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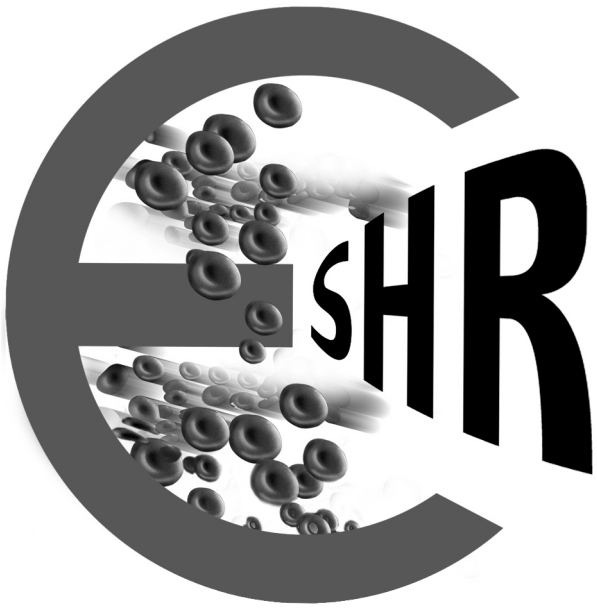

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Angiopoitin-2/Angiopoitin-1 Ratio in Acute Leukemia Patients with Febrile Neutropenia

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ABSTRACT

Background: Sepsis is a major cause of death in acute leukemia patients presented with febrile neutropenia (FN) despite the current use of modern antibiotics and resuscitation therapies. Septic shock is known to be one of the worst complications of sepsis. It frequently precedes multi-organ dysfunction syndrome and death. Septic shock Biomarkers can have an important place in predicting the severity of sepsis. Breakdown of the endothelial barrier is the hallmark of septic shock. Proteins that physiologically regulate endothelial barrier integrity as Angiopoitin-2 (Ang-2), Angiopoitin-1 (Ang-1) and vascular endothelial growth factor (VEGF) are thought to be candidate biomarkers of septic shock development.

Objectives: The aim of the present work was to evaluate the value of Ang-2/Ang-1 ratio as an early marker for prediction of occurrence of septic shock in acute leukemia patients with FN.

Patients and Methods: A prospective study was performed on inpatients with new onset FN from the Hematology Unit, Main University Hospital and Medical Research Institute. Levels of Ang-1 and Ang-2 were measured after the onset of neutropenic fever. Ang-2/Ang-1 ratio was calculated. Patients were categorized based on the occurrence of septic shock within 28 days as an outcome into two groups: Non-complicated FN patients (group A) and complicated FN patients (group B).

Results: High Ang-2/Ang-1 ratio was associated with septic shock development in acute leukemia patients (p= <0.001). Higher Ang-2/Ang-1 ratio was associated with lower Multinational Association for Supportive Care in Cancer (MASCC) score (p=<0.001) and higher CRP levels (p=0.011). Low Ang-1 and high Ang-2 levels were associated with higher mortality (p=0.035 & p=0.003).

Conclusions: High Ang-2 level and Ang-2/Ang-1 ratio are predictive of septic shock development in acute leukemia patients with febrile neutropenia. High Ang-2 and low Ang-1 levels in febrile neutropenic patients are markers of higher mortality.

Key Words: *Angiopoitin-1 – Angiopoitin-2 – Febrile neutropenia.*

INTRODUCTION

Febrile neutropenia (FN) frequently complicates chemotherapy in cancer patients, despite the intensive efforts in infection prevention. Myelosuppression, principally neutropenia, remains among the major toxicities of systemic cancer chemotherapy, particularly among patients with hematologic malignancies for which chemotherapeutic protocols are myelosuppressive by design. Septic shock can emerge as a fulminant complication, despite intensive treatment comprising broad-spectrum antibiotics and best supportive care **[1,2]**.

The septic response is an immensely complex cascade of events that involves inflammatory and anti-inflammatory processes, humoral and cellular reactions and circulatory abnormalities **[3]**. The establishment of sepsis diagnosis and evaluation of its severity is overwhelmed by the high variability and non-specificity of the signs and symptoms of sepsis **[4]**. Septic shock evolution is one of the worst complications of sepsis, frequently ending by multi-organ dysfunction syndrome and death. The Multinational Association for Supportive Care in Cancer (MASCC) prognostic model was introduced to help in predicting the clinical outcome of patients with FN **[5-7]**.

The fitness of inflammatory biomarkers in identifying patients with febrile neutropenia at high-risk of sepsis evolution continues to be explored; among them procalcitonin, C reactive protein, and others which are extensively studied **[8-10]**. The endothelial barrier integrity and the cellular and molecular mechanisms of its regu-

lation have been outlined where vascular endothelial growth factor-A (VEGF-A), Angiopoitin-1 (Ang-1), and Angiopoitin-2 (Ang-2) play pivotal roles in the physiological control of angiogenesis **[11]**.

Ang-1 has been shown to be a principal activator of the tyrosine kinase receptor Tie-2 (Tek) resulting in a downstream activation of the phosphatidylinositol 3'-kinase/Akt survival pathway, thereby promoting the survival of endothelial cells. Ang-2 is the typical antagonist of Ang-1 and hence, prevents Tie-2 activation. This action leads to vessel destabilization, an essential step in angiogenesis initiation by VEGF-A. Balanced and consecutive expression of angiopoietins and VEGF is needed for successful angiogenesis **[12,13]**.

Laboratory markers that can reliably early predict septic shock occurrence are deeply needed besides the clinical MASCC model. This need motorized many research efforts. In this regard, Ang-1 and Ang-2 were evaluated in the present work as possible early predictive markers for the occurrence of septic shock and mortality among acute leukemia patients with severe febrile neutropenia.

PATIENTS AND METHODS

Patients:

The present study was conducted on 36 patients diagnosed as acute leukemia with severe febrile neutropenia (FN) following induction or consolidation chemotherapy at the Hematology Unit, main University Hospital and Hematology department, Medical Research Institute, Alexandria University.

Inclusion criteria were: (1) Fever (T ≥38°C) for more than one-hour observation period or a single reading ≥38.3°C. (2) Chemotherapy-induced severe neutropenia (absolute neutrophilic count <0.5 x 10^9/L at the time of fever onset, and (3) Persistence of severe neutropenia until the time of blood sample collection. Exclusion criteria were: (1) Death during the study due to any condition other than septic shock, and (2) Chronic liver or kidney disease.

Patients were followed-up for a period of 28 days from the onset of FN. Sepsis diagnosis was established in the presence of two or more of the following: (1) Temperature ≥38°C for more than one hour. (2) Heart rate >90 beats/minute. (3) Respiratory rate >20 breaths/minute or PaCO2 <32mmHg. (4) A microbiologically proven or clinically evident source of infection. A diagnosis of septic shock was established when sepsis induced hypotension (systolic arterial pressure <90mmHg or dropping >40 mmHg from baseline) persisted despite adequate volume resuscitation. During the follow-up of patients, 10 patients died with a median survival time of 25.6 days (Fig. 1).

All subjects enrolled in this study signed a written informed consent before participation. Approval of the ethical committee of the faculty of medicine, Alexandria University was obtained. The procedures followed were according to the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Methods:

Acute leukemia patients with FN were stratified by calculation of the MASCC score at the onset of FN. Samples were withdrawn with the next routine sample collection requested for them. As per local protocol, the first routine sample collection requested for all admitted FN patients includes: Complete blood count (CBC), C reactive protein (CRP), liver function tests, kidney function tests and electrolytes. Blood and urine cultures were obtained and broad-spectrum antibiotics were initiated. All patients underwent chest radiography. Other imaging studies were performed when judged necessary by the attending physician.

Serum levels of Ang-1 and Ang-2 were measured by an individual blinded to patient outcomes using commercial enzyme linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Serum was stored at –80°C until analysis. Serum levels were recorded and Ang-2/Ang-1 ratio was calculated.

Statistical analysis:

Statistical analyses were performed using the software package SPSS/Win version 21 (SPSS Inc., Chicago, IL, USA). Qualitative data were presented as frequency and percentage. Chi square test was used to compare groups. Quantitative data were presented as median or

mean and standard deviation. For comparison between groups; *t*-test and Mann-Whitney test were used according the type of data. Correlation tests were performed using Spearman's rank test. A receiver operating characteristic (ROC) curve was used for determining cutoff values of Ang-2, Ang-1 and Ang-2/Ang-1 ratio as diagnostic parameters of septic shock development. Odds ratio was calculated for univariate risk estimation with 95% confidence interval. Kaplan-Meier test was used for overall survival analysis and the statistical significance of differences among curves was determined by log-rank test. A *p*-value equal to or less than 0.05 was considered statistically significant.

RESULTS

The present study was conducted on 20 males and 16 female patients with ages ranging from 18.0-72.0 years and a median of 38 years. Twenty-nine patients were diagnosed as AML and 7 patients had B-ALL. Seventeen out of the thirty-six patients developed septic shock.

Regarding inflammatory markers of sepsis, CRP was higher in patients who developed septic shock (mean level 89.35±43.92mg/dl) than patients with noncomplicated febrile neutropenia (mean level 53.32±22.37mg/dl) (p =0.006). Patients who developed septic shock had a lower MASCC score at onset of fever (ranging from 8 to 18 with a median of 16) compared to patients with non-complicated febrile neutropenia (ranging from 17 to 23 with a median of 21) (p-value=0.001).

Ang-2 serum level was higher in patients who developed septic shock compared to patients with non-complicated sepsis (p=0.034). Ang-1 levels were significantly lower in patients that developed septic shock compared to patients with noncomplicated FN (p=0.009). Therefore, the Ang2/Ang-1 ratio was higher in the septic shock-outcome group compared to patients with non-complicated FN (p<0.001; Table 1).

Estimation of the diagnostic accuracy of Receiver operating characteristics (ROC) curve showed that Ang-1 level <60ng/ml had 82.3% sensitivity and 68.4% specificity for the development of septic shock. Ang-2 level ≥600ng/ml had 84.21% specificity. Ang-2/ Ang-1 ratio >10 had 70.59% sensitivity and 89.47% specificity for the development of septic shock (Fig. 1).

The correlation between Ang-2, Ang-1 levels and different parameters was evaluated. Ang-2 level was positively correlated with CRP level (r=0.515, p<0.001) while Ang-1 was not correlated with CRP level. As regards MASCC score, Ang-2 showed negative correlation with MASCC score of the patients (r=–0.385,…p=0.020) while Ang-1 was positively correlated with MASCC score of the patients (r=0.399,…p= 0.016). Furthermore, higher Ang2/Ang-1 ratio was correlated with lower MASCC score (r=–0.509….p<0.001) and higher CRP levels (r=0.409….p=0.011). There were no significant correlations between Ang-2, Ang-1, or Ang-2/Ang-1 ratio on one side and either of complete blood count parameters, urea, creatinine, sodium, potassium, total bilirubin, direct bilirubin, albumin, ALT, AST, prothrombin time or activated partial thromboplastin time on the other side (Table 2).

The risk of development of septic shock was evaluated as regards the studied parameters. Significantly high risk was associated with the presence of comorbidity (p=0.046), hypokalemia (p=0.030), elevated CRP (p=0.013), high MASCC score (p=0.028), high Ang-2 (p= 0.046), and high Ang-2/Ang-1 ratio (p=0.007) (Table 3).

