Prognostic Implication of Erg Gene Expression in Adult Acute Myeloid Leukemia Patients with Normal Karyotyping

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Abstract: Back ground: The aim of the present work is to assess prognostic significance of ERG gene expression in AML with normal cytogenetic. **Subjects and Methods**: Cases were selected for analysis on the basis of sample availability (peripheral blood, bone marrow sample and presence of cytogentic, also ERG gene expression was evaluated using quantitative real-time PCR. **Results:** AML group was classified according to median ERG expression into high and low groups, median ERG expression was 1.575. The clinical outcome of AML patients in relation to ERG expression was that, all cases with low ERG expression achieved complete remission (CR) and all cases with refractory disease (RD) or induction deaths (ID) were in high expression group. **Conclusion:** High ERG expression is a bad prognostic factor for disease free survival (DFS) and overall (OS) in AML patients with normal cytogenetic.

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Key words: C-N AML patients, High ERG expression, Real time PCR.

1. Introduction:

Acute myeloid leukemia (AML) is a genetically heterogeneous clonal disorder characterized by the accumulation of acquired genetic alterations in the hematopoietic progenitor cells. These alterations disturb normal mechanisms of cell growth, proliferation and differentiation. This results in accumulation of leukemic cells in the bone marrow, ultimately replacing most of the normal hematopoietic cells and their functions, resulting in signs and symptoms of the disease. These include, most prominently, anemia, hemorrhage, infection and their consequences[(1)].

Cytogenetic abnormalities detected at diagnosis have long been recognized as predictors for clinical outcome in acute myeloid leukemia (AML). However, the largest cytogenetic subset of adult AML, approximately 45%, consists of patients with a normal karyotype[(2)].

In addition to genetic abnormalities, molecular markers were likely found to impact on clinical outcome of cytogeneticlly normal AML patients, because these markers are mutated or over expressed genes encoding proteins with potentially roles in leukomogenesis[(3)].

As such, a myriad of proteins have been suggested to be aberrantly regulated in this type of leukemia, including several proteins that belong to the E-twenty-six (ETS) family[(4)].

Ets- related gene (ERG), which in located on chromosome band 21q22, is frequently over expressed in AML patients[(5)].

The ERG protein is a member of the ETS-family and is known to bind to purine-rich sequences. ERG and other members of the same family are downstream regulators of mitogenic signal transduction pathways. They are key regulators of embryonic development, cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis [(6)]. Also ERG gene is a critical regulator of fetal hematopoietic stem cell (HSC) maintenance and as such required to sustain definitive hematopoiesis[(7)]. ERG gene signatures correlates well with the clinical characteristics of leukemia and is thought to contribute to disease progression [(8,9)]. In our work we assed the prognostic significance of ERG gene expression in AML patients with normal karyotype.

2. Subjects and Methods

Patients:

ERG gene expression was studied in bone marrow samples from 50 AML patients who had normal karyotype. The patients were 29 males and 21 females, their age ranged from 19 to 71 years (mean 44.7 $\pm$ 13.7). In addition, 10 apparently healthy volunteers with matched age (19 – 65) years mean (43.00 $\pm$ 13.5) and sex (4 females, 6 males) were included in the study after prior consent. The protocol of the study was approved by ethical committee of Benha Faculty of

medicine and written consents were obtained from all the participants.

They were selected from oncology center Mansoura university Hospital (OCMU). Cases were followed up for a period of 24 months. All cases subjected to: complete blood count, direct blood films were stained with leishman stain and examined for detection of blast cells, bone marrow aspiration, immunephenotyping by flowcytometery and real time PCR for ERG gene expression.

Methods:

RNA extraction and RT-PCR to measure ERG expression levels: Preparation of blood samples and analysis of ERG expression were performed. Total RNA extraction was performed using QIA amp RNA blood mini kit ***(QIAGEN, Hilden, Germany)*** and complementary DNA was synthesized from total RNA. Quantitative real time PCR was performed using Taqman gene expression master mix kit (Applied biosystem, Foster city, CA, USA) and ABI PRISM® 7000 sequence detection system.

