STUDY OF THE EXPRESSION LEVELS OF SURVIVIN GENE IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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ABSTRACT

Background: Acute myeloid leukemia (AML) is a genetically heterogeneous disease characterized by malignant clonal proliferation of immature myeloid cells. Survivin is a member of the inhibitor of apoptosis proteins (IAPs) gene family. It was found that knockdown of survivin was reported to induce apoptosis in leukemia cell lines and also potentiated the chemotherapeutic antileukemic effects.

Aim: Investigating survivin gene expression levels in de novo acute myeloid leukemia at the time of diagnosis, and the correlation between survivin expression at time of diagnosis and the achievement of complete remission after induction chemotherapy.

Materials and **Methods:** Real time quantitative polymerase chain reaction (PCR) was used and the results were evaluated with comparative Ct method for 35 AML cases controlled by 15 non AML patient.

Results: The present study showed that Survivin expression was significantly higher in cases than in controls ($p<0.001$). The mean expression level of survivin among patients with complete remission (28.64 ± 6.23) was significantly lower than those with induction failure (186.86 ± 229.71) ($p<0.001$). None of AML cases with high expression of Survivin above the median achieved complete remission. The age of AML cases positively correlated to survivin gene expression ($r= 0.615$, $p <0.001$).

Conclusion: Survivin expression analysis at time of diagnosis may aid in determining the prognosis of AML cases.

Key works: Survivin gene expression, Acute Myeloid Leukemia

Introduction: Acute myeloid leukemia (AML) is a genetically heterogeneous disease characterized by malignant clonal proliferation of immature myeloid cells in the bone marrow, peripheral blood, and occasionally other body tissues.[1,2] It is the most common acute leukemia in adults,[3] it accounts for 80 to 90 percent of cases in this group.[4] The number of new cases of acute myeloid leukemia was 4.1 per 100,000 men and women per year. Approximately 0.5 percent of men and women will be diagnosed with acute myeloid leukemia at some point during their lifetime.[5]

In Egypt, leukemia comprises 10% of all malignancies with AML representing 16.9%. [6] Acute myeloid leukemia is classified according to the FAB or WHO classification. In FAB classification (depending on the morphologic, cytochemical, and immunophenotypic features of the malignant cells) AML is classified to M0, M1, M2, M3, M4, M5, M6 and M7.[7] Whereas the WHO classification uses all available information including morphology, cytochemistry, immunophenotyping, incorporation with the underlying cytogenetic or molecular genetic abnormalities and clinical features to categorize the acute leukemias and provide a new classification which can be used in daily clinical practice.[8] The diagnosis of AML requires examination of both; peripheral blood samples and bone marrow aspirates/biopsies. The first clue to a diagnosis of AML is typically an abnormal result on a complete blood count. The hallmark of leukemia is the reduction or absence of normal hematopoietic element.[9,10] The peripheral blood usually shows anemia, thrombocytopenia, neutropenia and leukocytosis with the presence of blast cells in the circulation.[11] Examination of bone marrow is crucial to establish the diagnosis of AML. Bone marrow aspirates and biopsy samples demonstrate the characteristic replacement of normal marrow elements with the sheets of leukemic blasts.[12,13]

Survivin is a member of the inhibitor of apoptosis proteins (IAPs) gene family.[14-16] It is expressed in most human tumors but is largely undetectable in normal differentiated tissues and correlates with reduced tumor cell apoptosis in vivo, abbreviated patient survival, accelerated rates of recurrences, and increased resistance to therapy.[15] Survivin is a structurally and functionally unique protein of this family.[17,18] Overexpression of several IAPs has been detected in various hematological malignancies, including acute leukemias.[19-21] It was found that knockdown of survivin was reported to induce apoptosis in leukemia cell lines and also potentiated the chemotherapeutic antileukemic effects.[22] Thus, survivin could be used as an important molecular marker and target in a variety of cancer prognoses and therapeutics.

