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Prognostic Value of Gravin Versus Survivin Gene Expression in a Cohort of Egyptian Patients with Adult Acute Myeloid Leukemia

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Abstract
Despite recent progress in diagnosis and management, acute myeloid leukemia still remains a highly fatal disease which invites the needs for accurate predictors of clinical outcome. The aim of the present study was to explore gravin and survivin possible prognostic importance in adult patients with de novo AML by comparing their expression levels with initial tumor burden, response to induction therapy and overall survival. This study was conducted on 105 patients with de novo AML. RNA isolation from bone marrow aspirates or peripheral blood and cDNA preparation followed by quantitative real time RT-PCR were done to assess expression of gravin and survivin. Gravin expression was makedly down regulated (with a median of 0.02 in cases compared to 11.40 in controls) while survivin gene showed an over expression (with a median of 71.22 in cases compared to 0.12 in controls) in AML cases. There was a significant association between low gravin as well as high survivin expression and poor clinical outcome ($p < 0.001$). Overall survival and disease free survival were significantly lower in patients with low gravin and high survivin expression ($p < 0.001$). Survivin over expression and gravin down regulation were significantly associated with adverse clinical outcome and tendency to chemoresistance in acute myeloid leukemia and the degree of their expression derrangement has been found to be correlated with a lower complete remission rate and shorter overall and disease free survival which renders them as candidate prognostic markers and future targets for adjuvant immunotherapy.
Keywords- *Gravin, Survivin, expression, prognosis, AML, Egyptian patients.*

Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy characterized by the clonal expansion of myeloid blasts in the peripheral blood, bone marrow, and/or other tissues [1]. AML is the most common acute leukemia in adult. Despite recent progress in diagnosis and management, AML still remains a highly fatal disease. With intensive

induction therapy, complete remission rates between 65 and 75% are achieved in younger patients (<60 years). However, more than 50% of these patients will relapse, leading to an overall survival rate of only 30-40% after 5 years. Results are more unfavorable in patients older than 60 years [2]. Therefore, accurate predictors of clinical outcome can contribute to the design of appropriate treatment for individual patients [3].

The tumour suppressor gene gravin/ AKAP12 encodes a scaffolding protein and is a member of the cyclic AMP-dependent kinase-anchoring proteins (AKAPs). Evidence that AKAP12 might be a tumor and/or metastasis suppressor comes from its down regulation by specific oncogenes in cancer cell lines and human cancer tissues compared to normal controls. Besides its up regulation by treatments that suppress oncogenic growth by direct demonstrations showing that its re-expression suppresses *in vitro* and *in vivo* oncogenic growth, especially metastasis formation, or that its loss produces a tumor- or metastasis-prone condition [4]. Epigenetic silencing of AKAP12 transcription might occur in myeloid malignancies and showed that AKAP12 is a target of aberrant DNA methylation in AML cell lines and in primary haematopoietic cells of children with myelodysplastic syndrome or AML [5].

Survivin, a member of the inhibitors of apoptosis (IAP) protein family, is one of the most frequently up-regulated transcripts in cancer but is expressed at low or undetectable levels in many normal adult tissues. Its roles in regulating cell proliferation and cell death and its differential expression in many cancers make survivin a promising therapeutic target. Currently several clinical trials employing different approaches including antisense oligonucleotides, small molecule inhibitors and immunotherapy are in progress. Over expression of survivin has been identified in several hematologic malignancies [6]. Significantly higher levels of survivin were discovered in $CD34^{+}38^{-}$ AML stem/progenitor cells than in bulk blasts and total $CD34^{+}$ AML cells; and the higher levels of survivin were associated with a shorter overall and event-free survival. Furthermore, the expression of survivin correlated with the expression of multiple proteins involved in proliferation and cell survival [7].

The aim of the present study was to determine gravin and survivin expression levels in adult patients with de novo AML and to explore their possible prognostic importance by comparing their expression levels with initial tumor burden, response to induction therapy and overall survival.