As regards mortality during the 28 days follow-up, better survival rates were observed in patients with Ang-1 level ≥60ng/ml (p= 0.035), and Ang-2 level <600ng/ml (p=0.003). However, Ang-2/Ang-1 ratio <10 was not significantly associated with better survival (p= 0.086) (Figs. 2-4).

Table (1): Angiopoitin-2, Angiopoitin-1, and Angiopoitin-2/angiopoitin-1 ratio in 36 acute leukemia patients with febrile neutropenia as regards development of septic shock.

Parameter	Septic shock		
	No (n=19)	Yes (n=17)	p**
Angiopoitin-2: pg/ml	554.79± 378.04*	970.59± 681.58	0.034
Angiopoitin-1: pg/ml	255.58± 304.94	55.65± 28.02	0.009
Ang-2/Ang-1	5.78± 5.05	17.55± 11.39	<0.001

* Mean ± SD.
** Statistically significant: p≤0.05.

Table (2): Correlation between Angiopoiotin levels, Angiopoiotin-2/Angiopoitin-1 ratio and clinical and laboratory parameters in 36 acute leukemia patients in relation to septic shock.

	Angiopoitin-2						Angiopoitin-1						Ang-2/Ang-1					
	All cases		No septic shock		Septic shock		All cases		No septic shock		Septic shock		All cases		No septic shock		Septic shock	
	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*
Hemoglobin level	–0.251	0.140	–0.029	0.906	–0.485	0.048	0.031	0.860	0.195	0.423	–0.213	0.412	–0.316	0.061	–0.149	0.543	–0.478	0.053
WBCs count	0.021	0.905	0.092	0.708	–0.119	0.648	–0.387	**0.020**	–0.280	0.245	–0.680	**0.003**	0.253	0.136	0.311	0.195	0.310	0.227
Neutrophils count	0.017	0.923	0.130	0.595	–0.062	0.813	–0.174	0.310	–0.134	0.584	–0.292	0.256	0.136	0.428	0.224	0.356	0.180	0.489
Platelets count	–0.029	0.865	0.204	0.403	0.121	0.643	0.118	0.494	0.047	0.850	0.052	0.844	–0.136	0.428	–0.011	0.963	0.031	0.907
Urea level	0.169	0.326	0.188	0.440	0.263	0.308	0.089	0.607	0.247	0.307	0.123	0.638	0.078	0.649	–0.144	0.555	0.138	0.598
Creatinine level	0.067	0.698	0.189	0.437	0.199	0.445	–0.062	0.720	–0.240	0.323	0.031	0.906	0.100	0.561	0.193	0.429	0.131	0.618
Na level	–0.145	0.399	–0.100	0.684	–0.072	0.783	0.15	0.383	–0.040	0.870	0.361	0.155	–0.143	0.405	–0.014	0.955	–0.333	0.192
K level	0.082	0.633	0.597	**0.007**	0.039	0.881	0.019	0.913	–0.400	0.090	0.009	0.972	0.051	0.768	0.520	**0.023**	0.117	0.655
CRP level	0.515	**0.001**	0.471	**0.042**	0.404	0.108	–0.091	0.597	0.081	0.742	0.167	0.522	0.418	**0.011**	0.072	0.769	0.451	0.069
MASCC score	–0.385	**0.020**	–0.130	0.596	–0.235	0.364	0.399	**0.016**	0.371	0.118	–0.206	0.428	0.053	0.761	0.283	0.240	–0.152	0.560

Significant values were typed bold.

Table (3): Univariate logistic regression analysis of clinical and laboratory markers of sepsis in 36 acute leukemia patients as regards the development of septic shock.

Parameter	Odds ratio	*p*	95% C.I.	
			Lower	Upper
Gender	1.222	0.765	0.327	4.565
Age	1.001	0.957	0.962	1.042
Type of leukemia	1.150	0.603	0.679	1.948
Comorbidity	9.819	0.046*	1.039	92.777
Hyponatremia	0.982	0.849	0.810	1.190
Hypokalemia	0.171	0.030*	0.035	0.841
Elevated CRP	1.039	0.013*	1.008	1.071
High ESR	1.007	0.652	0.976	1.040
Positive blood culture	4.000	0.053	0.981	16.311
High MASCC score	0.234	0.028*	0.064	0.852
High Angiopoitin-2	1.002	0.046*	1.000	1.0003
Low Angiopoitin-1	0.986	0.110	0.969	1.003
High Ang-2/Ang-1 Ratio	1.269	0.007*	1.069	1.507

C.I.: Confidence interval. * Significant.

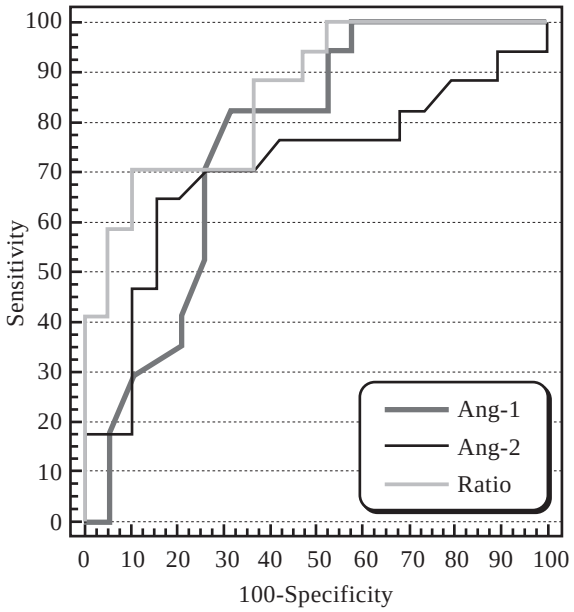

Fig. (1): Receiver operating characteristics curve of the development of septic shock in acute leukemia patients with febrile neutropenia as regards Ang-1 level <60pg/ml (specificity=68.4%), Ang-2 level >600pg/ml (specificity =84.2%), and Ang-2/Ang-1 ratio >10 (specificity=85.7%).

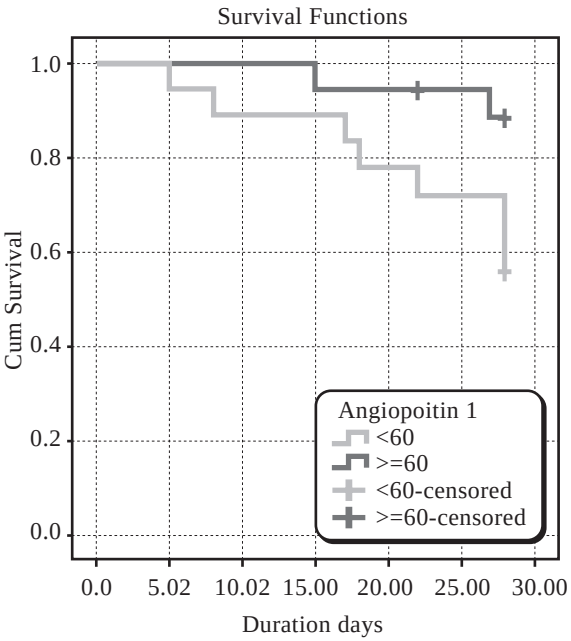

Fig. (2): Kaplan-Meier overall survival curve of acute leukemia patients with febrile neutropenia in septic shock according to Angiopoitin-1 level (p=0.035).

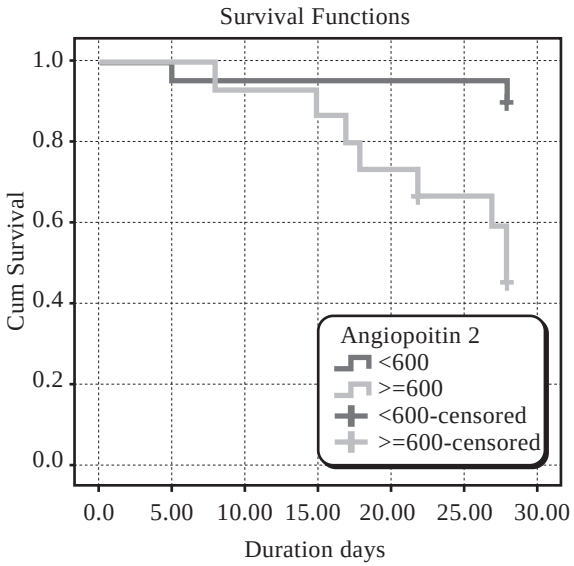

Fig. (3): Kaplan-Meier overall survival curve of acute leukemia patients with febrile neutropenia in septic shock according to Angiopoitin-2 level (p=0.003).

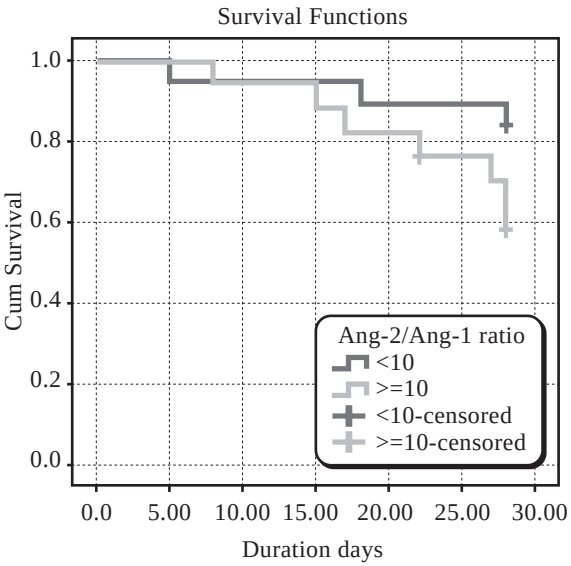

Fig. (4): Kaplan-Meier overall survival curve of acute leukemia patients with febrile neutropenia in septic shock according to Angiopoitin-2 / Angiopoitin-1 ratio (p=0.089).

DISCUSSION

Septic shock development is one of the worst complications of sepsis. Classically, it leads to multi-organ dysfunction syndrome and ends by death. Febrile neutropenic patients with hematologic malignancies present a high risk of septic shock **[14]**.

The most accepted risk stratification tool is the MASCC score. However, this score offers limited information for patients categorized as

high-risk (MASCC ≤21), the stage most acute leukemia patients with febrile neutropenia fit in. Accordingly, management of these patients remains a challenging situation for which the availability of informative biomarkers would provide better categorization and management **[15]**.

The aim of our study was to evaluate the role of Ang-1 and Ang-2 serum levels and Ang-2/Ang-1 ratio as endothelial barrier-stabilizing factors and as biomarkers of septic shock development in patients with acute leukemia suffering chemotherapy-induced febrile neutropenia.

Serum levels of Ang-2 were higher in patients who developed septic shock (p=0.009); besides higher Ang-2 was associated with higher CRP levels and lower MASCC score (p<0.001, p=0.020, respectively), while Ang-1 was lower in patients who suffered septic shock (p=0.034).

Ang-2/Ang-1 ratio was associated with septic shock development in our patients. Notably, it predicted septic shock development better than CRP, Ang-1, Ang-2, and MASCC scores.

In accordance with our results, Alves et al. **[16]** reported that acute leukemia patients who developed septic shock presented higher levels of Ang-2 and Ang2/Ang-1 ratio at the onset of fever compared to subjects with non–complicated sepsis (n=31). These levels correlated with sepsis severity scores.