Aim of the work: The aim of the present study is to investigate survivin gene expression levels in Egyptian patients with de novo acute myeloid leukemia at the time of diagnosis, and to evaluate the correlation between the expression levels of survivin at time of diagnosis and the achievement of complete remission after induction chemotherapy.

Materials and Methods. Subjects: The current study was carried out on fifty patients; thirty-five adult patients with de novo AML with age ranging from 18 to 65 years, and a control group consisting of fifteen hospitalized patients of matched age and sex with no malignant hematological disease to whom bone marrow aspiration is one of the required investigation. Patients were selected from the outpatient of the Alexandria University Hospitals. An informed written consent was obtained from each patient, and the study protocol was approved by the Alexandria Faculty of Medicine ethics committee.

The laboratory investigations analyzed included (1) examination of peripheral blood (PB) smears stained with Leishman's stain.[23] (2) examination of bone marrow (BM) aspirate smears (Leishman's stain).[24] (3) Blood chemistry investigations including ALT, AST, serum urea, serum creatinine, Serum alkaline phosphatase (ALP), Serum lactate dehydrogenase (LDH) and serum uric acid using the Dimension RxL Max chemistry auto-analyzer (Siemens, USA).[25] (4). Immunophenotyping by flowcytometry.[26] Immunophenotyping of the leukemic blast cells was performed on PB or BM samples using Miltenyi Biotec MACSQuant™ flowcytometry analyzer equipped with MACS Quantify software version 2.4. Monoclonal antibodies (DAKO-USA)[27] labelled with Fluorescein isothicyanate (FITC) or phycoerythrin (PE) were used for immunophenotyping. (5). Quantitative determination of the expression levels of Survivin gene by real time-PCR technique. [28-30]

Statistical analysis: The raw data were coded and transformed into coding sheets. The results were checked. Then, the data were entered into SPSS system files (SPSS package version 20). The following statistical measures were used; Descriptive statistics including frequency, distribution, mean, median, interquartile and standard deviation were used to describe different characteristics. Chi- square test (X2), Fisher Exact test and Mont Carlo correction were used to detect statistical difference between stigma and different factors. Mann Whitney U and Kruskal-Wallis H test to detect difference between small groups. Spearman correlation co-efficiency to measure the strength of correlation between healthcare discrimination and different factors. Survival analysis was conducted regarding gene expression of Survivin gene.
The significance of the results was at the 0.05 level of significance and confidence interval was 95%.

RESULTS: The current study was conducted on 35 newly diagnosed adult patients with de novo AML admitted to the hematology unit at Alexandria main university hospital and 15 patients as controls. The RBC count was significantly lower in cases than in controls ($p<0.001$). Platelet count was significantly lower in cases than in controls ($p<0.001$). BM blast percentage was significantly higher in cases than in controls. The mean of both LDH and ALP was significantly higher in cases than in controls ($p=0.003$, $p=0.027$) respectively (Table I). The median of survivin expression was significantly higher in cases than in controls ($p<0.001$) (Table II).

Fifteen (42.9 %) patients of the 35 AML cases enrolled in this study achieved a complete remission, while 20(57.1 %) had induction failure. There was no statistically significant difference found between AML patients with different FAB subtypes as regards the clinical outcome (MCp = 0.541) (Table III). The mean expression level of survivin among patients with complete remission (28.64 ± 6.23) was significantly lower than those with induction failure (186.86 ± 229.71) ($p<0.001$) (Table IV). Seventeen AML cases showed survivin expression levels below the median, while eighteen cases showed expression levels above the median. Among AML cases with survivin expression below the median, 15 cases (88.2 %) have achieved a complete remission, while only 2 cases (11.8 %) had induction failure. On the other hand, none of the eighteen AML cases with survivin expression above the median (100 %) have achieved a complete remission. The number of cases with survivin expression above the median who failed to respond to therapy was significantly higher than those with survivin expression below the median ($p<0.001$) (Table V). The age of AML cases positively correlated to survivin

gene expression (r= 0.615, p <0.001).Thus the older the patient, the lower the expression of gravin and the higher the expression of survivin (Figure 1). None of the correlations between survivin expression and the hematological parameters was statistically significant (Table VI). Overall survival (OS) and disease free survival (DFS) were higher in patients with survivin expression below median level (cumulative survival; 100.0%, 88.2%; 10, 12 months) than in patients with survivin expression above median (cumulative survival 82.2%, 27.8%; 12, 12 months), but without a statistical significance (p = 0.843, 0.970, respectively) (Table VII, Figures 2, 3).