Material and Methods

This study was conducted on 105 patients with de novo AML. Patients, who met the diagnostic criteria for AML, were selected from the hematology unit of Alexandria main university hospital between January 2014 and December 2015. The patients were 81 males and 24 females with age range from 18 to 65 years (median age 45). Forty five age and sex matched patients with hematological diseases to whom bone marrow examination is one of the required investigations were selected as a control group. They were 27 males and 18 females with age range from 20 to 60 years (median age 38). The selection of these patients was based on the following criteria: full history taking; thorough clinical examination; standard diagnostic methods, including cytomorphological, cytochemical, cytogenetic and immunophenotypic evaluation which was established using Becton Dickinson, FACS Calibur flow cytometer equipped with Cell Quest software (Becton Dickinson, San Diego, CA, USA). Positivity by flow cytometry was defined as an expression in at least 20 % of cells in the gated population of interest, compared to internal negative control cells. Inclusion criteria for the study were newly diagnosed AML with different FAB subtypes and normal karyotyping by conventional cytogenetics on bone marrow aspirate (BMA) at the time of diagnosis. To establish cytogenetically normal (CN-AML), 20 or more metaphase cells from the samples had to be examined to assure normal karyotypes. Patients with therapy-related AML were excluded from the study. RNA isolation from bone marrow aspirates or peripheral blood and

cDNA preparation followed by quantitative real time RT-PCR were done to assess expression of gravin and survivin in both cases and controls.

Then patients received the standard '3 + 7' induction chemotherapy protocol: doxorubicin (45 mg/m^2/day) for 3 days and cytarabine (100 mg/m^2/day as a continuous 24 h intravenous infusion) for 7 days. BMA was done between 21 and 28 days after the initiation of chemotherapy to demonstrate the morphological remission. Consolidation is comprised of three to four courses of high-dose cytosine arabinoside (3 g/m^2 every 12 h on days 1, 3 and 5; total, 18 g/m^2). Patients were followed up once every 3 months with clinical examination and complete blood counts. BMA was done if there was any doubt of a relapse on clinical examination or peripheral smear. Complete remission (CR) was a normocellular BM containing less than 5 % blasts and showing evidence of normal maturation of other BM elements, with neutrophil count of $\geq 1 \times 10^9$/L and a platelet count of $\geq 100 \times 10^9$/L. Overall survival (OS) was the time from diagnosis to the date of death. For patients achieving CR, disease free survival (DFS) was the time from the date of first CR to an event (death in first CR or relapse).

The study was approved by the medical ethics committee and informed consents were obtained from all participants involved in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 [(5)].

Gravin and Survivin Expression

Purification of total cellular RNA from human whole blood was done using the PureLink® RNA Mini Kit (Ambion by life technologies, USA). The concentration and purity of RNA were determined by measuring the absorbance at 260, 280 and 230 nm using Nano drop 2000 spectrophotometer (Thermo Scientific, USA). A260:A230 ratio greater than 1.9 and A260:A280 ratio greater than 2.1 indicates highly pure RNA. Reverse transcription (cDNA synthesis) was done using high capacity cDNA reverse transcription kit (Applied Biosystems, USA) by Biometra thermal cycler (Germany). Quantitative RT-PCR for gravin and survivin expression was performed using TaqMan® Universal Master Mix II (Applied Biosystems, USA). Thermocycling was carried out for 5 minutes at 95°C, followed by 45 cycles of denaturation for 30 seconds at 95°C and combined annealing/ extension for 30 seconds at 60°C. For gene expression quantification, we used the comparative Ct method. Expression data were normalized to the geometric mean of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to control the variability in expression levels by RT-PCR, using real-time cycler Rotor gene Q® (Qiagen, USA). Data analysis was done using the $2^{-\Delta\Delta CT}$ method as relative gene expression values.

Statistical Analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA). Comparisons between groups for categorical variables were assessed using Chi-square test (χ^2). Student t-test was used to compare two groups for normally distributed quantitative variables. Mann- Whitney test and Kruskal-Wallis test were used to compare two or more groups for abnormally distributed quantitative variables. Paired t-test and Wilcoxon signed ranks test were assessed for comparison between different periods. Spearman coefficient was used to correlate between quantitative variables. Kaplan–Meier test was used for survival analysis and the statistical significance of differences among curves was determined by log-rank test. Confidence interval was 95%. Significance of the obtained results was judged at the 5% level.