In addition, Fiusa et al. **[17]** aimed to validate the use of these biomarkers in a large cohort of cancer patients having chemotherapy-associated FN. Ang-2 concentrations were increased in patients with septic shock compared to non-complicated FN, whereas an inverse finding was observed for Ang-1, resulting in a higher Ang-2/Ang-1 ratio in patients with septic shock. Moreover, multivariate analysis confirmed the value of Ang-2/Ang-1 ratio as an independent determinant of septic shock development and mortality at 28 days.

Our results showed that an Ang-2/Ang-1 ratio of 10 was the optimal cut-off value identified by the ROC procedure with a sensitivity of 70.59% and a specificity of 85.7% for the development of septic shock, while the study of Fiusa et al. **[17]** defined Ang-2/Ang-1 ratio of 5 as an optimal cut-off value which can be attributed to different sample size.

The role of angiopoietins as prognostic markers in sepsis has been evaluated by Ricciuto et al. **[18]** and Mankhambo et al. **[19]**. Ricciuto et al. **[18]** demonstrated that high plasma Ang-2 levels correlated strongly with morbidity during the course of ICU admission. They extrapolated that Ang-2 has a pathogenic role in sepsis and contributes to organ dysfunction, likely through endothelial activation and subsequent vascular leak, which leads to circulatory and renal compromise. In addition, they reported higher serially measured levels of Ang-2 in non-survivors relative to survivors (p=0.022). However, they did not report any association between admission levels of Ang-2 and 28 day mortality (p= 0.42).

Prior studies have reported an association between admission levels of Ang-2 and mortality in sepsis **[20,21]**. In agreement with this, high Ang-2 and low Ang-1 levels were found to be associated with higher mortality in the present study.

In conclusion, high Ang-2 level and Ang-2/Ang-1 ratio are predictive of septic shock development in acute leukemia patients with febrile neutropenia. High Ang-2 and low Ang-1 levels in febrile neutropenic patients are markers of higher mortality.

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Leukemic Cell/Microenvironment Cross Talk in Acute Myeloid Leukemia: Effect on CXCL12

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ABSTRACT

Background: Mesenchymal stromal cells (MSCs) secrete an array of signaling molecules; among these molecules is the chemokine CXCL12 whose fine-tuned concentration in bone marrow niches is important for maintaining stem cell characteristics. In acute myeloid leukemia, leukemic stem cells occupy the stem cell niche and react with the stromal cells at the expense of normal hematopoietic stem cells resulting in aberrant malignant niches. This crosstalk influences leukemogenic process and response to therapy.

Objectives: This work aims to demonstrate the effect of acute myeloid leukemia cells on mesenchymal stromal cells' production of CXCL12.

Subjects and Methods: MSCs were cultured from 20 AML bone marrow aspirates and 10 non neoplastic bone marrows as a control group. CXCL12 gene expression in cultured mesenchymal cell was evaluated using real time PCR and determination of the level of CXCL12 protein in BM plasma was performed using ELISA.

Results: CXCL12 gene expression was elevated in MSCs in AML cases in comparison to the control group; in addition, the level of CXCL12 in BM plasma was significantly higher in AML cases in comparison to the control group.

Conclusion: These findings suggest that MSCs is an active component in AML microenvironment through increasing the expression and levels of CXCL12 chemokine, which plays an important role in leukomogenesis.

Key Words: *Acute myeloid leukemia – Bone marrow microenvironment – CXCL12 – Mesenchymal stromal cells.*

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by the accumulation of clonal undifferentiated leukemic cells in the bone marrow (BM) and/or peripheral blood. Leukemic cells exhibit uncontrolled proliferation, blockade of differentiation capabilities, and reduced apoptotic ability. Despite initial responsiveness to standard chemotherapy, the prognosis of AML patients remains poor, considering the high relapse rates **[1-4]**.

Bone marrow niches, defined as the cellular and molecular micro-environmental components that regulate stem cell functions, control the balance between quiescence, self-renewal, and differentiation both in normal and malignant conditions. In acute myeloid leukemia, crosstalk between stromal cells and the leukemic stem cells (LSCs) within the bone marrow niches affects the leukemogenesis initiation and progression and influences the prognosis. This crosstalk provides a suitable environment through which LSCs acquire a drug-resistant phenotype and thus can evade the apoptotic signaling induced via chemotherapeutic drugs resulting in drug resistance or relapse status **[1,5-9]**. Several critical signaling pathways are involved in these interactions, among them is the CXCL12-CXCR4 signaling pathway **[10-12]**.

Stromal cell-derived factor-1 (SDF-1/CXCL12) is a CXC chemokine, which maintains the quiescent hematopoietic stem cell (HSC) pool via CXCL12-CXCR4 signaling pathway in bone marrow niches **[12,13]**. It is continuously secreted through marrow stromal cells. The vascular bone marrow niche includes heterogeneous stromal cells that are characterized by high CXCL12 expression, among these cells: CXCL12-abundant reticular (CAR) cells **[8,12]**, leptin receptor+ stromal cells and nestin-GFP+ stromal cells **[8,14]**. CAR cells, as mesenchymal progenitors that can differentiate into both

adipogenic and osteogenic cells in vitro, are the major source of CXCL12 in the bone marrow. Loss of CAR cells is associated with marked reduction in CXCL12 levels in the bone marrow **[11,15]**. CAR cells are in direct contact with HSCs and are important components of HSCs niches through which HSCs maintain their self-renewal capabilities **[12,15,16]**.

Association of CXCL12/CXCR4 with AML pathogenesis was indicated in several studies in which levels of CXCR4 expression on CD34+ cells were found as a negative prognostic indicator of the overall survival and disease-free survival **[17,18]**. The interactions between the LSCs and the bone marrow stromal cytokines/ chemokines and adhesion molecules have been claimed to be the cause of drug resistance and disease relapse in AML. Several studies reported that stromal elements protect AML from chemotherapy-induced apoptosis; CXCL12-CXCR4 pathway has been recognized as a critical mediator of this protection **[19-21]**. Moreover, CXCL12, through binding and activation of its receptor CXCR4 on leukemic cells, facilitates leukemia cell trafficking and homing in the bone marrow, and maintains leukemic cells in close proximity to the stromal microenvironment that continuously sends anti-apoptotic and pro-survival signals such as activation of the PI3K (phosphoinositide 3-kinase)/AKT and MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) axes **[22-24]**.

A better understanding of the interactions between LSCs and bone marrow stromal cells is required. Furthermore, focusing on stromal cells gene expression patterns that contribute to LSCs maintenance and quiescence may provide new therapeutic modalities to overcome drug resistance of current chemotherapeutic agents. This could be done through targeting these important interactions and disturbing the protective effect of the stromal cells on the LSCs **[25-27]**.

The aim of this work is to demonstrate the effect of acute myeloid leukemia cells on mesenchymal stromal cells production of CXCL12 through the evaluation CXCL12 gene expression in cultured mesenchymal cell using real-time PCR and determination of the level of CXCL12 protein in BM plasma using ELISA.

SUBJECTS AND METHODS

Subjects:

Twenty de novo AML cases were included in this study. Patients were diagnosed and selected among cases referred to Al-Kasr Al-Ainy Hospital, Faculty of Medicine, Cairo University. Samples were taken at time of initial diagnosis, before induction therapy. Diagnosis of AML was based on standard morphology, cytochemistry and immuno-phenotyping of leukemic blast cells. Patients' clinical and laboratory information were reviewed from their medical records. Ten control cases were included in this study; they were patients undergoing bone marrow aspiration for other indications than malignancy e.g. before splenectomy for hypersplenism or immune thrombocytopenia. An informed written consent was obtained from each patient or parents/guardians before enrollment. The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in human patients. The ethical committee of Clinical and Chemical Pathology department, Faculty of Medicine, Cairo University approved the study. Patients' characteristics are summarized in Table (1).

Table (1): Clinical and laboratory characteristics of the studied groups.

Parameter	AML group N=20	Control group N=10
Age (years):		
• Median (range)	39.5 (10-62)	31.5 (20-56)
Gender:		
• Male: N (%)	10 (50%)	3 (30%)
• Female: N (%)	10 (50%)	7 (70%)
TLC (10^9/L):		
• Median (range)	70 (34 -163)	6 (4-8.5)
Blast percentage in bone marrow:		
• Median (range)	74.5 (34-85)	–
FAB classification: N (%)		
• M2	2 (10%)	
• M3	6 (30%)	
• M4	7 (35%)	
• M5	4 (20%)	
• M6	1 (5%)	

Methods:

Sample collection:

1-2 ml heparinized bone marrow samples were collected from the posterior iliac spine of

patients and controls; samples were processed within 24 hours of collection for mononuclear cell separation and cell culture. One ml citrated bone marrow sample was withdrawn for CXCL12 detection by ELISA.

Separation of human MNCs from bone marrow:

Heparinized marrow samples were diluted with double volume of RPMI with Lglutamine (Lonza, Cologne, Germany). Mononuclear cells were separated by Ficoll hypaque (Biowest, France). Cells were re-suspended in 2ml complete culture medium (CCM); Dulbecco's modified Eagle medium (DMEM) with lg/l glucose (Lonza, Cologne, Germany), 10% fetal bovine serum (FBS) (Lonza, Cologne, Germany), 1% L-Glutamine (Biowest, France) and 2% antibiotic-antimycotic (penicillin, streptomycin and amphotericin B) (Gibco, Eggenstein, Germany). Then the cells were counted **[28]**.

Culture and expansion of human MSCs:

The cells were plated in 25cm^2 tissue culture flask containing 5ml CCM with a density of 1.2X10^6 cells/cm^2. The flasks were incubated at 37ºC in 5% humidified CO2. After 48 hours, non-adherent cells were discarded. Five ml of fresh CCM was added to the flask and the flasks returned to the incubator. The cells inside the flask were examined every other day under inverted microscope. Every third day the medium was discarded, the cells rinsed with RPMI and fed with a fresh 5ml CCM until 80% confluency was reached. MSCs were characterized and identified by their plastic adherence and by their spindle fibroblast like morphology under the inverted microscope **[29]**.

MSC colonies were washed once with RPMI and harvested using Trypsin (Thermo Fisher Scientific Inc). Cells were cultured for three additional passages, tested for viability using trypan blue and cryopreserved using DMSO (Thermo Fisher Scientific Inc) and placed in cryo tubes at –80ºC for future experiments **[30]**.

Detection of CXCL12 gene by real time RT-PCR:

Thawing of cryopreserved cells:

Cells were rapidly thawed in water bath at 37°C over 2min. and transferred to a 50ml sterile falcon tube. Thawing media, HBSS1X (18ml, Thermo Fisher Scientific Inc) and FBS (2ml), was added after filtration (10x amount of cryopreserved cell suspension) without delay by drop wise addition **[21]**. Cells were centrifuged at 1500rpm for 5min at 4ºC, supernatant was removed and cells were re-suspended in remaining solution. Ten ml iced cold medium were added and viability determined. Cells were spun down at 1200rpm for 5min at 4ºC and the cell pellet used for RNA extraction.

CXCL12 gene expression by real time RT-PCR:

RNA extraction from the cultured MSC was done using QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany). cDNA was formed using reverse transcription kit (Applied Biosystem, USA). CXCL12 was detected through using Taqman gene expression assay for CXCL12 and β-actin as a house keeping gene (Applied Biosystem, USA) using Real time 7300 (Applied Biosystem, USA).