Table I: Demographic data and laboratory investigations of cases and controls

	Cases (n =35)		Control (n = 15)		Test of sig.	P
	No	%	No	%		
Sex						
Male	27	77.1	9	60.0	χ^2=1.531	^{FE}p= 0.304
Female	8	22.9	6	40.0		
Age (years)						
Min. – Max.	18.0 – 65.0		20.0 – 60.0		t=0.278	0.783
Mean ± SD.	42.0 ± 13.43		40.87 ± 12.74			
Median	45.0		38.0			
HB(g/dl)						
Min. – Max.	4.80 – 13.0		8.0 – 19.50		t=2.017	0.058
Mean ± SD.	9.40 ± 1.91		11.17 ± 3.15			
Median	9.50		10.0			
RBC (10^6/µl)						
Min. – Max.	1.45 – 4.49		2.80 – 6.30		t=4.523*	<0.001*
Mean ± SD.	3.20 ± 0.68		4.23 ± 0.85			
Median	3.14		4.10			
WBC(10^3/µl)						
Min. – Max.	0.92 – 142.70		2.10 – 10.50		Z=0.847	0.397
Mean ± SD.	32.74 ± 44.41		5.92 ± 2.33			
Median	10.0		5.90			
Platelets(10^3/µl)						
Min. – Max.	8.0 – 134.0		75.0 – 480.0		Z=4.563*	<0.001*
Mean ± SD.	59.91 ± 36.81		218.13 ± 119.55			
Median	56.0		230.0			
Blast percent in BM						
Min. – Max.	24.0 – 95.0		0.0 – 5.0		Z= 3.021	0.002*
Mean ± SD.	58.0 ± 21.0		1.82 ± 1.21			
Median	57.0		2.0			
ALT (U/L)						
Min. – Max.	25.0 – 102.0		36.0 – 64.0		Z=0.095	0.924
Mean ± SD.	51.14 ± 18.50		49.07 ± 9.62			
Median	51.0		47.0			
AST(U/L)						
Min. – Max.	16.0 – 88.0		18.0 – 35.0		Z=1.283	0.200
Mean ± SD.	33.49 ± 14.73		27.87 ± 5.67			
Median	31.0		29.0			
Urea(mg/dl)						
Min. – Max.	11.0 – 90.0		16.0 – 42.0		0.127	0.899
Mean ± SD.	30.17 ± 15.13		28.87 ± 8.72			
Median	29.3		28.0			
Creatinine(mg/dl)						
Min. – Max.	0.60 – 3.60		0.60 – 1.0		1.176	0.240

Mean ± SD.	0.94 ± 0.52	0.88 ± 0.12		
Median	0.80	0.90		
LDH (U/l)				
Min. – Max.	101.0 – 250.0	103.0 – 161.0		
Mean ± SD.	152.17 ± 45.47	123.73 ± 17.79	3.176*	0.003*
Median	124.0	118.0		
ALP (U/l)				
Min. – Max.	54.0 – 144.0	59.0 – 130.0		
Mean ± SD.	102.37 ± 28.14	85.13 ± 22.33	2.307*	0.027*
Median	97.0	84.0		
Uric acid (mg/dl)				
Min. – Max.	3.0 – 7.0	3.70 – 7.10		
Mean ± SD.	4.85 ± 1.21	5.30 ± 1.19	1.210	0.232
Median	4.20	5.0		