Results

Demographic findings, initial peripheral blood counts and tumor burden parameters in cases compared to the control group are shown in table (1). Gravin expression was makedly down regulated

while survivin gene showed an over expression in leukemic blasts of AML cases. The difference in gravin as well as survivin expression between patients and controls was statistically significant (p <0.001). There was a significant association between both gravin as well as survivin expression and demographic data (p <0.001 for age, p = 0.001 for sex) table (2). A significant negative correlation was found between gravin expression and age (r_s = -0.608, p <0.001) as well as bone marrow blasts percentage (r_s = -0.250, p = 0.010) table (3). Conversely, a significant positive correlation was detected between gravin expression and platelet count (r_s = 0.277, p = 0.004). On the other hand, there was a significant positive correlation between survivin expression and age (r_s = 0.615, p <0.001) as well as bone marrow blasts percentage (r_s = 0.242, p = 0.013). On the contrary, a significant negative correlation was found between survivin expression and platelet count (r_s = -0.281, p = 0.004). There was a significant association between gravin expression and the clinical outcome of AML patients where it was highest in patients who achieved a complete remission and lowest in relapsed patients and those with induction death (p <0.001) table (4). Another significant relation was detected between survivin expression and induction outcome where it was markedly lower in patients with continuous complete remission than in relapsed patients and highest in those with induction death (p <0.001). Overall survival (OS) and disease-free survival (DFS) times according to gravin and survivin expression are shown in table (5), figure (1). OS and DFS were lower in patients with low gravin expression (cumulative survival; 70.6, 0.0 %) than in patients with high gravin expression (cumulative survival; 100, 83.3 %) with a statistical significance (p <0.001). OS and DFS times were higher in patients with low survivin expression (cumulative survival; 100, 88.2 %) than in patients with high survivin expression (cumulative survival; 72.2, 0.0 %) with a statistical significance (p <0.001).

Table (1): Comparison between the two studied groups according to the different parameters

	Cases (n =105)	Control (n = 45)	p
Age (years)	42 ± 13.3	40.9 ± 12.4	0.627
<60	93(88.6%)	42(93.3%)	0.554
≥60	12(11.4%)	3(6.7%)	
Sex			
Male	81(77.1%)	27(60%)	0.032
Female	24(22.9%)	18(40%)	
WBC(10^9/l)	10(0.9 – 142.7)	5.9(2.1–10.5)	0.140
Hb(g/dl)	9.4 ± 1.9	11.2 ± 3.1	0.001
Platelets(10^9/l)	56(8 – 134)	230(75 – 480)	<0.001
LDH (U/l)	152.2 ± 45	123.7 ± 17.4	<0.001
ALP (U/l)	102.4 ± 27.9	85.1 ± 21.8	<0.001
Uric acid (mg/dl)	4.9 ± 1.2	5.3 ± 1.2	0.035
Gravin expression	0.02(0.01 – 5.62)	11.40(4.86 – 16.69)	<0.001
Survivin expression	71.22(21.04 - 1124.24)	0.12(0.01 – 5.05)	<0.001

Normally quantitative data was expressed in mean ± SD and was compared using student t-test, abnormally distributed data was expressed in median (Min. - Max.) and was compared using Mann Whitney test, Qualitative data were described using number and percent and was compared using Chi square test.*: Statistically significant at $p \leq 0.05$

Table (2): Relation between demographic data with Gravin and Survivin relative quantitative expression in cases

Age/Sex	Gravin	p	Survivin	p
<60	0.02(0.01–5.62)	<0.001	55.90(21.04–269.61)	<0.001
≥60	0.02(0.01–0.02)		221.71(82.98–1124.24)	
Male	0.02(0.01–0.8)	0.001	82.98(25.19–1124.24)	0.001
Female	0.05(0.01–5.62)		40.5(21.04–244.68)	

Abnormally distributed data was expressed in median (Min. - Max.) and was compared using Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table (3): Correlation between Gravin and Survivin relative quantitative expression in cases with different parameters in cases

	Gravin		Survivin	
	r_s	P	r_s	p
Age (years)	-0.608*	<0.001	0.615*	<0.001
WBC (10^9/l)	-0.133	0.177	0.121	0.219
Hb(g/dl)	0.171	0.080	-0.170	0.084
Platelets (10^9/l)	0.277*	0.004	-0.281*	0.004
LDH (U/l)	-0.096	0.328	0.086	0.383
ALP (U/l)	-0.167	0.088	0.168	0.086
UricAcid (mg/dl)	0.171	0.081	-0.168	0.086
Blast (%)	-0.250*	0.010	0.242*	0.013

r_s: Spearman coefficient
*: Statistically significant at $p \leq 0.05$

Table (4): Relation between different clinical outcomes with Gravin and Survivin relative quantitative expression in cases