Data were presented as the comparative threshold cycle (Ct); the numerical value of the CT is inversely related to the gene expression in the reaction (i.e., the lower the CT, the greater the expression of the gene). Each sample was normalized with the housekeeping gene (β-actin) using the formula Δ Ct = Ct of CXCL12-Ct of β-actin. Ct was used to determine the gene expression relative to a normal control (calibrator). Transcription level of CXCL12 gene was represented by the following formula 2 to the power of –ΔΔCt as fold change from the control group Therefore; a 2-ΔΔCT value of more than 1 is considered as high gene expression in comparison to the control and a value of less than 1 is considered low expression of the gene **[31]**.

CXCL12 protein level in BM plasma using ELISA technique:

CXCL12 protein levels were detected in bone marrow plasma using ELISA technique (Wuhan USCN Business Co., Ltd, USA), Reading of samples was done using standard curve. Detection range of the kit was 0.156-10ng/mL.

Statistical analysis:

Data was analyzed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Numerical data was expressed as median and range. Qualitative data was expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For not normally distributed quanti-

tative data, comparison between two groups was done using Mann-Whitney test (non-parametric *t*-test). Spearman-rho method was used to test correlation between numerical variables. All tests were two-tailed. A *p*-value <0.05 was considered significant.

RESULTS

CXCL12 gene expression in cultured MSCs in AML patients:

The expression of CXCL12 in cultured MSCs of AML cases was elevated in comparison to the control group (median fold change: 71.35, IQ range: 8.83-437.24). The highest expression levels of CXCL12 were detected among M4 patients, followed by M3 then the M5 subtype (Table 2).

There was no statistically significant correlation between gene expression levels of CXCL12 in MSCs and age of the patients (*r*= 0.052, *p*-value=0.844), TLC (*r*=–0.196, *p*-value =0.450) nor the blast percentage in the BM of AML group (*r*=–0.149, *p*-value=0.569).

There was no significant correlation between expression of CXCL12 gene in MSCs in BM using RT-PCR and the level of CXCL12 protein in BM plasma (*r*=0.081, *p*-value 0.756).

CXCL12 level in BM plasma:

The level of CXCL12 in BM plasma was significantly higher in AML de novo cases than in the control group (*p*-value <0.001). The level of CXCL12 in BM plasma of AML cases ranged from 0.6 to 10.8ng/ml (median: 6.9ng/ml). While for the control group, it ranged between 0.1 and 1.2ng/ml (median: 0.7ng/ml). As regards the level of plasma CXCL12 in different AML FAB subtypes: The median level of CXCL12 was highest among M3 cases followed by M4 cases. The least value was observed in the M5 subtype (Table 2).

No statistically significant correlations were found between the level of CXCL12 and either the age of the AML patients (*r*=0.149, *p*-value =0.532), TLC (*r*=0.19, *p*-value=0.939), or the blast counts in the bone marrow (*r*=0.092, *p*-value=0.699).

No statistically significant difference in the level of CXCL12 between males and females in AML group (*p*-value=0.393) as in males the levels ranged between 0.6 and 10.8ng/ml with median value of 5.9ng/ml, while in females levels ranged between 0.8 and 10.8ng/ml with median value of 8.9ng/ml.

Table (2): CXCL12 gene expression level in cultured MSCs and CXCL12 BM plasma level among AML subtypes.

Parameter	CXCL12 gene expression ($2^{-\Delta\Delta Ct}$) in cultured MSCs*	CXCL12 in BM plasma ng/ml*
FAB subgroup:		
M3 (No=6)	180 (36-507)	10.6 (0.8-10.8)
M4 (No=7)	389 (21-612)	5.3 (0.6-10.8)
M5 (No=4)	99 (50-184)	3.1 (0.9-10.8)

* Median (range).

DISCUSSION

During AML pathogenesis, leukemic cells progressively hijack and alter the normal hematopoietic niche where normal HSCs settle **[32,33]**. Growing evidence suggests that leukemia cells induce dramatic modulations in hematopoietic and non-hematopoietic cells within the niche at the genetic levels. These changes contribute to the appearance of leukemic niche that supports the leukemogenesis and blocks the normal hematopoiesis. However, the detailed underlying mechanisms remain largely unknown **[33-35]**.

Many researchers supported the idea of interactions between leukemic cells and bone marrow microenvironment so it is rational to give the assumption that MSCs in acute myeloid leukemia exhibit molecular changes due to their exposure to the leukemic cells **[36-37]**. These changes, in turn, may represent an essential component of disease relapse and drug resistance characterizing AML disease **[38]**.

In this study, we demonstrated an increased expression of CXCL12 mRNA in the MSCs in AML patients in comparison to the control group. This is in accordance with Civini et al., who proved that leukemic cells affect the gene-profiling pattern in stromal mesenchymal cells that lead to the expression of multiple cytokines and chemokines including CXC12. The study also noted that this effect on mesenchymal cells differs according to the degree of stemness of the leukemic cells resulting in the heterogeneity of clinical presentation among leukemia sub-

types **[39]**. Moreover, Kim et al., demonstrated that leukemia stem cells from de novo AML patients can induce extensive alterations in the mesenchymal niche, resulting in an altered expression of crosstalk molecules, including CXCL12 **[40]**.

In another study, Lopes et al., determined cytokine expression profile of MSCs from patients with myelodysplastic syndrome (MDS), AML with myelodysplasia-related changes (MRC) and de novo AML, in comparison to healthy control. They found that MSCs in AML-MRC showed a significant increase in IL6 expression, whereas de novo AML MSCs presented a significant increase in the expression levels of different cytokines including VEGFA, CXCL12, RPGE2, IL1β, IL6, and IL32. They suggested that the difference in pathogenesis of AML-MRC and de novo AML may extend into MSCs within the leukemic niche **[41]**.

These results indicate that crosstalk between the leukemia cells and MSCs is a dynamic process. MSCs affect leukemia cells and leukemia cells change MSCs genetic profile to be in favor of leukemogenesis.

In this study, CXCL12 protein level in the BM plasma of AML patients was significantly elevated at initial diagnosis in comparison to the control group. However, we did not find a significant correlation between the level of CXCL12 protein and the blasts percent in the bone marrow or with the peripheral blood total leucocytic count. This is in accordance with Alinkovich et al who found that the concentrations of CXCL12 in the peripheral blood and bone marrow plasma samples from AML patients were significantly elevated in comparison to the normal group. Furthermore, they found that CXCL12 was able to enhance in vitro survival of AML cells **[42]**. Also, Wen et al., studied the level of plasma CXCL12 in 48 children with acute leukemia and 20 with non-hematologic malignancies as a control group and showed that the level of CXCL12 in the peripheral plasma of acute leukemia group was significantly higher than that of the control group **[43]**.

In our study, although CXCL12 expression in MSCs and the level of CXCL12 in bone marrow plasma of AML cases were higher than the levels in the control group, they are not correlated to each other statistically. This indicated that MSCs contribute to CXCL12 secretion but they are not the sole source of CXCL12 in bone marrow of AML cases. It has been shown that vascular endothelial cells, stromal fibroblasts, and osteoblasts are contributor sources of CXCL12 **[10,44]**.

The highest expression of CXCL12 gene was detected among our M4 AML cases followed by M3 AML cases, while the level of CXCL12 in BM plasma was highest in promyelocytic AML (FAB: M3) followed by M4 and M5 AML. Mohle et al., observed differential expression of CXCR4, CXCL12 receptor, in different FAB subtypes being higher in AML (M3) and myelomonocytic AML (M4/5), while its expression is low in AML (M0, M1, and M2) **[45]**. Concentrations of CXCL12 protein in the bone marrow plasma in AML patients could be sufficient to provide survival and anti-apoptotic signals to AML cells. Few data are available regarding the role of CXCL12 in different AML subtypes. Heterogeneity of the alteration in mesenchymal stromal cell function in different subtypes of AML at initial diagnosis might be the contributing factor to their heterogeneous presentation and the further heterogeneous post-treatment outcome, and hence may serve as a potential prognostic factor **[40]**. In addition, correlation of tumor cell aggressive phenotype and metastasis with the levels of CXCL12 expression was documented in different studies, among different types of tumors **[46-48]**.

Further studies on larger groups of different AML subtypes are recommended to further elucidate the differential role of CXCL12 in different FAB subtypes pathogenesis.

In conclusion, our study demonstrated that bone marrow mesenchymal stromal cells react to myeloid leukemic cells by increasing the expression of CXCL12 and CXCL12 protein level. This alteration of CXCL12 may contribute to the creation of a BM niche more favorable to host leukemia stem cells.

Conflict of interest: The authors declare that they have no conflict of interest.

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Expression of Insulin-Like Growth Factor-Binding Protein-2 and 7(*IGFBP2* and *IGFBP7*) Genes in Acute Myeloid Leukemia

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ABSTRACT

Background: Variations of the insulin-like growth factor (IGF) receptors and ligands are well defined in different hematological and solid neoplasms, providing a possible anti-neoplastic molecular target.

Objectives: This study aims to determine the expression level of human *insulin-like growth factor binding protein2* and *7 (IGFBP2* and *IGFBP7)* genes in adult AML patients and to asess outcome accordingly.

Patients and Methods: A case control study carried out on 35 adult De novo AML patients who were managed at the National Cancer Institute, Cairo University (NCI), as well as 20 age and gender matched healthy controls. Detection of *IGFBP2* and *IGFBP7* genes was done using real time RT-PCR. Expression of both genes was correlated with the clinical and Laboratory data, complete remission rate and overall survival.

Results: There was a higher *IGFBP2* and *IGFBP7* genes expression in AML patients compared to control. This difference in expression was statistically significant for *IGFBP7* (p=0.036) but not for *IGFBP2* (p=0.1). There was a fare positive correlation between *IGFBP2* and *IGFBP7* gene, (r=0.478, p=0.004). High expression of both *IGFBP2* and *IGFBP7* was significantly associated with lower complete remission rate (p=0.008) and shorter overall survival (p=0.001 and p=0.012 respectively).

Conclusion: High expression of *IGFBP2* and *IGFBP7* genes was associated with poor prognosis in AML cases. Moreover, both genes may present a potential molecular target or a new therapeutic modality for treatment strategy and management of AML.

Key Words: *IGFBP-2 – IGFBP-7 – Acute Myeloid Leukemia (AML).*

INTRODUCTION

Acute myeloid leukemia (AML) is a complex heterogeneous disease in clinical course, cytogenetic and molecular genetics. Understanding the molecular patho-biology in acute leukemia has dramatically improved over the last years **[1]**.

The identification of aberrant signaling pathways in AML and cross-talks has clarified mechanisms of disease pathogenesis and has directed to the development of new targets. Further characterization of prognostically relevant signaling pathways might improve risk-assessment and development of therapeutic strategies for AML patients **[2]**.

Despite being part of the physiological machinery for growth and development, Insulin-like growth factor (IGF) gained special interest over the last decade because of its oncogenic potential **[3]**.

Variations of the insulin-like growth factor (IGF) receptors and ligands are well defined in different hematological and solid neoplasms, providing a possible anti-neoplastic molecular target **[4]**.

The IGF pathway has 2 growth factors ligands (IGF-I and IGF-II), their 2 corresponding receptors (IGF-IR and IGF-IIR) and a group of IGFBPs (IGFBP1-7) **[5]**.

The functional activity of IGF-I and IGF-II is regulated by 7 high-affinity IGF binding proteins (IGFBP1-7), which act as carrier proteins modulating bioavailability and half-life of the IGFs. In addition, some authors suggested that IGFBPs promote cell growth independently of IGFs **[2]**.

