RESEARCH ARTICLE

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Expression of Enhancer of Zeste Homolog 2 (*EZH2*) Gene in Acute Myeloid Leukemia

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Abstract

Background: Enhancer of Zeste Homolog 2 (*EZH2*) is the catalytic subunit of the chromatin modifying enzyme polycomb repressive complex 2 (PRC2). As a complex, these proteins selectively silence target genes through trimethylation of histone 3 at lysine 27. *EZH2* is strongly oncogenic. It has been observed in various malignancies which makes it an interesting therapeutic target. Whether it functions as a tumor suppressor or oncogene in acute leukemia is not settled. **Aim of this work:** This study aimed at determining the expression levels of *EZH2* gene in a cohort of adult Egyptian patients newly diagnosed with acute myeloid leukemia (AML). **Materials and methods:** The present study included 45 de novo AML patients and 40 healthy subjects of matched age and sex as a control group. All study participants were subjected to complete blood count (CBC), bone marrow examination, immunophenotyping, conventional cytogenetic studies and Detection of *EZH2* gene expression levels by real time quantitative polymerase chain reaction (RQ-PCR). **Results:** *EZH2* was significantly downregulated in AML patients compared to controls ($p<0.001$). There was no significant difference in *EZH2* level when considering age, sex, bone marrow blasts count, cytogenetic studies, type or site of infection. Low *EZH2* expression was associated with higher mortality (31 patients, 68.9%). **Conclusions:** Low *EZH2* expression is prevalent in Egyptian AML patients subsequently; it is suggested to function as tumor suppressor gene rather than an oncogene. Moreover, *EZH2* downregulation is associated with resistance to chemotherapy and high mortality rate.

Keywords: *EZH2*- AML- gene expression- chemoresistance

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous disorder characterized by complex genomic alterations that contribute to disease biology and prognosis. Cytogenetic analysis is a powerful prognostic tool for risk stratification of AML patients. However, 50% of de novo AML patients have a normal karyotype. That's why molecular screening of genetic defects is crucial for the risk stratification and choice of the therapy in AML. The discovery of novel discriminative biomarkers remains of utmost importance to provide new outcomes definitions and therapeutic targets (DiNardo and Cortes, 2016; Moarii and Papaemmanuil, 2017).

There is a great advance in the significance of epigenetic dysregulation and its role in AML pathogenesis in the past decade. Post-translational histone modification by chromatin modifying enzymes is a key mechanism in gene transcription regulation. One of the chromatin modifying enzymes is the Polycomb Repressive Complex 2 (PRC2) which consists of multiple core and regulatory proteins. The histone methyl transferase Enhancer of zeste homolog 2 (*EZH2*) is the catalytic subunit of the PRC2. It is located at 7q36.1 and acts as an epigenetic writer that trimethylates histone H3 at lysine 27 (H3K27me3) which influences DNA transcription. Its primary function is to promote transcriptional repression. The expression of more than 200 tumor suppressors genes are repressed by *EZH2*. There are physical and functional links between the epigenetic silencing enzymes DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and *EZH2* histone methyltransferase (Tan et al., 2014; Rinke et al., 2020).

The activity of *EZH2* is vital for stem cell development, including hematopoiesis through its cellular proliferation function. It modulates H3K27me3 marks at promoters of tumor suppressor genes and alters pro-proliferation protein activity. *EZH2* dysregulation is strongly oncogenic by directing cells toward a cancer stem cell (CSC) state and by epigenetic silencing of tumor suppressor genes. This makes targeting *EZH2* an interesting and attractive therapeutic target against different cancers.

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Specific *EZH2* small molecules inhibitor may reduce CSC self-renewal thus eliminating the oncogenic addiction of tumor cells toward this protein. Interference with the tumor-suppressor role of wild-type *EZH2* must be avoided (Lund et al., 2014; Tan et al., 2014).

New findings suggest that *EZH2* may act as a transcriptional gene activator rather than a gene silencer. This means that *EZH2* may have a dual role as an oncogene and as tumor-suppressor gene. *EZH2* is overexpressed in many cancers such as cancer prostate, breast, and bladder. However, its role in hematological malignancies is complex.