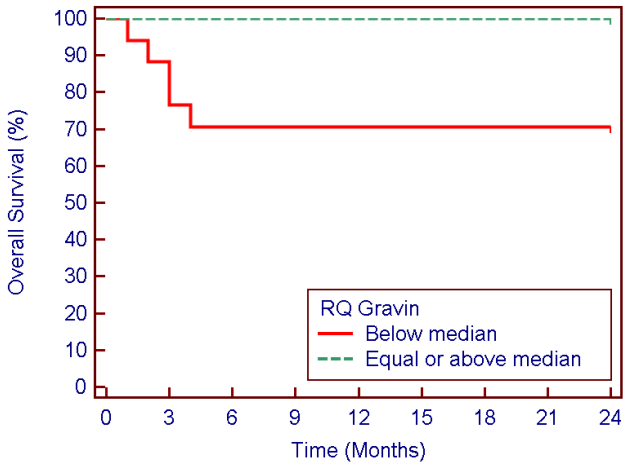
Outcome	Gravin	p	Survivin	p
Complete remission (n = 45)	0.05(0.03 – 5.62)	<0.001	25.9(21.0-42.7)	<0.001
Relapse (n = 45)	0.015(0.01 – 0.023)		116.6(55.9-268.9)	
Death (n = 15)	0.011(0.010 – 0.012)		244.7(160.3 - 1124.2)	

Abnormally distributed data was expressed in median (Min. - Max.) and was compared using Kruskal Wallis test
*: Statistically significant at $p \leq 0.05$

Table (5): Overall and disease free survival with Gravin and Survivin relative quantitative expression in cases

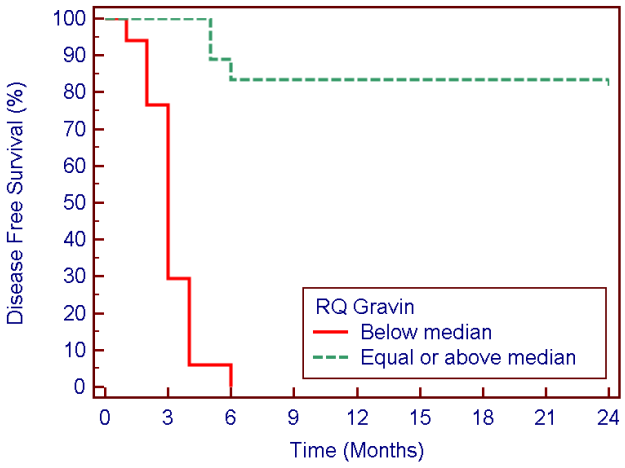
	Gravin				Survivin			
	Overall survival		Disease free survival		Overall survival		Disease free survival	
	Low expression	High expression	Low expression	High expression	Low expression	High expression	Low expression	High expression
Mean	17.71	-	3.12	20.89	-	18.06	21.82	3.22
95% CI of mean (UCI - LCI)	15.03-20.39	-	2.82-3.42	19.03-22.75	-	15.50-20.62	20.19-23.46	2.92-3.53
Cumulative survival (%)	**70.6**	**100**	**0.0**	**83.3**	**100**	**72.2**	**88.2**	**0.0**
Log rank								
χ^2	18.463*		107.953*		16.354*		108.730*	
p	<0.001*		<0.001*		<0.001*		<0.001*	

Cumulative survival: Cumulative proportion surviving at 24 months. 95 % CI: 95 % confidence interval, OS Overall survival, DFS disease-free survival
UCI: Upper Confidence Interval , LCI: Lower Confidence Interval,*: Statistically significant at $p \leq 0.05$