Relative quantification of ERG gene normalized to reference (GADPH) gene using the $2^{-\Delta\Delta CT}$ (Livak) method.

i. The cycle threshold (CT) of the target (ERG) gene was normalized to that of the reference (GAPDH) gene, for both the test sample(AML) and the calibrator (Normal) sample:

$\Delta CT(test) = CT(test) - CT(ref.)$

$\Delta CT(calibrator) = CT(calibrator) - CT(ref.)$

ii. The ΔCT of the test sample was normalized to the ΔCT of the calibrator:

$\Delta\Delta CT = \Delta CT(test) - \Delta CT(calibrator)$

iii. The expression ratio was then calculated

Normalized expression ratio = $2^{-\Delta\Delta CT}$

Statistics:

The mean objective was to evaluate the impact of ERG expression on clinical outcome. A set of 50 patients were initially divided into 2 groups of high and low expression according to median ERG expression. Analysis of data was done by **SPSS** (Statistical package for social science) program.

Student t-test and Mann-whitmey test were used (for non parametric data). Chi square test was used to compare groups (for qualitative data). Kaplan Meier test was used for survival analysis and statistical significance of differences among curves was determined by Log – Rank test.

3. Results:

AML patients comprised of 29 males (58%) and 21 females (22%). While the control group comprised of 6 (60%) males and 4 (40%) females. Among AML cases (8) were M_1 (16%), (14) were M_2 (28%), (14) were M_4, (28%) (10) were M_5 (20%), (4) were M_6 (8%), non of cases included in present study were M_3FAB subtype.

Comparison between ERG expression and laboratory data: No statistically significant differences were found between each laboratory parameter and ERG expression groups (P> 0.05 for each; table 1).

Table (1): Laboratory data of the studied cases according to ERG expression groups.

Laboratory data		Low expression group (n=24)	High expression group (n=26)	*P*
TLC ($X10^9$/l)	Median	60.500	29.200	0.147
	Range	3.70-210.00	2.70-173.00	
Hb (g/dl)	Median	8.250	8.1	0.551
	Range	5.10-11.40	3.4-11.9	
Platelets ($X10^9$/l)	Median	36.5	63.000	0.123
	Range	97-323.0	53.0-67.00	
Peripheral blasts (%)	Median	69.5	62.0	0.217
	Range	24-90	23-93	
Marrow blasts (%)	Median	81.0	75.0	0.617
	Range	27-98	26-100	
LDH (IU/L)	Median	855.00	691.00	0.078
	Range	198-4362	216-2431	
Uric acid (mg/dl)	Median	5.45	5.65	0.803
	Range	2.6-15.2	2.9-11.2	

Relation of ERG expression in AML patients with clinical outcome: all cases with low ERG expression achieved complete remission, while only 50% of high expression group achieved CR, all cases with RD or ID were in high expression group (P< 0.001; table 2).

Table (2): Relation of ERG expression in AML patients with clinical outcome.

	Low expression group (n=24)		High expression group (n=26)		*P*
	No. of patients	%	No. of patients	%	
CR	24	100.0	13	50.0	<0.001
RD	0	0	4	15.4	
ID	0	0	9	34.6	

Prognostic value of ERG expression: patients with low ERG expression showed significant better OS and DFS (80%, 80.4% respectively) than those with high ERG expression (17.1%, 27.4% respectively) (P= 0.001 for OS and P= 0.042 for DFS; table 3).

Table (3): Survival times according to ERG expression in AML patients.

Survival	Low expression group (n=24)				High expression group (n=26)				*P* Log Rank (Mantel-Cox)
	Cumulative Survival (%)	Median *(months)*	CI 95%		Cumulative Survival (%)	Median *(months)*	CI 95%		
OS	80.0	31.000	27.054	34.946	17.1	18.000	10.687	25.313	0.001
DFS	80.4	30.000	6.126	15.874	27.4	11.000	24.932	35.068	0.042

Cumulative Survival: Cumulative proportion surviving at 24 months. CI 95%: Confidence interval at 95%, OS: Overall survival. DFS: Disease free survival.

Multivariate analysis for disease free survival and overall survival dependant parameter studied with other covariates.

Multivariate analysis showed that high ERG expression is a bad prognostic factor for DFS and OS (P = 0.045, hazard ratio (HR) = 2.731, 95% CI= 1.044 – 5.474; P= 0.002, HR = 2.938, 95% CI = 1.162 – 4.139 respectively).

Bone marrow blasts were considered as bad prognostic factor for OS (P = 0.049, HR = 1.023, 95% CI = 1.162 – 4.139), otherwise, multivariate analysis didn't show any significant difference in DFS and OS regarding any covariate (P> 0.05; table 4).