Most studies suggest that *EZH2* loss rather than overexpression is more associated with MDS. *EZH2* mutations have been described in 10–13% of poor prognosis myelodysplasia-myeloproliferative neoplasms (MDS/MPN), 13% of myelofibrosis (MF), and 6% of MDS (Lund et al., 2014; Stomper et al., 2021). However, the prevalence and prognostic value of *EZH2* mutations in individuals with AML is still uncertain. In this context, this study aimed at determining the expression levels of *EZH2* gene in a cohort of Egyptian patients with acute myeloid leukemia.

Materials and Methods

The current study was conducted on 45 newly diagnosed acute myeloid leukemia patients aged 18 - 40 years admitted to Hematology Unit, Alexandria Main University Hospital. Forty healthy subjects of matched age and sex were included as a control group. AML-M3 was excluded from the study. Patients received induction regimen in the form of 3+7 protocol. According to standard criteria, complete remission (CR) was defined as less than 5% bone marrow blasts, an absolute neutrophil count of 1.0×10^9/L or more, a platelet count of 100×10^9/L or more, no blasts in the peripheral blood and no extramedullary leukemia. Therapeutic failures were classified as either refractory disease (RD) or early death, which was death before treatment. Relapse was defined as more than 5% BM blasts unrelated to recovery from the preceding course of chemotherapy or new extramedullary leukemia in patients with previously documented CR.

The study was approved by the Research Committee of Alexandria University. Written informed consent was obtained from all subjects before enrollment in the study.

All patients were subjected to routine investigations, and radiological assessment if needed. CBCs were performed on the automated cell counter ADVIA 2120 hematology system (Siemens Healthcare Diagnostics, USA). Bone marrow aspirate was done under aseptic technique according to the standard procedure. Immunophenotyping was analyzed using Becton Dickinson, FACS Calibur flow cytometer equipped with BD CellQuest Pro software (BD biosciences, San Jose, CA, USA). Cytogenetic analysis was carried out for AML patients only not controls.

EZH2 gene expression analysis

EZH2 gene expression was done using Real Time Quantitative PCR (RQ-PCR) of bone marrow aspirate mononuclear cells (BM-MNCs) using QIAamp® RNA blood Mini kit (Qiagen, USA). Relative quantitation of *EZH2* gene expression was normalized to the endogenous gene Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH). RQ-PCR for *EZH2* expression was performed by Rotor gene Q® real time PCR system (QIAGEN, USA) using The SensiFAST™ SYBR® No-ROX Kit (ThermoFisher, USA).

Relative quantitation was expressed by a comparative Ct method where the amount of target, normalized to an endogenous reference and relative to a calibrator, was given by: $2^{-\Delta\Delta Ct}$ (Huggett et al., 2005; Abo Elwafa et al., 2019).

Statistical analysis of the data

Data were analyzed using IBM SPSS software package version 23.0. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Significance of the obtained results was judged at the 5% level. Data were expressed as a minimum, maximum and median values. Qualitative data were compared using Chi-square test while quantitative data were compared using Mann Whitney test for 2 groups and Kruskal Wallis test for more than 2 groups. Correlation between *EZH2* and different variables were done using Spearman correlation. A receiver-operating characteristic curve (ROC) was generated. The area under the curve (AUC) and 95% CI was calculated when appropriate. P value <0.05 was considered significant.

Results

Characteristics of study participants

Clinical and laboratory characteristics of the study participants are summarized in Table1.

EZH2 expression in AML patients and controls

AML patients had statistically significant downregulation of *EZH2* expression compared to the control group as it ranged from 0.01 to 0.49 with a median of 0.12, while in control group it ranged from 0.64 to 3.15 with a median of 1.1 (p=<0.001*) (Table 1).

EZH2 expression and FAB subtypes and cytogenetics risk groups

The median values of *EZH2* expression in different FAB subtypes including M0, M1, M2, M4 and M5 were 0.49, 0.14, 0.26, 0.08, 0.11and 0.12 respectively. There was no statistical significant difference in *EZH2* expression among different FAB subtypes (p=0.317).