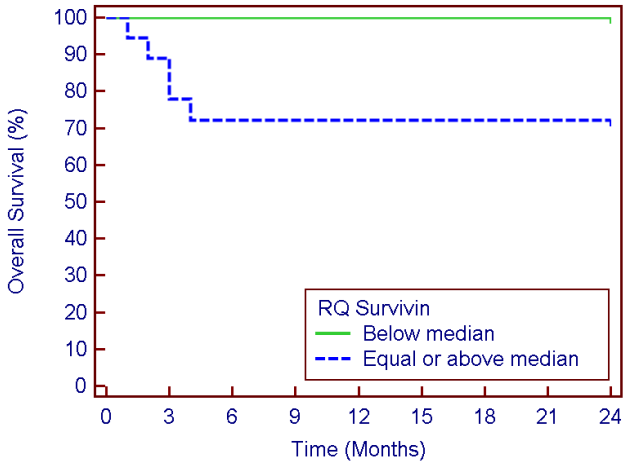
A

Overall survival with Gravin



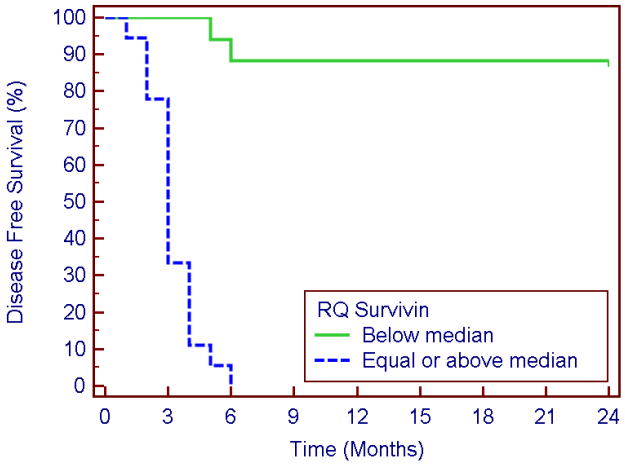
B

Disease free survival with Gravin



C

Overall survival with survivin



D

Disease free survival with survivin

Figure (1): Kaplan-Meier survival curves for overall and disease free survival with Gravin and survivin expression

Discussion

In the present study, gravin expression was significantly lower in AML cases in comparison to control samples (p<0.001). In harmony with our results, Mostafa et al found that the relative quantitative expression of gravin gene was 16-folds lower in patients than in controls [8]. It has been revealed that the expression of gravin was down-regulated in all patients with de novo AML, all CML patients studied and at all phases of the disease concluding that the gravin gene was consistently down-regulated in myeloid malignancies and in several cases showed absent expression.

On the contrary, survivin expression was significantly higher in AML cases in comparison to control samples (p<0.001). This agrees with Sun et al who revealed that survivin expression in AML patients was higher than that of controls (P < 0.001) [9].

Moreover, survivin mRNA was detected in all the cell lines and most of the acute leukemia patients. However in normal peripheral blood and anemia samples, expression of survivin gene was not detected. A quantitative analysis of the expression of the anti-apoptotic gene survivin in malignant haematopoietic cells revealed that survivin mRNA levels were demonstrable in all malignant cell lines, but no survivin mRNA expression was detected in normal leukocyte fractions. Furthermore, in a study on hematopoietic cells transformed by FLT3-ITD (FMS-like tyrosine kinase 3-internal tandem duplication) and frequently expressed in AML, Abe et al demonstrated that the FLT3-ITD mutations increased survivin expression which was associated with enhanced or accelerated cell proliferation [10].

The discrepancy in the expression levels of gravin and survivin between AML patients and controls also agrees with Yildirim et al. who observed that the expression levels of gravin were decreased in 77.5% of AML cases as compared to the control group [11]. Moreover, Azzazi M et al. found that survivin gene was constitutively expressed in AML cases, but was barely detectable in the control group (p< 0.001) [12]. Zaimy et al. found that survivin was one of the genes which were over-expressed in AML and have antiapoptotic properties [13].

Considering the demographic data, the present study identified a significantly lower expression of gravin and a significantly higher expression of survivin in males than in females (p=<0.001). Meanwhile, a significant negative correlation was found between gravin expression and age and a significant positive correlation was found between survivin expression and age (p <0.001). These results are in agreement with another study, which reported a significant positive correlation between survivin expression and age but not with the patients' gender [12]. These data point out that the older the patient, the lower the expression level of gravin and the higher the expression level of survivin and that was significantly predominating in the studied male patients. Wilhelm et al demonstrated that epigenetic silencing of AKAP12 has been linked to the process of tumorigenesis. They found that AKAP12α promoter showed DNA hypermethylation in juvenile myelomonocytic leukemia samples, which was associated with decreased AKAP12α expression and correlated with older age at diagnosis, elevated levels of fetal hemoglobin and poor prognosis [14].