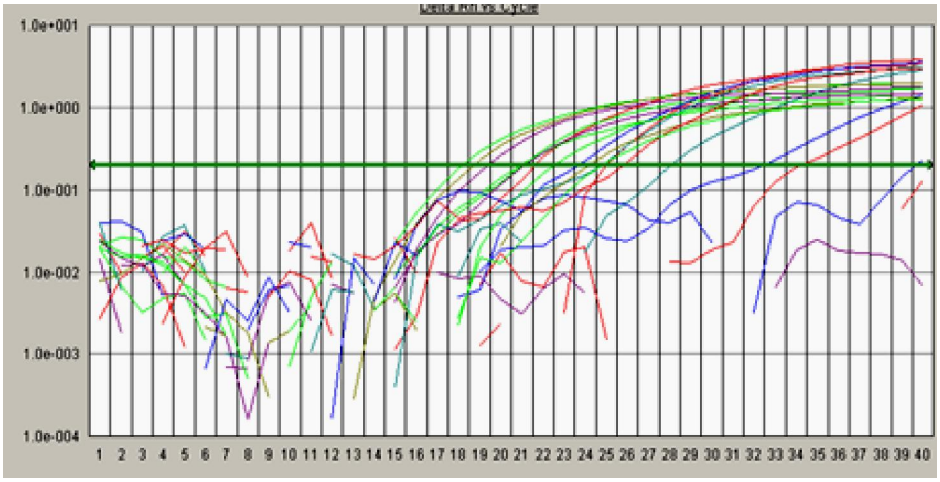

Fig. (1): Log florecence plotted against cycle number for (ERG) gene expression

Table (4). Multivariate analysis for disease free survival and overall survival dependent parameters studied with other covariates (multivariate analysis).

Covariates	DFS				OS			
	P	HR	95% CI		*P*	HR	95% CI	
Age (years)	0.460	1.019	0.969	1.073	0.158	1.026	0.990	1.064
TLC ($x10^9$/L)	0.632	1.002	0.993	1.012	0.726	1.001	0.994	1.009
BM blasts (%)	0.132	1.027	0.992	1.063	0.049	1.023	1.000	1.047
ERG expression	0.045	2.731	1.044	5.474	0.002	2.938	1.162	4.139

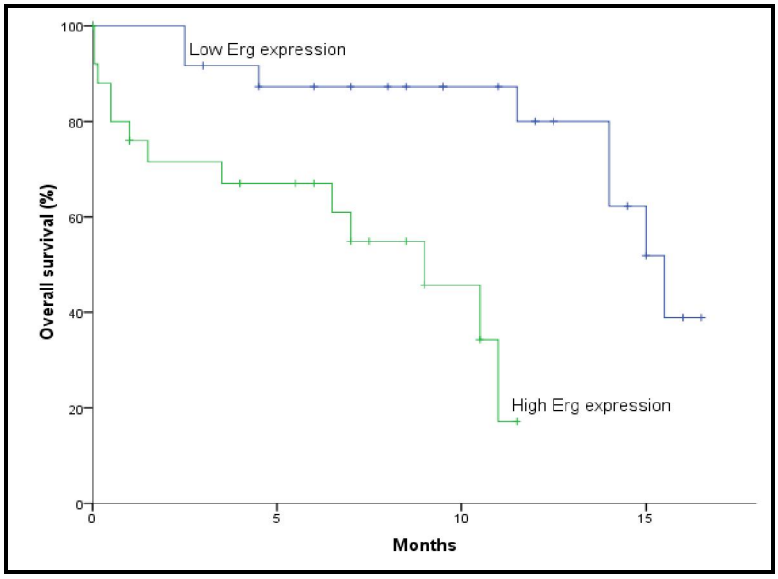

Fig (2): Overall survival according to ERG expression in AML patients.