The median expression values of *EZH2* in cytogenetic risk groups; favorable, intermediate and high were 0.2, 0.11 and 0.8 respectively showing no significant association between *EZH2* expression and cytogenetic risk stratification system (p=0.441).

EZH2 expression and type of infection

The median value of *EZH2* concentration in patients with bacterial, fungal, combined bacterial viral and combined fungal viral infections were found to be 0.08, 0.05, 0.23 and 0.23 respectively. There was a statically significant association between *EZH2* concentration and

Table 1. Clinical and Laboratory Data of the Studied Subjects

Parameter	Cases N=45	Controls N=40	p- value
Age (years)			0.057
Median	33	29	
Min-max	19.0-40.0	19.0-40.0	
Sex			
Males	30 (66.7%)	22 (55%)	0.271
Females	15 (33.3%)	18 (45%)	
FAB subtype			
M0	1 (2.1 %)		
M1	4 (8.9 %)		
M2	4 (8.9 %)		
M4	4 (8.9 %)		
M5	32 (71.2 %)		
Chromosomal abnormality			
Normal karyotype	28 (62.2 %)		
Monosomy 7	3 (6.7%)		
t (9;22)	1(2.2%)		
t (8;21)	4 (8.8%)		
Complex karyotype	3 (6.7%)		
Cytogenetics risk groups			
Favorable risk	4 (8.9%)		
Intermediate	28 (62.1%)		
High	7 (15.6%)		
Hb (g/dl)			<0.001*
Median	7	13.9	
Min-max	4.0-11.0	12.0-16.0	
Platelets (x109/l)			<0.001*
Median	28	312	
Min-max	10.0-64.0	170.0-425.0	
WBCs (x109/l)			<0.001*
Median	87	6.6	
Min-max	10.0-300.0	4.0-9.7	
Bone marrow blasts (%)			
Median	70		
Min-max	25.0-97.0		
EZH2			
Median	0.12	1.1	<0.001*
Min-max	0.01-0.49	0.64-3.15	

p, p value for Mann Whitney test for comparing between the two studied group; *, Statistically significant at $p \leq 0.05$

type of infection (p=0.028) with combined infections showed the highest values (Table 2).

EZH2 expression and CT findings

Regards CT findings, 16 patients had GGO, 9 patients had consolidation, 18 patients had micronodules and 2 patients had clear chest. The median value of *EZH2* expression in patients with CT finding; GGO, consolidation, micronodules and clear chest were 0.24, 0.08, 0.08 and 0.13) respectively. There was no significant association between *EZH2* expression levels and CT findings (p=0.059).

EZH2 expression and response to treatment

Complete response was attained in 6 patients (13.3%), partial response in 5 patients (11.1%) and 6 patients were resistant to chemotherapy (13.3%). The highest median value of *EZH2* was 0.19 and was found in patients with partial response, while the lowest median value was 0.06 in relapsed and refractory patients. The mean value of *EZH2* in patients who received induction only and patients in complete remission was found to be 0.15 for both. Despite these results, they didn't reach the level of statistical significance and there was no significant

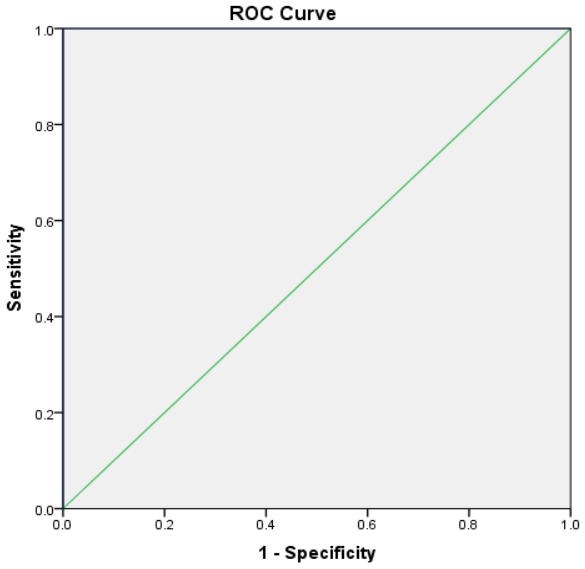

Figure 1. ROC Curve Analysis for EZH2 Expression Levels for Discriminating AML Patients from Controls