IGFBP2, one of the major IGFBPs, is aberrantly expressed in a variety of solid tumors, lymphomas and in patients with acute lymphoblastic leukemia (ALL) and AML **[6,7]**. Although the functional role of IGFBP2 in acute leukemia and other types of cancer is still unknown, overexpression of IGFBP2 has been associated with higher tumorigenicity and therapy resistance **[6]**. In preclinical studies, IGFBP-2 administration could improve tumor metabolism (inhibition of adipogenesis and enhanced insulin sensitivity), while blocking of IGFBP-2 blockade could prevent neoplastic growth and development of metastasis **[8]**. Unlike IGFBP1 to IGFBP6, IGFBP7 binds insulin with 500-folds higher affinity than insulin-like growth factor I (IGF-I) **[9]**.

The study of *IGFBP7* in a variety of solid malignancies, including breast, lung, prostate, colorectal, and liver cancer has suggested a role of a tumour suppressor gene **[10-14]**. High IGFBP7 was also associated with induction chemotherapy failure in acute lymphoblastic leukemia **[1]**. In this study we assessed the expression of human *insulin-like growth factor binding protein 2 and 7* (*IGFBP2* and *IGFBP7*), as potential oncogenes implicated in AML and correlated the results with the different clinical and laboratory features as well as response to treatment and overall survival.

PATIENTS AND METHODS

Patients:

A case-control study was conducted on 35 consecutive newly diagnosed AML patients who presented to the Medical Oncology Department, National Cancer Institute (NCI), Cairo University over a period of 6 months. The age ranged from18-68 with a median of 45 years. Nineteen out of 35 (54%) were males and 16/35 (46%) were females.

Twenty age and gender-matched healthy individuals were included as control group. The study was approved by the ethical committee of NCI [Institutional Research Board (IRB)], Cairo University. All subjects were aware by the nature of the study and gave a written informed consent.

Methods:

Pre-treated BM samples were available from 35 AML patients and 20 healthy controls. Bone marrow mononuclear cells (BMMCs) were extracted from 1-2ml EDTA samples, (they were obtained by gradient density centrifugation using Ficoll-Hypaque 1077 (Sigma). Cell pellets were preserved at –80°C until RNA extraction was performed. Extraction of total RNA from mononuclear cells was carried out using Prep Ease RNA Spin kit 78766, 50 preps, (Affymetrix), following the manufacturer's instructions. The concentration and purity of RNA was measured at 260 & 280 & 230nm using Nano Drop 2000/ 2000c spectrophotometer (Thermo Scientific, USA). Ratio of A260/A280=1.8-2.1 and A260/ A230=1.8-2.1 indicates highly pure RNA.

Extracted RNA was reverse transcribed into complementary DNA (cDNA) using TaqMan® RNA reverse Transcription Kit (Applied Biosystem, USA) according to the TaqMan RNA Assay protocol. The PCR reactions were carried out in a total volume of 20ul (10ulmix+10ul extracted RNA), the thermal cycle reaction program was as follows: 10 minutes at 25ºC (incubation), 37ºC for 2 hours for reverse transcription, 85ºC for 5 seconds to stop the reaction. The main PCR cycles were repeated 40 times (denaturation at 95ºC for 15 seconds, annealing extension at 60ºC for 1m).

The Primers and Probes sequences used were as follows:

- *IGFBP2* Forward primer: 5'CATCACCTTGGCCTGGAG
- *IGFBP2* Reverse primer: 5'GGATGTGCAGGGAGTAGAGG
- *IGFBP2* probe: 5'-FAM- CCTGCCAGGACTCCCTGCCAAC-TAMRA
- *IGFBP7* Forward primer: 5' CATCACCCAGGTCAGCAAG
- *IGFBP7* Reverse primer: 5'TCACAGCTCAAGTACACCTG
- *IGFBP7* probe:6-FAM-TGCGAGCAAGGTCCTTCCATAGT-TAMRA-30

The "comparative threshold method" ($2^{-\Delta\Delta CT}$ method) was used to calculate the relative expression levels of a target (*IGFBP2* and *7*) relative to a reference control using the Ct data. The amount of target was normalized to the endogenous housekeeping gene *GAPDH* and

evaluated relative to the calibrator (healthy control). The formula used was as follow:

$2^{-\Delta\Delta}$ CT, where ΔΔ Ct = Δ Ct (sample) - Δ Ct (calibrator), and Δ Ct is the Ct of the target gene subtracted from the Ct of the housekeeping gene.

$$\Delta\Delta \text{ CT} = \Delta\text{CT sample} - \Delta\text{CT calibrator}$$
$$\text{Fold Change} = 2^{-\Delta\Delta\text{CT}}$$

Response rate at day 28:

Complete remission (CR) in our patients detected on day 28 post induction is defined as morphological recovery of the BM and blood counts (i.e., neutrophils ≥1,500/L and platelets ≥100,000/L), and no circulating leukemic blasts, BM cellularity more than 20% with maturation of all cell lines, no Auer rods, <5% BM blasts and without extramedullary leukemia. Relapse was defined by ≥5% BM blasts, reappearance of circulating leukemic blasts, or development of extramedullary leukemia **[15]**.

Statistical methods:

Data was 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 (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative not normally distributed data, comparison between two groups was done using Mann-Whitney test (non-parametric *t*-test). Spearman-rho method was used to test correlation between numerical variables. Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. A *p*-value <0.05 was considered significant.

RESULTS

We evaluated the expression of *IGFBP2* and *IGFBP7* genes in 35 de novo AML patients. They included 19 males and 16 females with an age range of 18-68, a mean of 41.5±15.6 and a median of 45 years. Twenty healthy age and gender matched subjects were included as a control.

Taking the median value as cut off for both *IGFBP2* and *IGFBP7* genes, 18/35 (51%) were high expressers as compared to 17/35, 49% low expressers.

Correlation between IGFBP2 and IGFBP7 genes:

Good positive correlation was found between *IGFBP2* and *IGFBP7* gene, (r=0.478, p=0.004, Fig. 3).

Both genes had higher expression level in AML patients compared to control that achieved statistical significance for *IGFBP7* (p=0.036) but not for *IGFBP2* (p=0.1).

As regards *IGFBP2* gene, we found that the gene expression level in AML patients (median value=2.05) was higher than gene expression level of control group (median value=1.0), however this difference was not statistically significant (p-value=0.1). While in *IGFBP7* gene, we found a statistically significant difference in *IGFBP7* gene expression level between the AML group and control group (median values were 2.11 and 0.97, respectively, p-value =0.036).

Categorization of patients according to gene expression levels:

The median value of gene expression was taken as a cut off (2.05 for *IGFBP2* and 2.11 for *IGFBP7*). Within each category 17 patients (49%) had values below (low expressers) and 18 (51%) had values above the median (high expressers).

High and low *IGFBP2* and *IGFBP7* expressers showed comparable clinical and haematological characteristics (Tables 1,2).

Follow-up of patients:

In case of *IGFBP7*, 6/35 patients died before day 14 of treatment. A significant positive association between blast count at day 14 with *IGFBP2* and *IGFBP7* gene expression was encountered (p=0.008 and 0.002, respectively, Table 3).

Response rate at day 28:

After the first course of induction, CR was achieved in 11/35 (31%) patients and CR was more associated with low expression of both genes (p=0.008, Table 4, Fig. 1a,b).

Overall survival:

In our study, the median follow-up period was 1.9 month (range 0.1-11.1 month). Overall cumulative survival was 13.3% with a median of 1.9 month (95% CI: 1-2.8m).

Low expressers of both genes showed significantly higher survival rate (p=0.001 and 0.012, respectively; Fig. 2a,b).

Table (1): *IGFBP2* expression in 35 Adult AML cases in relation to clinical and haematological characteristics.

Parameter	Low expressers No. 17 (%)	High expressers No. 18 (%)	p-value
Sex:			
Male	7 (41.2%)	12 (66.6%)	0.130
Female	10 (58.8%)	6 (33.3%)	
TLC x10^9/L:			
Median (range)	26 (3.5-181)	20.5 (1.3-265)	0.732
Hb: gm/dl*	7.2 (4.4-10)	7.4 (3.8-10.4)	0.660
PLTx10^9/L*	37 (11-315)	26.5 (6-257)	0.232
Splenomegaly:			
Yes	4 (23.5%)	3 (16.7%)	0.691
Hepatomegaly:			
Yes	4 (23.5%)	1 (5.6%)	0.177
CD34 expression:			
+Ve	6 (35.3%)	10 (55.5%)	0.229
CD117:			
+Ve	11 (64.7%)	6 (33.3%)	0.063
Aberrant lymphoid markers:			
+Ve	6 (35.3%)	3 (16.7%)	0.264
FAB classification:			
M0, M1&M2	15 (88%)	14 (77%)	0.658
M4	2 (12%)	4 (22%)	

TLC: Total leukocyte count.
Hb : Haemoglobin.
Plt : Platelet count.
* Median (range).

Table (2): *IGFBP7* expression in 35 Adult AML cases in relation to clinical and haematological characteristics.

Parameter	Low expressers	High expressers	p-value
Gender:			
Male	11 (64.7%)	8 (44.4%)	0.229
Female	6 (35.3%)	10 (55.5%)	
TLCx10^9/L:*			
Median (range)	13.3 (3.5-119)	27.5 (1.3-265)	0.732
Hb: gm/dl:*			
Median (range)	7 (4.4-10.4)	7.3 (3.8-10)	0.660
PLTx10^9/L:*			
Median (range)	25 (12-315)	29 (6-257)	0.546
PB blasts:			
Median (range)	55 (28-94)	72.5 (6-94)	0.351
BM blasts:			
Median (range)	73 (30-95)	72 (30-93)	0.782
Splenomegaly:			
Yes	4 (23.5%)	3 (16.6%)	0.691
Hepatomegaly:			
Yes	4 (23.5%)	1 (5.9%)	0.177
CD34:			
+Ve	6 (35.3%)	10 (55.5%)	0.229
CD117:			
+Ve	11 (64.7%)	6 (33.3%)	0.063
Aberrant lymphoid markers:			
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FAB classification:			
M0, M1&M2	15 (88%)	14 (77%)	0.658
M4	2 (12%)	4 (22%)	

TLC: Total leukocyte count. Plt: Platelet count.
Hb : Haemoglobin. * Median (range)

Table (3): *IGFBP2* and *IGFBP7* expression in relation to response to treatment; day 14 blasts in 35 Adult AML patients.

	Low expressers	High expressers	p-value
Day 14 blasts IGFBP2:			
No.	16/35	13/35	0.008
Median (range)	4 (1-42)	15 (4-60)	
Day 14 blasts IGFBP7:			
Median (range)	6 (1-42)	22 (2-60)	0.002

Table (4): *IGFBP2* and *IGFBP7* expression rate in relation to response to treatment at day 28.