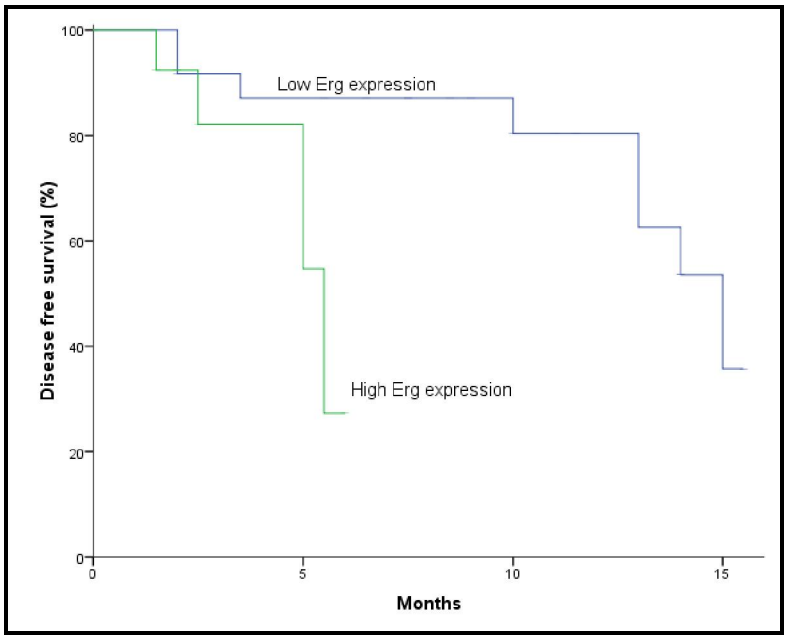

Fig. (3): Disease free survival according to ERG expression in AML patients.

4. Discussion

ERG (Ets related gene 1) gene belongs to erthroblast transformation specific (ETs) family of transcription factor, and is located on chromosome 21q22[13]. ERG gene plays an important role in early hematopoiesis and hematopiotic stem cell maintance[14,7,15]. It encodes transforming proto-oncogene expressed in haematopoietic stem cell and endothelial cells[16] and are involved in key steps regulating cell proliferation, differentiation and apoptosis[17].

ERG gene constitutes a powerful oncogen both in solid organ and hematological maligancies[18]. ERG is a critical factor protecting HSCs from differentiation. Specifically, loss of ERG gene accelerates HSC differentiation by > 20- fold, thus leading to rapid depletion of immunophenotypic and functional HSCs[15].

The present study was conducted on 50 adult newly diagnosed AML patients with normal karyotype attending at Mansura and Benha University Hospitals and 10 apparently healthy control group with matched age and sex. Patients with secondary AML and AML patients under chemotherapy were excluded.

ERG gene expression was analyzed in bone marrow aspirate from patients using real time PCR and was used to evaluate prognostic impact in AML patients. Cases were followed up for 2 years and classified into; complete remission, refectory disease and induction death.

In the present study there were highly significant decrease in hemoglobin concentration and platelets count in AML group compared to control group, while highly significant increase in TLC, LDH in AML group compared to control group ($P < 0.001$ for each).

AML cases classified according to median expression into; high ERG gene expression 24 cases (48%) and low ERG gene expression 26 cases (52%).

In the present study no statistically significant difference was found between number of cases in low and high ERG expression regarding age group. Similar result was found by ***Marcucci et al.(2005) and Metzeler et al. (2009)***[10&19] and in contrast to what reported[20] who approved a statistically significant difference was found between ERG gene expression groups with age. As they found that high ERG expression were included more in the group of patients ≥ 45 years. So increase sample size with more age variation may confirm or exclude such difference.

In the present study there were no statistical significant difference were found between cases with low and high ERG expression regarding sex. This finding go on line with another study performed by ***Metzeler et al. (2009) and Rashed et al. (2015)***[19&20], who stated that no statistical significant difference were found between cases with low and high ERG expression regarding sex.

In the present study there were no statistical significant difference were found between cases with low and high ERG expression regarding lymphadepathy, splenomegaly and hepatomegaly. This finding go on line with another study performed by ***Rashed et al. (2015)***[20] who stated that no statistical significant difference were found between cases with low and high ERG expression with lymphadepathy, splenomegaly and hepatomegaly.

The study found that, there was no statistical significant difference between hemoglobin and platelet count regarding ERG expression groups ($P > 0.05$). This is supported by ***Baldus et al. (2006); Metzeler et al. (2009); Schwind et al. (2010) and Rashed et al. (2015)***[21,19,11,20]. As they reported also no statistical significant difference between hemoglobin and platelet count regarding ERG expression groups.

In the present study there were no statistical significant difference between high ERG gene expression with TLC and PB and BM blasts ($P > 0.05$). Our study agreed with ***Rashed et al. (2015)***[20] and in contrast to ***Schwind et al. (2010)***[11] who reported that statistically significant difference was observed between TLC, PB and BM blasts regarding ERG expression groups. Difference in sample size and technique used to asses such gene may affect these difference.