Table II: Comparison between the studied groups according to survivin relative quantitative expression

	Cases (n = 35)		Control (n = 15)		Test of sig.	p
	No.	%	No.	%		
Survivin						
Low expression	17	48.6	8	53.3	χ^2= 0.009	0.997
High expression	18	51.4	7	46.7		
Min. – Max.	21.04 – 1124.24		0.00021 – 5.045		Z=5.557*	<0.001*
Mean ± SD.	119.05 ± 189.25		1.0 ± 1.60			
Median	71.25		0.123			

χ^2: Chi square test
Z: Z value for Mann Whitney test
*: Statistically significant at $p \leq 0.05$

Table III: Comparison between AML patients with different FAB subtypes as regards the clinical outcome

n=35	Outcome				χ^2	p
	Complete Remission (n = 15)		Induction Failure (n=20)			
	No.	%	No.	%		
FAB						
M1	2	13.3	1	5.0	4.431	^{MC}p = 0.541
M2	5	33.3	4	20.0		
M3	1	6.7	0	0.0		
M4	4	26.7	6	30.0		
M5	3	20.0	8	40.0		
M6	0	0.0	1	5.0		

χ^2: Chi square test
^{MC}p: Monte Carlo for Chi square test for comparing between group A and B
SD: Stranded Divination
* Statistically significant at $P \leq 0.05$

Table IV: Survivin expression in AML patients with different clinical outcome

	Survivin				χ^2	p
	Low expression (n =17)		High expression (n = 18)			
	No	%	No	%		
Outcome						
Complete remission	15	88.2	0	0.0	27.744*	<0.001*
Induction Failure	2	11.8	18	100.0		

χ^2: Chi square test
*: Statistically significant at $p \leq 0.05$

Table V: Comparison between survivin expression in AML patients with different clinical outcomes

		N	Median	Min. – Max.	Mean ± SD.	Z	p
Outcome	**Complete remission**	**15**	25.90	21.04 – 42.66	28.64 ± 6.23	5.000*	<0.001*
	Induction failure	**20**	122.76	55.90 – 1124.24	186.86 ± 229.71		

Z, p: Z and p values for Mann Whitney test for comparing between the two groups
*: Statistically significant at $p \leq 0.05$

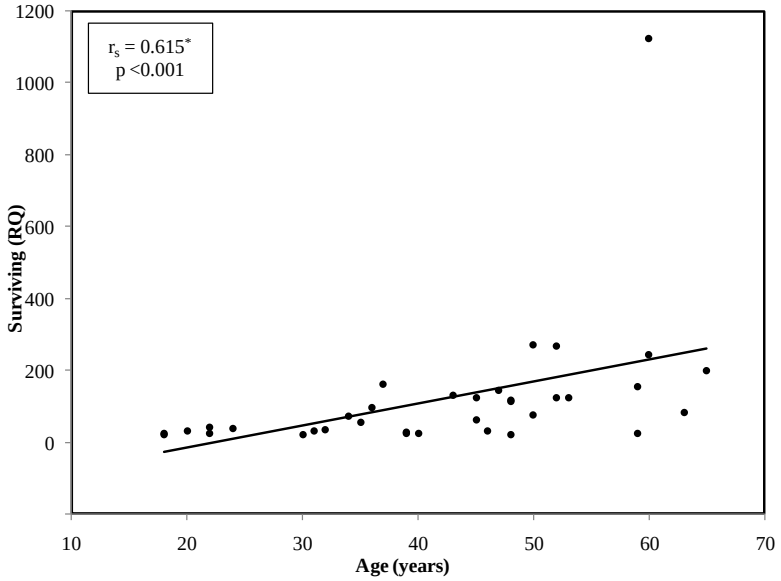

Figure (1): Correlation between survivin expression and age in AML cases.