IGFBP2	Complete response (n=11)	No response (n=24)	*p*-value
Low expression	9/11 (81.8%)	8/24 (33.3%)	
High Expression	2/11 (18.2%)	16/24 (66.7%)	
IGFBP7	Complete response (n=11)	No response (n=24)	0.008
Low expression	9/11 (81.8%)	8/24 (33.3%)	0.008
High Expression	2/11 (18.2%)	16/24 (66.7%)	

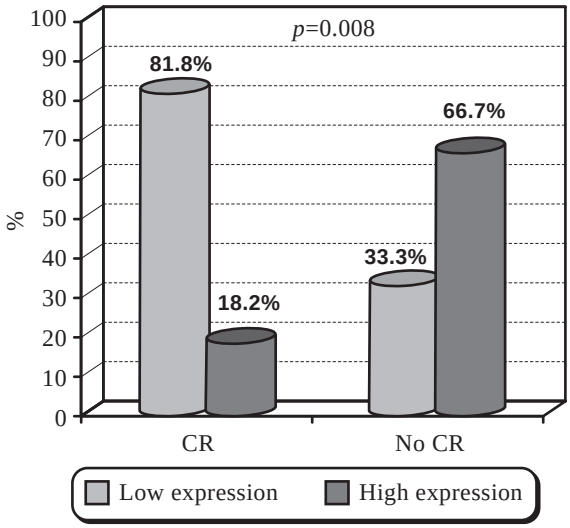

Fig. (1-A): IGFBP2 expression in relation to response to treatment at day 28.

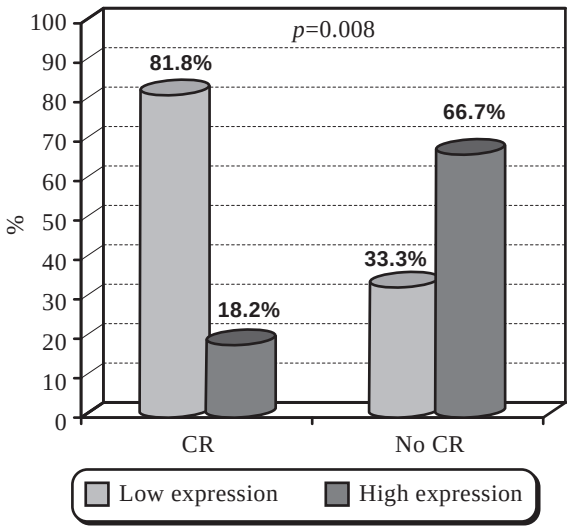

Fig. (1-B): IGFBP7 expression in relation to response to treatment at day 28.

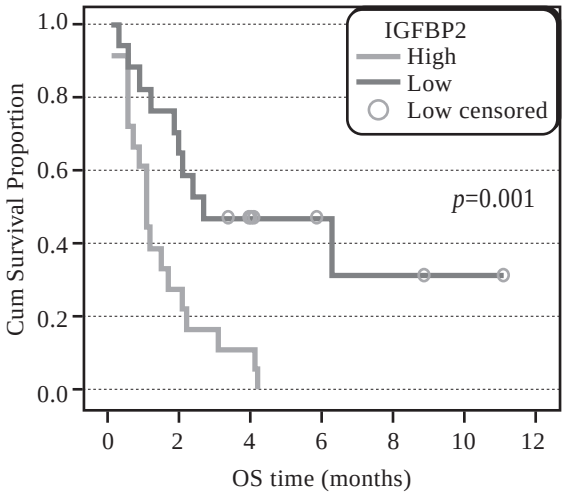

Fig. (2-A): Kaplan Meier curve of overall survival for high and low IGFBP2 expressers.

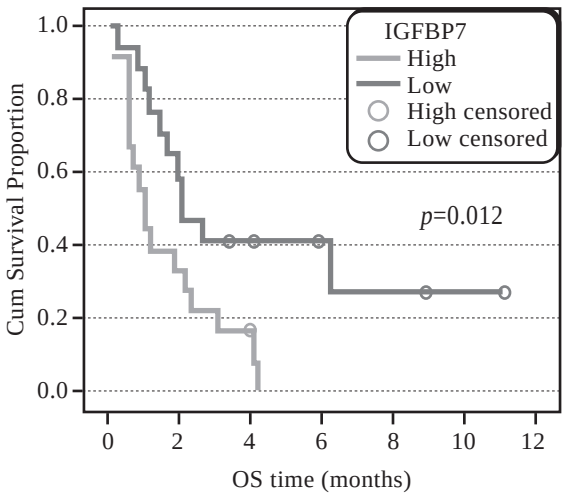

Fig. (2-B): Kaplan Meier curve of overall survival for high and low IGFBP7 expressers.

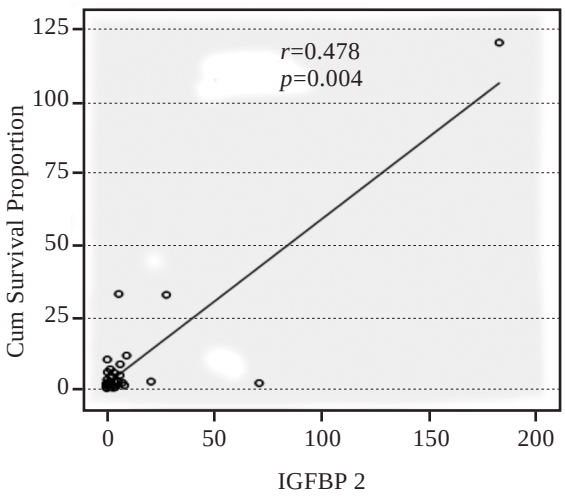

Fig. (3): Correlation between IGFBP2 and IGFBP7 genes.

DISCUSSION

In our study, we measured the level of mRNA expression of both *IGFBP2* and *IGFBP7* genes by real time RT-PCR in BM samples of 35 de novo adult AML patients and 20 age and gender matched healthy controls. We evaluated their level of expression in relation to clinical and hematological parameters, response to treatment and survival.

The frequency of *IGFBP2* gene expression was higher in AML patients than the control group. However, this difference could not reach a statistically significant level (median: 2.05 vs. 1; p=0.1). In line with our results, significantly higher expression levels of both genes were encountered in AML patients **[2,7]**.

Moreover, IGFBP2 serum protein levels were significantly higher in AML patients compared to controls **[2]**. In the current study, a level of significance might be reached by increasing sample size.

In our study, there was no association between *IGFBP2* expression and either age or initial TLC. This is in accordance with Kühnl et al. **[2]**.

We did not either found any association of *IGFBP2* expression with other hematological or clinical parameters including FAB subtypes. In contrast to the latter, Kühnl et al. **[2]** reported an association between high expression and M4/M5 FAB subtype.

Differences in sample size and differences in relative frequency of FAB subtypes may explain that differences in results.

In the current study high *IGFBP2* expression was significantly associated with a lower CR rate (p=0.008). This is in agreement with Kühnl et al. **[2]** who reported significant association of high *IGFBP2* expression with a higher incidence of primary resistant disease (p=0.02) and a lower CR rate (p=0.01). This is also in line with others **[16]** who reported that serum IGFBP-2 level was significantly lower in responders than in non-responders both at diagnosis and after induction cycle of chemotherapy. On the same line another study **[7]** reported that high *IGFBP2* expression was a predictor of higher relapse rate in pediatric AML.

In the current study, lower *IGFBP2* expression was significantly associated with better overall survival (p=0.0013). This is in contrast to Kühnl et al. **[2]** who reported no relationship between *IGFBP2* expression and OS (p=0.53).

On the other hand, Huang et al. **[17]** and Lin et al. **[18]** concluded from their study on newly diagnosed glioma patients using an IGFBP-2 ELISA kit that IGFBP-2 levels are significantly correlated with recurrence and DFS in patients with astrocytoma grade IV. Low *IGFBP2* expression had an obvious benfit in overall and disease-free survival, whereas those with high *IGFBP2* expression have worse median survival.

This controversy in results is likely due to different ethnic population, different sample size and/or exposure to different carcinogens in different environments (gene environmental interaction) and also gene to gene interaction in cancer.

IGFBP2 has strong oncogenic potential promoting proliferation, survival, and migration of leukemic cells. Chen et al. **[19]** found that deletion of *IGFBP2* decreased extramedullary leukemic infiltration, suggesting that IGFBP2 is required for the migration of AML cells out of bone marrow. For this reason, the development of IGFBP2 therapy that affects leukemic stem cells with minimal effect on normal stem cells is a potentially promising therapeutic cancer treatment **[20]**.

In the current study, *IGFBP7* gene expression showed significantly higher frequency in AML patients as compared to the control group (p=0.036). This is in agreement with previous reports both in adult **[1]** and paediatric AML **[21]**.

In our study, no association was encountered between *IGFBP7* gene expression and any hematological or clinical parameters at diagnosis.

However, high *IGFBP7* expression was significantly associated with a lower CR rate (p=0.008) and worse overall survival (p=0.012). In contrast, Verhagen et al. **[22]** reported the association of high *IGFBP7* expression with a better outcome in AML patients. Heesch et al. **[1]** concluded that high expression of *IGFBP7* was found to be associated with a more immature phenotype of early T-ALL, inferior survival, and resistance against chemotherapy in T-ALL.

Also, Tomimaru et al. **[23]** reported improved OS rate in patients with *IGFBP7* expression was significantly lower than that in patients with positive *IGFBP7* expression in hepatocellular carcinomas (p=0.0063). Benassi and colleagues **[24]** concluded that not only tissue expression of *IGFBP7* is highly prognostic in poor metastasis-free survival, but in parallel, the determination of serum protein levels might contribute to soft tissue sarcoma diagnosis. Jiang et al. **[25]** found that expression of *IGFBP7* correlated inversely with overall Glioblastoma survival rates. This controversy in *IGFBP7* expression in leukemia and glioblastoma cell lines is in disagreement with other solid tumors findings. This may be due to different mechanisms of action of IGFBP7. One of these possibilities is that cell signaling pathways that result in senescence or apoptosis due to IGFBP7 are not present or functioning in hematopoietic or glioma cells. Another proposed mechanism by Cao et al., **[26]** is that deletion of IGFBP7 leads to activation of IGF1 which in turn stimulates the FGF4-FGFR1-ETS2 pathway in tumor-associated endothelial cells and induce chemo-resistance and malignant stem cells formation.

In the current study, we found a good positive correlation between the expression of both *IGFBP2* and *IGFBP7* genes. High expression of one gene is accompanied by high expression of the other. *IGFBP2* and *IGFBP7* genes have functions both dependant and independent of IGF/IGF receptor signalling. An appropriate understanding of their mechanism of function is required. Moreover, *IGFBP2* and *IGFBP7* may present a potential molecular target or a new therapeutic modality for treatment strategy and management of AML.

In conclusion, more studies with larger sample size are needed to establish and confirm the function and clinical significance of *IGFBP2* and *7*. The role of *IGFBP2* and *IGFBP7* as prognostic molecular markers and potential therapeutic tool in management of AML needs to be confirmed.

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Expression of Homeobox Gene in Relation to Different Molecular Aberrations Associated with Adult Acute Myeloid Leukemia

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ABSTRACT

Background: Homeobox (*HOX*) gene expression is linked with definite cytogenetic and molecular abrasions in acute myeloid leukemia (AML) patients. This association may be a direct result of transcriptional process or may be related to the differentiation stage at which the leukemic cell is arrested.

Objectives: Our aim was to detect the *HOX* genes expression pattern in leukemic patients is related primarily to the differentiation stage of myeloid cells or emerged during the process of malignant transformation.

Patients and Methods: We examined the bone marrow (BM) of 38 adult patients with AML and BM of 20 healthy individuals. We sorted the various stages of myeloid differentiation and correlated them to their morphological counterpart of AML. The expression of mRNA of *HOX* genes (*HOXA* and *HOXB*) in BM was detected using Quantitative real time-PCR.