As regarding the FAB sub-classification, no statistical significant difference was found between number of cases in each FAB subtype according to ERG gene expression ($P > 0.05$). This result is approved by ***Eid et al. (2010)***[12] who stated that no statistical significant difference was found between number of cases in each FAB subtype according to ERG gene expression. This was in contrast to ***Baldus et al. (2006); Loughran et al. (2008); Metzeler et al. (2009) and Rashed et al. (2015)***[21,14,19,20], as they proved that ERG gene mainly belongs to M0, M2, M3 FAB subtype and less expressed in more differentiated subtypes of AML.

On the other hand, in the present study there was no statistically significant difference between low and high expression groups regarding number of positive versus negative cases in each immunophenotype markers ($P > 0.05$). This result go in line with ***Metzeler et al. (2009) and Rashed et al. (2015)***[19,20] who stated that no statistically significant correlation between ERG gene expression with any of immunophenotyping markers.

The clinical outcome of studied cases showed thirty seven patients (74%) achieved complete remission (CR), 4 cases (8%) were refractory, 9 cases (18%) were died during induction therapy. All cases with low ERG gene expression achieved complete remission, all cases with refractory disease or induction death were in high expression ($P< 0.001$). That result support that ERG expression is a valuable predictor for clinical outcome in patient with AML. Our finding is constant with ***Marcucci et al. (2005); Metzeler et al. (2009) and Schwind et al. (2010)***[10,19,11].

Regarding the overall survival, there was a statistical significant difference between ERG expression level and over all survival (OS). The study found that patient with low ERG expression predict longer over all survival and disease free survival ($P = 0.001$; $P = 0.04$) respectively. Similarly, ***Metzeler et al. (2009); Eid et al. (2010); Schwind et al. (2010) and Rashed et al. (2015)***[19,12,11,20] found that high ERG gene expression showed significant shorter OS and DFS ($P <0.05$).

In the present study as regarding multivariate analysis for age, TLC, BM blasts and ERG gene expression the study found that high ERG gene expression is a bad prognostic factor for DFS (P< 0.05) and OS (P = 0.002). Also bone marrow blasts were considered as a bad prognostic factor for OS (P < 0.05) not for DFS. As regards, age and TLC, the multivariate analysis revealed that they didn't show any significant difference in DFS and OS (P > 0.05).

This result are compared to the result of ***Schwind et al. (2010)***[11] who approved that expression level of ERG gene was the only factor associated with OS upon multivariate analysis (P = 0.03).

However, ***Metzeler et al. (2009)***[19] investigated whether ERG expression levels are useful to refine the risk stratification for patients with CN-AML who were already classified according to NPM_1 and FLT-ITD status. Low molecular risk group was (NPM1 mutant or FLT_3 ITD) negative while high molecular risk group was (NPM wild-type or FLT_3 -ITD). They stated that patients with FLT-ITD and high ERG expression level should be classified as high risk group because their outcomes resemble those of patients with a complex aberrant karyotype. In contrast, patients with NPM_1 mutation, low ERG level and absent FLT_3-ITD, as well as CEBPA, can be considered as low risk group. So ***Metzeler et al. (2009)***[19] approved that high ERG expression was the marker with the strongest impact on survival.

However, ***Marcucci et al.(2005)***[10] stated that in AML carrying t(16; 21), ERG was found rearranged with FUS, linking ERG with myeloid leukemogenesis. FUS is a member of the TET family of RNA-binding proteins. Gene rearrangements involving ETS members are often characterized by a TET-related transactivation domain at the N terminus and ETS DNA binding and protein-protein interaction domains at the C terminus. This structure likely increases the oncogenic activity of the resulting chimeric transcription factors by redirecting them to specific targets.

However, ***Diffner et al. (2013)***[18] approved that ERG expression in AML is associated with activity of the ERG promoters and +85 stem cell enhancer and a heptad of transcription factors that regulate genes in HSCs. Gene expression signatures derived from ERG promoter-stem cell enhancer and hepated activity are associated with clinical outcome when ERG expression alone fails.

On the other side, ***Schwind et al. (2010)***[11] reported that low ERG gene expression is associated with up-regulation of topoisomerase I and miRNA-148a and down-regulation of DNA-methyl-transferases which, by its turn, may lead to new therapeutic strategies in AML patients.

In conclusion, our preliminary results for the analysis of the expression level of ERG genes in bone marrow samples obtained from Egyptian AML patients showed that ERG gene is specific significant molecular markers in survival and prognosis.

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