Table VI: Correlation between survivin expression and the different hematological parameters in the studied AML cases

Hematological profile	Survivin (RQ)	
	r_s	p
Hb (g/dl)	-0.170	0.330
WBC (10^3/µl)	-0.106	0.545
RBC (10^6/µl)	0.064	0.714

Haematocrit	0.121	0.489
Platelets (10^3/µl)	-0.281	0.102
BM Blast %	0.242	0.161

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

Table VI: Survival times according to survivin expression in AML patients

	Survivin expression below median				Survivin expression equal or above median				
			95 % CI				95 % CI		
	Cumulative survival (%)	Median (Months)	UCI	LCI	Cumulative survival (%)	Median (Months)	UCI	LCI	p log-rank (Mantel-Cox)
OS	100.0%	10	7.98	12.02	82.2%	12	1.3	22.7	0.843
DFS	88.2%	11	9.1	12.9	27.8%	12	8.5	15.5	0.97

Cumulative survival: Cumulative proportion surviving at 12 months. 95 % CI: 95 % confidence interval, OS Overall survival, DFS disease-free survival
UCI: Upper Confidence Interval
LCI: Lower Confidence Interval
*: Statistically significant at $p \leq 0.05$

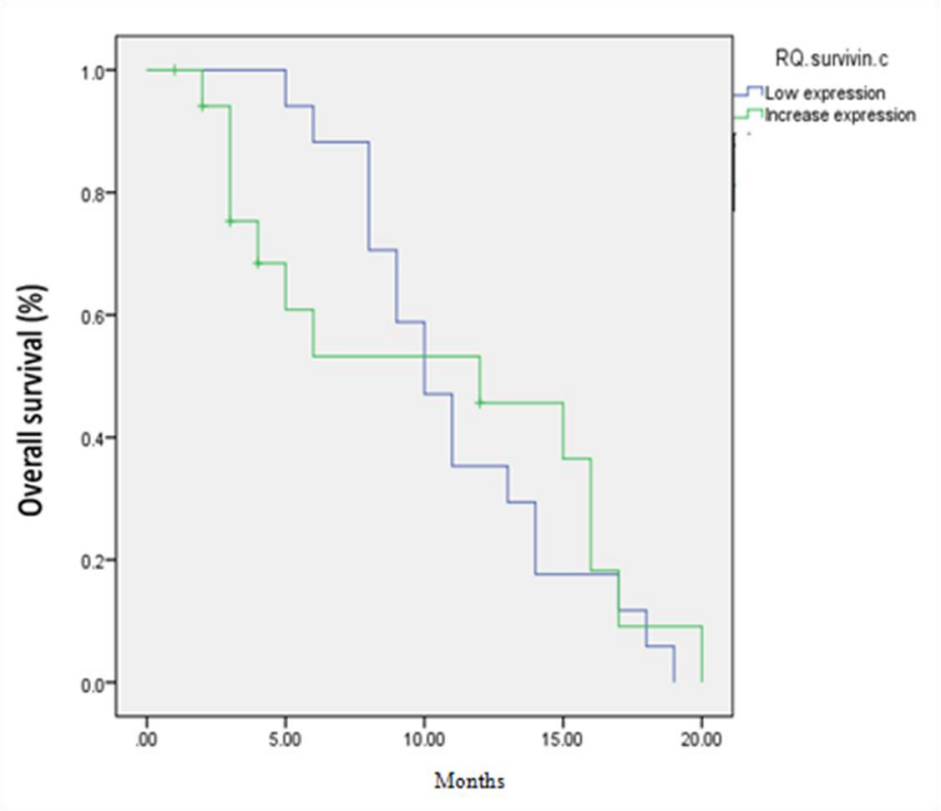

Figure (2): Overall survival according to survivin expression in AML patients

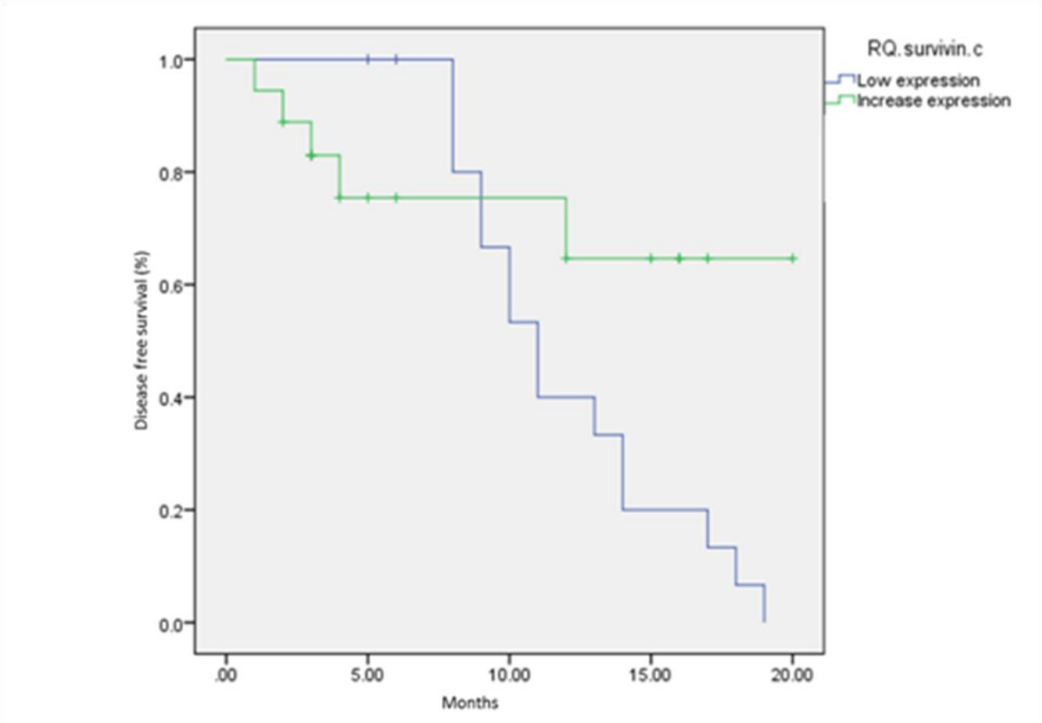

Figure (3): Disease free survival according to survivin expression in AML patients

Discussion: AML is the most common acute leukemia in adults, [12] it accounts for 80 to 90 percent of cases in this group.[3] AML is a curable disease; the outcome for an