Table 2. Distribution of the Studied AML Patients According to Type of Infection and EZH2 Expression

EZH2	Bacterial (n=9)	Fungal (n=10)	Bacterial & viral (n=7)	Fungal & viral (n=19)	p value
Median	0.08	0.05	0.23	0.23	P=0.028*
Min-Max	0.03-0.19	0.03-0.22	0.04-0.44	0.01-0.49	

p, p value for Kruskal Wallis test; *p value significant at level<0.05

Table 3. Distribution of the Studied AML Patients According to Response to Treatment and EZH2 Expression

EZH2	Relapse (n=2)	Refractory (n=6)	Partial Response (n=5)	Induction Only (n=26)	CR (n=6)	p value
Median	0.06	0.06	0.19	0.15	0.15	p=0.370
Min-Max	0.03-0.08	0.04-0.44	0.04-0.49	0.01-0.49	0.03-0.32	

p, p value for Kruskal Wallis test; CR, Complete remission; *p value significant at level<0.05

Table 4. Distribution of the Studied AML Patients According to Survival and EZH2 Expression

EZH2	Alive (n=14)	Died (n=31)	p value
Median	0. 9	0.14	<0.001*
Min-Max	0.1-1.7	0.01-0.2	

p, p value for Mann Whitney test for comparing between the two studied group; *, Statistically significant at p ≤ 0.05

Table 5. Analysis of ROC Curve for EZH2 as a Predictor of AML

Cut off point of EZH2	AUC	p value	Sensitivity (%)	Specificity (%)	PPV (%)	VPV (%)
< 0.49	1	<0.001*	95	100	100	100

AUC, area under the curve; *p-value, significant at level<0.05; PPV, Positive Predictive Value; NPV, Negative Predictive Value

association between *EZH2* expression levels and patient response to treatment (p=0.370) (Table 3).

EZH2 expression and survival

There was a statistically significant association between lower *EZH2* expression levels and poor survival as the median value *EZH2* expression in alive patients was 0.9 while in patients who had died was 0.14. (p<0.001*) (Table 4).

EZH2 as a predictor of AML

The diagnostic performance of changes in *EZH2* expression levels was analyzed by the receiver operating characteristic curve (ROC) and the associated area under the curve (AUC). AUC values greater than 0.9 indicating excellent discriminating power, greater than 0.8 indicating a very good discriminative power and greater than 0.7 indicating a good discriminative power. Analysis of ROC curve revealed that *EZH2* expression at a cutoff level of <0.49 was a predictor of AML with 95.0% sensitivity and 100.0% specificity from healthy controls (Table 5, Figure 1).

Discussion

EZH2 expression levels were significantly lower in newly diagnosed AML patients compared to healthy control group with patients having a median expression level less than 0.49 were discriminated as AML (sensitivity: 95.0% and specificity: 100.0%) (Ntziachristos et al., 2016). To our knowledge, this is the first study to analyze the relative expression levels of *EZH2* in AML patients. Previous research on *EZH2* in AML revealed loss-of-function mutations of *EZH2* in about 1–2% of de novo AML patients. Inactivation of other PRC2 components e.g., loss-of-function mutations of SUZ12 and EED are equivalent to *EZH2* loss and can lead to reduced PRC2 histone methyltransferase activity in vitro. However, they occur at a lower frequency compared to *EZH2* mutations (Hu et al., 2018). This may denote that quantitative expression data give better results and perhaps reveals previously undiagnosed cases associated with atypical mutations that need more advanced techniques (Zhang et al., 2019).

Although a previous study deny an association between *EZH2* mutations in MDS and progression of MDS to AML, Sashida and Iwama, (2017) we determined low *EZH2* expression levels in one patient with AML secondary to MDS as well in 2 patients with myeloblastic crisis of chronic myeloid leukemia. However, further assessment of more cases is needed to confirm this finding.

There was a significant inverse correlation between *EZH2* expression levels and hemoglobin values in our AML cases. No significant correlation between *EZH2* expression and other CBC parameters, bone marrow blast count or liver and kidney function tests was found in the present study.

