Skokowa J, Cario G, Uenalan M, et al. (2006):** LEF-1 is crucial for neutrophil granulocytopoiesis and its expression is severely reduced in congenital neutropenia. Nat Med;12(10):1191-1197.
- **Skokowa J and Welte K. (2007):** LEF-1 is a decisive transcription factor in neutrophil granulopoiesis. Ann N Y Acad Sci.;1106:143-151.
- **Spaulding C, Reschly EJ, Zagort DE, et al.(2007):** Notch1 co-opts lymphoid enhancer factor 1 for survival of murine T-cell lymphomas. Blood.110(7):2650-2658.
- **Wang Y, Krivtsov AV, Sinha AU, et al. (2010):** The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. Science.327 (5973): 1650-53.
- **Widelitz R. (2005):** Wnt signaling through canonical and non- canonical pathways recent progress. Growth Factor; 23: 111-6.
- **World Medical Association. Declaration of Helsinki (2008):** Ethical Principles for Medical Research Involving Human Subjects, the 59th WMA General Assembly, Seoul, South Korea.
- **Ysebaert L, Chicanne G, Demur C, et al.(2006):** Expression of beta-catenin by acute myeloid leukemia cells predicts enhanced clonogenic capacities and poor prognosis. Leukemia. 20(7):1211-1216.
- **Zhou F, Zhang L, van Laar T, van Dam H, Ten Dijke P (2011):** GSK3beta inactivation induces apoptosis of leukemia cells by repressing the function of c-Myb. Mol Biol Cell 22: 3533–3540.