In the current study, it was found that gravin and survivin expression showed no statistically significant correlations with hemoglobin concentration (p= 0.080, p=0.084) or WBC count (p=0.177, p=0.219) respectively. On the other hand, there was a significant positive correlation between gravin and platelet count (p= 0.004) and conversely a significant negative correlation between survivin and platelet count (p= 0.004). These results are in accordance with data reported by Azzazi M et al who noticed that there was no significant correlation between survivin expression and WBC count, hemoglobin, PLT count or bone marrow blasts [12]. Furthermore, Yildirim et al showed no correlation between gravin expression values and WBC count, hemoglobin or platelet count in acute leukemia patients [11]. Regarding bone marrow blasts, there was a statistically significant negative correlation between gravin expression and BM blast percentage (p= 0.010). On the contrary, a significant positive correlation was found between survivin expression and BM blasts (p= 0.013). Likewise, Sadek et al reported a statistically positive

correlation between survivin expression and bone marrow blasts [15]. As regards remission after induction chemotherapy, it was considered that failure of achieving blast clearance from bone marrow aspirates after 1 or 2 weeks of induction chemotherapy a poor prognosis. The response to induction chemotherapy has been evaluated by assessing the degree of residual leukemic infiltration in the bone marrow after 14 days of chemotherapy. In the present study, due to bone marrow aplasia present on day 14 and poor general condition of the patients, therefore the evaluation of response to induction chemotherapy was carried out on day 28 where only 45 patients (42.9%) have achieved a complete remission whereas 60 patients (57.1%) failed to respond to induction therapy. There was a statistically significant association between gravin expression and the clinical outcome of AML patients where it was highest in patients who achieved a complete remission and lowest in relapsed patients and those with induction death (p <0.001). In agreement with these results, Mostafa et al found that gravin showed a significantly lower expression in patients who failed to respond to therapy than those who achieved a complete remission (p<0.001) [8]. Similarly, Raslan H et al detected a significant difference in gravin expression in patients with CR in comparison to those with induction failure (p = 0.032) [16]. As for the risk of relapse, it has been reported that hypermethylation of tumour suppressor genes (TSGs) might be involved in the relapse of childhood ALL. Moreover, Agrawal et al reported that increased levels of (TSGs) methylation in the bone marrow of patients with acute leukemia in clinical remission provide a powerful indicator for a high risk of leukemia relapse in these patients [17].

On the other hand, a significant relation was detected between survivin expression and induction outcome where it was markedly lower in patients with continuous complete remission than in relapsed patients and highest in those with induction death (p <0.001). Concordant with our results, another study by Azzazi M et al had reported that the difference in survivin expression level between patients who achieved a complete remission and those who failed to respond to induction therapy was statistically significant (P = 0.005) [12]. It was also observed that complete remission in survivin-positive AML patients was significantly lower than that reported in survivin-negative patients (P = 0.018). Carter et al. recently profiled survivin expression in samples from 511 newly diagnosed AML patients and found that survivin levels predict poor clinical outcomes in AML [18]. Similarly, Smith et al revealed that high expression of survivin was associated with poor clinical outcome in pediatric AML [19]. This indicates that lower gravin expression and over expression of survivin were associated with an unfavorable response to induction chemotherapy which coincides with the detected correlation in this study with lower platelet count and higher BM blasts.

The present study demonstrated that OS and DFS were lower in patients with low gravin expression than in patients with high gravin expression with a statistical significance (p <0.001). It was reported that the expression levels of gravin were reduced in accelerated phase and blast crisis as compared with chronic phase, and thus, they suggested that a progressive reduction in the expression levels of gravin may be associated with rapid progression and short survival. In contrast, Yildirim et al hypothesized that OS was longer in cases with lower gravin expression [11]. Nevertheless, Mostafa et al found that gravin expression was not correlated to OS or DFS, and this was confirmed by multivariable analysis [8]. The current study also revealed that OS and DFS times were higher in patients with low survivin expression than in patients with high survivin expression with a statistical significance (p <0.001). This goes in harmony with Huang et al who demonstrated that higher levels of survivin correlated with a worse overall survival in AML patients [20].

Conclusions

Survivin over expression and gravin down regulation were significantly associated with adverse clinical outcome and tendency to chemoresistance in acute myeloid leukemia and the

degree of their expression derrangement has been found to be correlated with a lower complete remission rate and shorter overall and disease free survival which renders them as candidate prognostic markers and future targets for adjuvant immunotherapy.

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Literature search, study design, data interpretation and writing.

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Data analysis.

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Data collection.

Conflict of interest:

Authors have no conflict of interest.

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