individual patient depends on a number of prognostic factors including age at diagnosis and white cell count at presentation. However, the genetic abnormalities in the tumour are the most important determinant. [31, 32] Advances in molecular oncology have revealed various roles that oncogenes play in the development of cancer. Survivin has been identified as one of the top 4 transcripts among 3.5 million human transcriptomes uniformly up-regulated in cancer tissues but not in normal tissues.[33]

The present case-control study was conducted on fifty subjects, thirty five (27 males, 8 females) adult de novo AML patients before starting induction of chemotherapy and fifteen volunteers as a control group (9 males, 6 females), both groups were assayed using real time quantitative PCR to analyze mRNA expression for survivin gene. In our study, no significant difference was found between AML cases and controls as regards haemoglobin concentrations or white blood cell count (WBC), also there was no significant difference regarding alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum urea, serum creatinine and serum uric acid concentrations.

Unlike us, the study done by Sadek H et al. [34] which included 14 adult patients with untreated de novo AML patients had significant difference between AML cases and controls as regards hemoglobin concentrations (p=0.0001), WBC (p=0.002) and uric acid concentrations (p=0.0001). In contrast to our results, a study done by Raslan H et al. [35] which was conducted on 40 leukemia patients of which 14 had AML, and 10 healthy volunteers included as controls, reported a statistically significant difference was found when comparing AML cases and control group regarding haemoglobin level and WBC and serum uric acid level.