Results: The AML cases were divided into five groups according to the presence of common molecular genetic abnormalities. The differentiation stage of the blasts was not statistically significant affecting *HOX* gene expression while it is more related to genetic and molecular abrasion associated with AML. *PML-RARa* fusion gene showed depressive effect on HOX gene expression, regardless the *FLT3/ITD* mutational status.

Conclusions: We concluded that the *HOX* gene expression in AML patients is associated with molecular and genetic aberrations, rather than with the differentiation stage of leukemic cells.

Key Words: *Homeobox genes – Acute myeloid leukemia – Myelopoiesis staging – Molecular abnormalities.*

INTRODUCTION

The family of *homeobox* genes code for many transcription factors. There are many *HOX* genes clustered into four genomic regions (*HOXA, B, C* and *D* clusters) found on four chromosomes (chromosomes 7, 17, 12 and 2, respectively). Each cluster carries 9-11 genes **[1,2]**.

The expression of *HOX* genes occurs maximally in the stem and early hematopoietic cells during hematopoiesis. Then their expression progressively decreases with maturation till it reaches minimum expression in fully differentiated hematopoietic cells **[3,4]**. This process is firmly under control of regulators; the disruption of regulators leads to transformation to leukemic cell **[5,9]**.

The potential oncogenic effect of *HOX* genes was studied thoroughly for the last two decades. A number of *HOX* genes abnormal expression, either directly (e.g., *NUP98-HOX* fusion) or by their upstream regulators (e.g., *MLL* rearrangements), had been describedin Leukemia patients **[10,13]**. Also it was found that their over expression in leukemia patients is a poor prognostic factor where they induce myeloproliferation and with additional cytogenetic insult may cause leukemia **[14,16]**.

In the current study, we tried to answer the question of either the *HOX* genes expression in leukemic patients is related primarily to the differentiation stage of myeloid cells or emerged during the process of malignant transformation. The expression of *HOX* genes was studied in relation to the morphological stage, cytogenetic and molecular characteristics of leukemic cells in adult patients of acute myeloid Leukemia (AML).

PATIENTS AND METHODS

BM samples:

We collected samples of BM from 38 patients newly diagnosed as AML from 2014 to 2017 at Dammam University Hospital, Kingdom of Saudi Arabia. After University Institutional Review Board (IRB) approval and patient's signature of informed consent, BM samples were processed to separate mononuclear cells using Ficoll-Paque Plus (GE Healthcare Life Sciences, Uppsala, Sweden) and stored at –80°C.

Twenty control BM samples were collected from BM transplantation donors.

Methods:

Different developmental stages of hematopoiesis were collected using Fluorescence Activated Cell Sorter (FACS) Aria (BD, San Jose, CA, USA). The different stages of myeloid lineage differentiation were identified by surface markers listed in Table (1).

From both patient and control groups, RNA was prepared with RNeasy Mini Kit (Qiagen, Hilden, Germany) and transcribed to cDNA by the iScript kit (Bio-Rad, Hercules, CA, USA).

Gene expression was measured by the iCycleriQ System (BioRad, Hercules, CA, USA); it was used to quantify the HOXA and HOXB gene expression in both groups. The PCR primer make up was settled for the detection of different *HOX* genes subtypes (*HOXA3, A4, A5, A6, A7, A9, A10, B2, B4, B5, B6,* and *B7*) and patient samples (*HOXA1, A3, A4, A5, A6, A7, A9, A10, A11, A13, B1, B2, B4, B5, B6, B7, B8,* and *B9*) **[17,18].** We quantified the ABL1 gene, which is known to be stably expressed during the developmental stages of myeloid lineage cells as internal control.

We selected 14 patients who were negative for the presence of the main molecular abnormalities to be examined for the presence of the gene mutation of the *NPMI, NRAS, KRAS, CEBPa, c-KIT* and *FLT3* genes (Table 2). The analysis was performed by Real Time PCR followed by sequencing **[19,22].**

According to previously published studies, the maturation stages were identified (Table 1) **[23,25].**

Statistical analysis:

The statistical significance among the subgroups was done by Mann-Whitney and Kruskal-Wallis tests with Dunn's multiple comparison post-test. Cluster analysis, was done to identify the subgroups with similar gene expression patterns using the Genesis software (Institute for Genomics and Bioinformatics, Graz University of Technology (IGB-TUG, Graz, Austria). All data is represented in tables, graphs and expressed in mean and median with calculation of statistical significance.

RESULTS

For better assessment of the *HOX* gene expression throughout developmental stages, granulocytic and monocytic lineages were pooled together, so we classified the myeloid differentiation into four stages (stage 1=G1+M1, stage 2=G2+M2, stage 3=G3+M3 and stage 4=G4+M4). *HOXA* and particular *HOXB* (HOXB2 and HOXB4) genes expression gradually decreased during the process of maturation (Fig. 1).

According to French-American-British classification (FAB) AML subtypes certain *HOX* gene expression patterns was observed. M3 FAB was the subtype with lowest expression *HOXA* and *HOXB* genes when compared with other FAB subtypes. In contrast, AML M5 was the highest expression of *HOXA* genes. According to molecular genetics, significant differences were also found among subgroups where cases that carry *PML-RARa* fusion gene showed the lowest levels of *HOXA* and *HOXB* genes; on the other hand cases that carry *MLL* gene aberrations express the highest levels (Fig. 2).

According to FAB subtypes and genetic aberrations, *HOX* gene expression was significantly lower in AML M4 with *CBFb-MYH11+* rearrangement and AML M2 with the *RUNX1-RUNX1T1+* rearrangement patients when compared with patients lacking such rearrangement (Fig. 3).

In our study, patients who have mutated *FLT3/ITD* and *PML-RARa*, showed very low levels while those with *FLT3* mutation only,

showed higher expression levels of *HOX* genes (Fig. 4).

We sorted 20 normal BM subpopulations according to different myelopoiesis stages and compared the subpopulations with matched AML FAB subtypes (Table 1). When we compared promyelocytes (ID=G2) with AML M3, we found different *HOX* gene expression patterns. Also, a significant difference in the median of *HOX* genes expression was found when we compared between AML M4 and M5 subtypes with their matched normal subpopulations (Fig. 2).

Table (1): Subpopulations of healthy BM cells **[23,25]**.

Normal sorted myeloid lineage populations			FAB Counterparts
Granulocyte Lineage subpopulations:			
G1	Myeloid progenitor	CD117+ CD15- CD11b-	AML M1 AML M2
G2	Promyelocyte	CD117+ CD15+ CD11b-	AML M3 AML M2
G3	Promyelocyte-Myelocyte	CD117- CD13++ CD16-	
G4	Myelocyte	CD117- CD13dim CD16-	
Monocyte lineage subpopulations:			
M1	Monoblast	CD34+ SSc++ HLA-DR+ CD33-	AML M1-M0
M2	Myelo/monoblast	CD34+ SSc++ HLA-DR+ CD33+	AML M2
M3	Promonocyte	CD34- FSc and Ssc corresponding to monocyte CD33+ Cd14-	AML M4-AML M5a
M4	Monocyte	CD34- FSc and Ssc corresponding to monocyte CD33+ Cd14+	AML M5b

Table (2): Clinical, hematological and cytogenetic / molecular data of 38 acute myeloid leukemia patients.

Parameter	N	%
Male/Female	20/18	53/47
Age: years - median (range)	47 (28-54)	
FAB Subtypes:		
M0	3	8
M1	3	8
M2	8	22
M3	7	18
M4	9	23
M5	8	21
Cytogenetics/Molecular genetics:		
t(15;17) PML-RARa	7	18
t(8;21) RUNX1-RUNX1T1	6	16
inv(16) CBFb-MYH11	3	8
t(11q23) MLL rearrangements	8	22
Cytogenetically Normal **1**	14	36
NK-AML	7	18
FLT3/ITD	5	13
NPMI1 (mut)	2	5

1 Patients negative for the presence of four chromosomal translocations typical of AML.

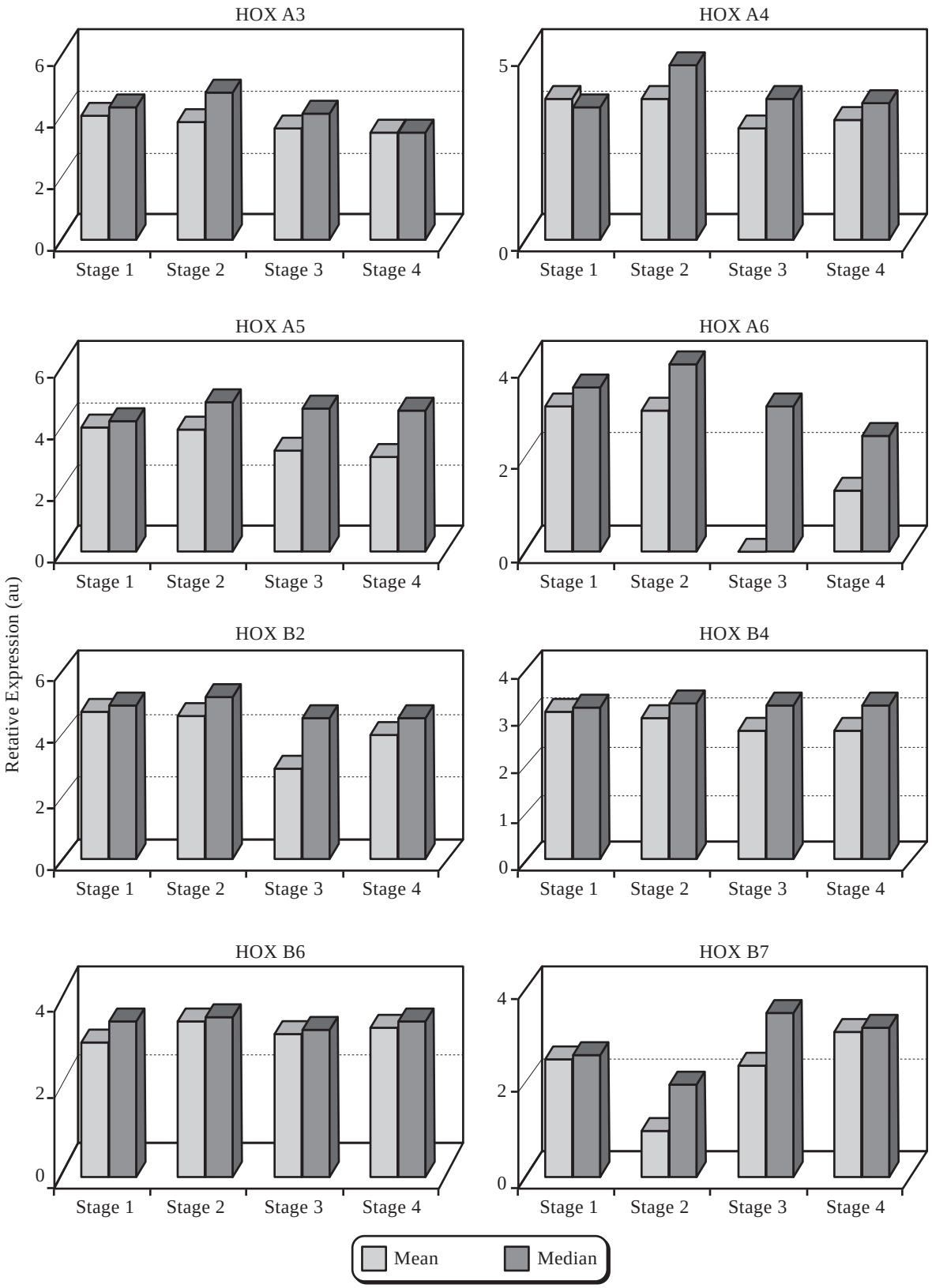

Fig. (1): Expression of different *HOXA* and *HOXB* genes in healthy BM. The myeloid differentiation was classified into four stages (stage 1=G1+M1, stage 2=G2+M2, stage 3=G3+M3 and stage 4=G4+M4).