EZH2 is a downstream target of P38alpha/JNK pathway which belongs to MAPK family. P38 regulates p53 and *EZH2* through JNK. P38 negatively modulates p53 physiologically while it positively regulates p53 in cancer cell line due to signal transduction dysregulation in cancer cells. This may explain why JNK/*EZH2* signaling serves as a prosurvival mechanism in erythroblasts independent of EPO in physiological condition and is associated with anemia in leukemic cases (Rinke et al., 2020).

There was no significant association between *EZH2* and any cytogenetic risk or chromosomal abnormality including normal karyotype (28 cases), monosomy 7 (3 cases) and t 8,21 (4 cases). There is previous evidence for contribution of *EZH2* mutations in the pathogenesis of AML with t(8;21) in children (Tan et al., 2014). However, the incidence of such mutation still needs assessment in a larger pediatric cohort (Sashida and Iwama, 2017).

EZH2 was low in AML patients with infection. There was no significant relation between the site or the type of infection whether bacterial or fungal and *EZH2* expression level in our patients. Also the grade of CT lung changes were not related to *EZH2* level. This means that low expression level of *EZH2* in leukemic patients is not protective against infection as expected (Castelli et al., 2019). Perhaps chemotherapy induced bone marrow suppression in leukemic patients suppresses the activation of inflammatory cells and release of cytokines in the circulation and infection sites, and inhibits replenishing of circulating neutrophil and monocyte pool from the bone marrow as occurs in normal persons (Ntziachristos et al., 2016; Hu et al., 2018).

EZH2 was found to be associated with poor response to therapy in our study. Complete response was achieved only in 6 patients. The mortality rate was 68.9% (31 patients). Previous studies reported that secondary AML patients who carry *EZH2* mutations have a worse prognosis compared to patients with wild-type *EZH2* (Rinke et al., 2020). This cannot be explained by loss of chromosome 7 or 7q deletion in AML which is associated with an adverse prognosis, in part as a result of haploinsufficiency or loss of *EZH2* (Inaba et al., 2018). This denotes that *EZH2* is a driver rather than a bystander (A, 2019).

EZH2 dysregulation is strongly oncogenic by

directing cells toward a cancer stem cell (CSC) state and by epigenetic silencing of tumor suppressor genes. This makes targeting *EZH2* an interesting attractive therapeutic target against different cancers including acute leukemia. (Wen et al., 2017)

Specific *EZH2* small molecules inhibitor may reduce CSC self-renewal thus eliminating the oncogenic addiction of tumor cells toward this protein. However, interference with the tumor-suppressor role of wild-type *EZH2* must be avoided (Tan et al., 2014)و

In conclusion, downregulated expression of *EZH2* is prevalent in Egyptian acute myeloid leukemia patients. Subsequently, it is suggested to function as a tumor suppressor gene rather than an oncogene. *EZH2* low gene expression is inversely correlated with Hb level while it has no relation with age, sex, blasts percent in bone marrow, conventional cytogenetics and, type or site of infection. *EZH2* downregulation is associated with resistance to chemotherapy and high mortality rate.

Author Contribution Statement

All authors have contributed to the current work. Reham A. Abo Elwafa performed laboratory investigations of the participants' samples. Nahla A. M. Hamed and Ashraf El Ghandour contributed substantially to the conception and design of the study. Nahla A. M. Hamed, Ashraf El Ghandour, Omar Ghallab and Mohamed Rezk carried out the acquisition, analysis and interpretation of data and patient management. Mohamed Rezk drafted the manuscript, and Reham A. Abo Elwafa performed the critical revision and corrections of the manuscript. All the authors approved the final version submitted for publication and take responsibility for the statements made in the published article.

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Declarations

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Faculty of Medicine, Alexandria University. All methods in the current research were carried out in accordance with relevant guidelines and regulations. An informed written consent was obtained from all patients before enrollment in the study.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

Neither of the authors discloses any potential or actual conflict of interest. No financial or nonfinancial benefits have been or will be received from any party related directly or indirectly to the subject of this article.

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