According to our study, red blood cell count (RBC) and platelets count were significantly lower in AML cases than in controls ($p<0.001$), agreeing with findings of Sadek H et al. [34] who found significant difference between AML cases and controls as regards RBC (p=0.0001), platelets (p=0.0001) (290). like us, Raslan H et al.[35] found a statistically significant difference between AML cases and controls regarding mean platelet count. Here, we found that the means of both lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were significantly higher in AML cases than in controls. In concordance with our results, Raslan H et al.[35] showed that significant difference was detected between AML patients and controls regarding LDH values.

Regarding the median of survivin expression, we found that it was significantly higher in AML cases than in controls ($p<0.001$). This comes in consistency with results found by Azzazi M et al.[36] who conducted their study on 120 patients with untreated de novo AML and showed that survivin gene expression in AML patients was significantly higher when compared with control group ($P < 0.001$). Like our study, the study done by Sun et al.[37] which included 63 newly diagnosed AML patients revealed that survivin expression in AML patients was higher than that of controls ($P < 0.01$). Zhu et al.[38] conducted their study on 48 AML cases and found that survivin expression was significantly higher in AML patients than that of controls. Also, Mori et al.[39] examined 31 AML patients for the expression of survivin by reverse transcriptase–PCR (RTPCR) and revealed that the expression of survivin was found in all AML cases, although none of the healthy controls showed survivin expression.

Concordant with our results, the results found by Azzazi M et al.[36] who declared that difference in survivin expression level between patients who achieved CR and those who did not achieve CR was statistically significant ($P = 0.005$). Also, the study done by Ibrahim et al.,[29] who conducted their study on 30 patients with de novo AML and showed that 81.2% of patients with survivin expression failed to achieve CR. Zhu

et al.,[38] also showed that CR rate in survivin-positive AML patients was found to be significantly lower than that reported in survivin-negative patients (P = 0.018). (207) Further data reported by Sun et al.,[37] showed that survivin-positive patients had lower CR and higher relative relapse rates; however, this was not statistically significant.

Adida et al.[40] showed that the difference in CR or survival in adult AML patients expressing high levels of survivin versus those with low levels of survivin was not significant, which is contrary to our study, where the mean expression level of survivin among patients with CR (28.64 ± 6.23) was significantly lower than those with induction failure (186.86 ± 229.71) ($p<0.001$). This difference in results might be due to the high level of quality of health services and patient follow up.

As regards the survival analysis of survivin, the OS and DFS times were higher in patients with survivin expression below median level (cumulative survival; 100.0%, 88.2%; 10, 12 months) than in patients with survivin expression above median (cumulative survival 82.2%, 27.8%; 12, 12 months), but without a statistical significance (p = 0.843, 0.970, respectively). However, literature mentioned a trend for a shorter overall survival in survivin-positive patients when compared with the survivin-negative group (P =0.15 by the log-rank test). The median overall survival was 20.5 months vs. 43.9 months in the survivin-positive and survivin-negative groups respectively.[37]

Also, Mori et al.[39] demonstrated that the leukemia-free survival rate at 35 months was significantly lower in patients with survivin expression than in patients without survivin expression (P < 0.02 for acute leukemia and P <0 .03) for AML. This is supported by Azzazi M et al., [36] where the difference in mean overall survival (OS) between AML patients with positive survivin expression (mean: 15 days) and AML patients with negative surviving expression (mean: 222.2 days) was statistically significant (log-rank: 3.940, P = 0.047). Results of Ibrahim et al.[29] went in the same direction where patients with over expression of survivin showed induction failure 81.2% as well as, shorter median survival time (30 days) compared to patients with normal controls (150 days). Moreover, Netterwald [41] conducted a study on 511 newly diagnosed AML patients had reported that higher survivin levels predicted shorter OS (p=0.016) and disease-free survival (p=0.023) survival.

Conclusion: Survivin expression is higher in AML cases than controls ($p<0.001$). Survivin expression is lower in AML patients with complete remission than in patients with induction failure ($p<0.001$), thus it would be worthy to evaluate the expression level of survivin gene as prognostic marker in various myeloid neoplasms.

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