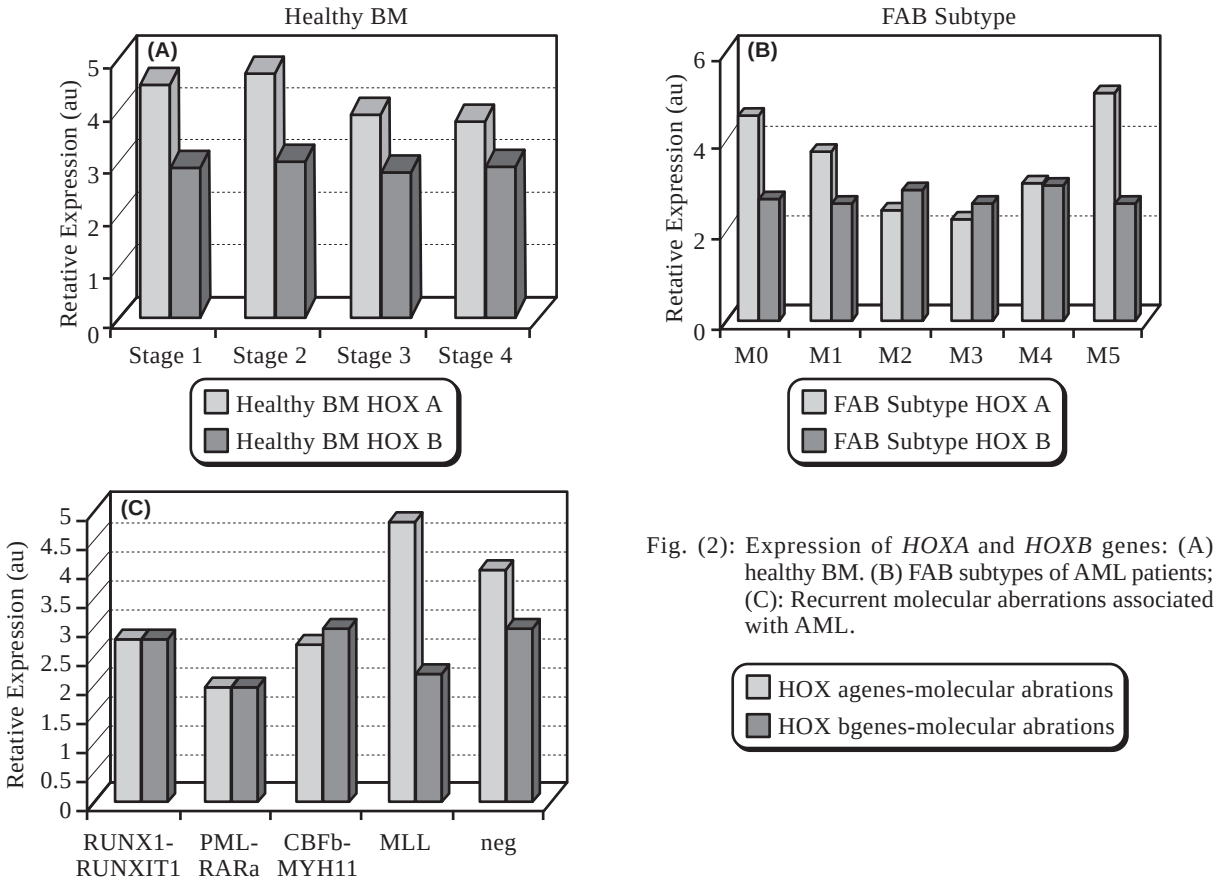

Fig. (2): Expression of *HOXA* and *HOXB* genes: (A) healthy BM. (B) FAB subtypes of AML patients; (C): Recurrent molecular aberrations associated with AML.

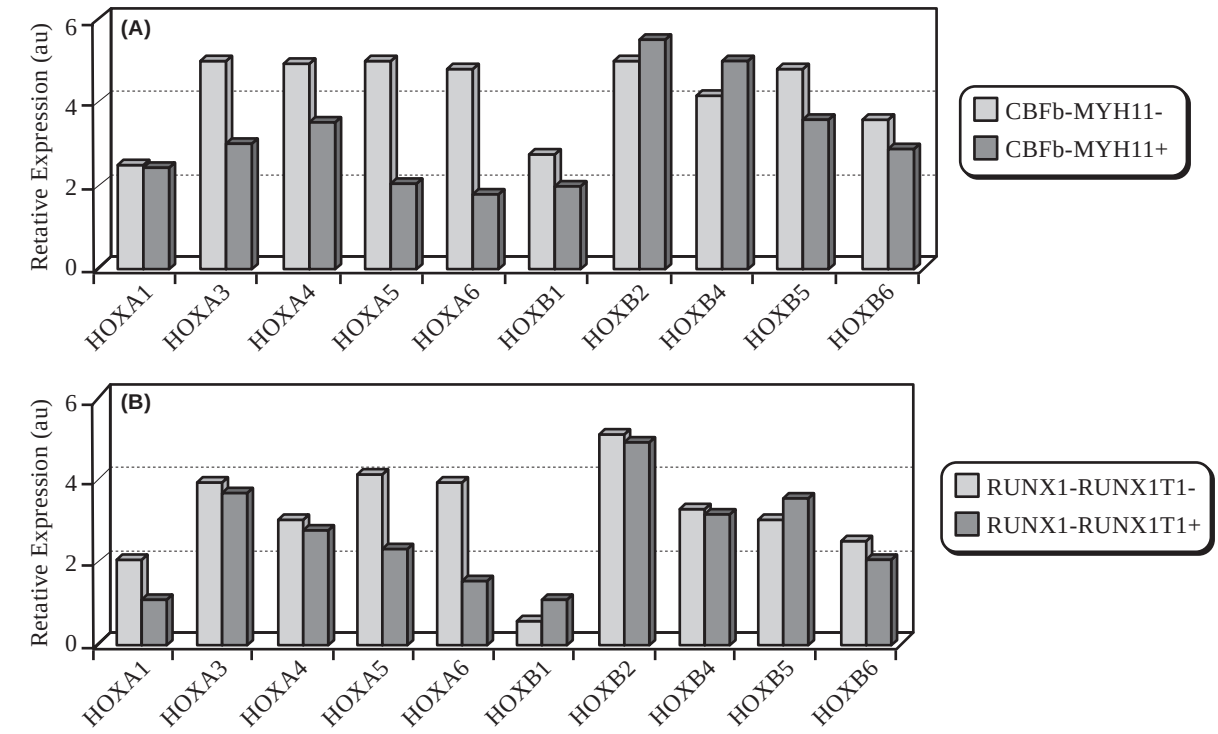

Fig. (3): Expression of different HOXA and HOXB genes with: (A) AML M4 patients with CBFb-MYH11 +/–. (B) AML M2 patients with RUNX1-RUNX1T1+/–. Statistical significance: *≤0.05, **≤0.01.

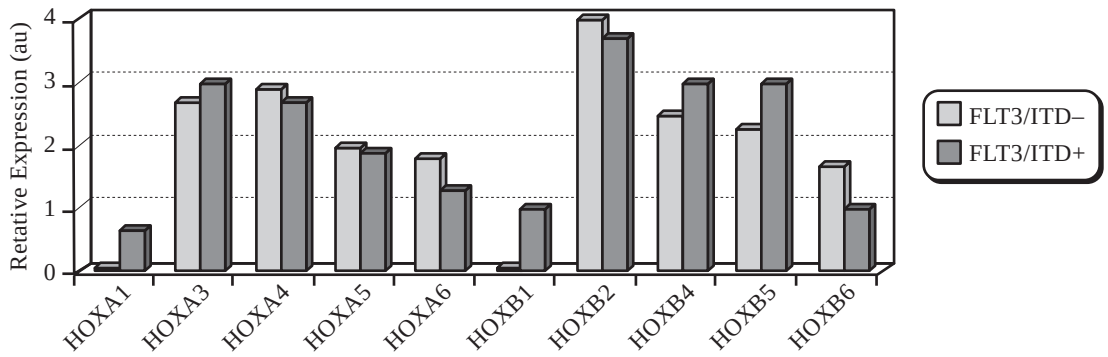

Fig. (4): Expression of different HOXA and HOXB genes in PML-RARa+ AML M3 patients with concurrent FLT3/ITD+ and FLT3/ITD–. Statistical significance: *≤0.05, **≤0.01.

DISCUSSION

HOX genes are potent regulators of normal hematopoiesis, both during embryonic development and in adult life. However, the role of *HOX* genes in the process of leukemogenesis has not yet been proven **[26-28]**.

Abnormal expression of *HOX* genes was reported in most leukemia patients; however it is unclear whether it is a primary cause or a consequence of differentiation block **[29]**. Also the effect of the molecular abnormality present in leukemic cells and its relation to *HOX* genes expression was addressed **[30,31]**. In this study we sorted normal BM subpopulation according to the stage of differentiation and compared between the expression of *HOX* genes in the normal BM cells and their matched counterpart in AML patients*******. We found that at the early stages of hematopoiesis, the expression of *HOX* genes was higher and gradually decreased with maturation, which proves and matches with the claim that in the early stages of hematopoiesis, *HOX* genes play strong regulation roles **[27]**.

A different expression of *HOX* genes was found in leukemic cells other than that found in the normal matched counterpart at the same differentiation stage. This proves that the presence of abnormal of *HOX* gene expression is not enough to explain the developmental stage at which the cells have been arrested. Previous reports have documented that *HOX* gene expression in ALL patients' subgroups was different in malignant cells from their matched normal counterparts' **[15]**. Collectively, our results support the hypothesis that the abnormal control of *HOX* genes has a role in the pathogenesis of leukemia and malignant transformation.

In the current study, the molecular abnormality associated with the AML patient's subtypes found to affect the *HOX* genes expression. This was most clearly found in AML M3 patients with positive *PML-RARa* fusion gene where they expressed the lowest level of *HOX* genes. Also AML patients with other gene rearrangements such as *RUNX1-RUNX1T1+*, *CBFb-MYH11+* and *MLL* showed unique *HOX* gene expression patterns. This agrees with reports telling that MLL rearrangements have a prominent impact on HOX gene expression **[32-37]**.

Moreover, *FLT3/ITD+PML-RARa+* patients showed statistically significantly lower expression of *HOX* gene than those having *FLT3/ITD+* mutation without *PML-RARa* fusion protein **[23,38]**. Thus *PML-RARa* fusion gene has a more dominant role over *FLT3/ITD* in the process of neoplastic transformation.

In conclusion, particular differentiation stages are not the only factor affecting *HOX* genes expression patterns in AML malignant cells. The specific molecular aberration associated with AML is the main and primary factor affecting the pattern of expression of *HOX* genes.

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