Prognostic impact of Annexin A1 expression in acute myeloid leukemia

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Objective The aim was to assess expression of Annexin A1 (ANXA1) on malignant myeloid blast cells and its correlation with clinical outcome, overall survival (OS), and other prognostic factors among adult Egyptian patients with acute myeloid leukemia (AML).

Patients and methods A total of 60 patients with de novo AML were treated and followed up in Ain Shams University Hospitals, Hematology Unit and compared with 20 age-matched and sex-matched normal healthy controls. Expression of ANXA1 was detected by flow cytometry in bone marrow samples.

Results Patients with AML had significant higher mean ANXA1 expression than mean ANXA1 expression in control at D0.
The mean ANXA1 level in the favorable cytogenetics group was significantly higher than the mean ANXA1 level in the unfavorable cytogenetics group. ANXA1 was positive in 76.7% patients with AML and negative in 23.3% patients with AML.
There was a significant difference between ANXA1-positive group and ANXA1-negative group of patients with AML regarding different outcomes. In ANXA1-positive group, 52.2% of patients with AML achieved complete remission (CR), whereas in ANXA1-negative group, 14.3% of patients with AML achieved CR. Highest mean ANXA1 expression was in the group of patients who had CR. High ANXA1 expression was associated with longer OS.

Conclusion ANXA1 is significantly expressed in Egyptian patients with de novo AML, and its expression associated with favorable prognostic effect on clinical outcome and longer OS.

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Introduction

Acute myeloid leukemia (AML) is a malignant disorder of hemopoietic stem cells with variable outcomes and characterized by clonal proliferation of myeloid precursors and accumulation of leukemic blasts in bone marrow (BM), ultimately resulting in various cytopenias owing to BM failure [1].

AML is the most common type of acute leukemia in adults, accounts for ~80% of cases, and is a highly heterogeneous and fatal disease [2].

Annexin A1 (ANXA1) is a member of the Annexin family and belongs to calcium-dependent phospholipid-binding protein. It was previously found that ANXA1 was an intracellular inflammation-related factor that regulates the anti-inflammatory effects of glucocorticoids, and it has also been shown to be involved in the regulation of cell proliferation, differentiation, and apoptosis, as well as development or progression of tumors [3].

The role of ANXA1 in tumors is paradoxical, as ANXA1 appears to behave either as a tumor suppressor or an oncogenic gene. As the mechanism of ANXA1 in cancer progression has not been still completely clarified, more studies are required to investigate the detailed action mechanisms of this protein in tumors [4].

However, few studies have been conducted until now concerning the role of Annexins in AML. These studies have shown that ANXA1 expression analysis may help to understand the development of different phenotypes and the biological functions of ANXA1 in AML. The primary aim of the present study was to assess the expression level of ANXA1 in Egyptian adult patients with AML.

Patients and methods

A total of 60 Egyptian patients with untreated de novo AML were included in this study, who were treated

and followed up in Hematology Unit of Ain Shams University Hospitals for 1 year. Informed consent was taken from all participants before their inclusion in the study.

Patients comprised 26 (43.3%) males and 34 (56.7%) females. Their age ranged between 18 and 59 years, with a mean of 36.08±11.2 years. All patients were newly diagnosed and did not receive any treatment for AML. The control group included 20 age-matched and sex-matched normal healthy volunteers, comprising eight (40%) males and 12 (60%) females. Their age ranged between 18 and 58 years, with a mean of 36.6±11.7 years.

All patients were treated according to Ain Shams Hematology Unit's ongoing induction and consolidation regimens. Patients were followed up for one and a half year.

Apart from AML-M3, patients received induction chemotherapy under a 3+7 regime: (a) daunorubicin at 25 mg/m^2/day for 3 days and (b) cytarabine (Ara-c) at 200 mg/m^2/day for 7 days of continuous intravenous infusion.

(a) Patients who entered into remission received four courses of high-dose Ara-c as consolidation. This was Ara-c 1.5–3.0 g/m^2 on 2-h infusion every 12 h on days 1, 3, and 5.
(b) For unfavorable risk group, allogeneic BMT was carried out if a suitable donor was available.
(c) If the patient did not enter into remission, this protocol was repeated. If there was no or minimal response, patients were shifted to high-dose chemotherapy.
(d) Patients with AML-M3 received induction with all-transretinoic acid (ATRA) at 45 mg/m^2 daily and idarubicin at 12 mg/m^2 intravenous on days 2, 4, 6, and 8.
(e) Patients received consolidation therapy consisting of ATRA at 45 mg/m^2/day for 15 days together with idarubicin at 5 mg/day on days 1–4 (consolidation 1), mitoxantrone at 10 mg/m^2/day on day 1 (consolidation 2), and idarubicin at 12 mg/m^2/day on day 1 (consolidation 3).
(f) Patients received maintenance treatment consisting of ATRA at 45 mg/m^2/day for 15 days every 3 months plus 6-mercaptopurine at 90–100 mg/m^2/day plus methotrexate at 15 mg/m^2/week, all for 2 years.

Patients with AML in relapse and patients younger than 18 years or older than 60 were excluded from the study.

Methods

All patients were subjected to thorough assessment of history, complete clinical examination, and routine radiological and laboratory investigation, including the following:

(1) Complete blood counts on Coulter LH 750 cell counter (Coulter Electronics, Hialeah, Florida, USA) with examination of peripheral blood smears stained with Leishman stain.
(2) PB samples and BM aspiration samples were collected at diagnosis from the patients, whereas BM samples were obtained from the control group.
(3) BM aspiration with morphological examination of Leishman stained smears.
(4) Flow cytometric immunophenotyping was performed on BM samples using a standard panel of monoclonal antibodies (MoAb) on Coulter Epics XL 4-color flow cytometer (Coulter Electronics).
(5) Karyotyping and other specific cytogenetic analysis that have favorable prognosis results were done with respect to t (8;21), t (15;17), and inversion 16.
   (a) Favorable cytogenetics were assigned if the patients were positive for t (8;21), t (15;17), and inversion 16.
   (b) Unfavorable cytogenetics were assigned if the patients were negative for t (8;21), t (15;17), and inversion 16.

Detection of Annexin A1 by flow cytometric immunophenotyping

Detection of ANXA1 by flow cytometric immunophenotyping in myeloid blast cells was done by flow cytometry on BM samples at day 0 (D0) and D28 and then every 6 months.

(1) Specific isotypic controls for FITC and PE-conjugated MoAb are used.
(2) Purified anti-ANXA1 primary antibody and FITC anti-mouse IgG1 secondary antibody were supplied from Biolegend (San Diego, California, USA).
(3) BM aspirate samples were processed on the same day of sample collection. They were counted using Coulter Cell Counter, and the total leukocytic count was adjusted to be around 5.0×10^9/l using phosphate buffered saline (PBS) 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4 (commercially available from Sigma, St Louis, Missouri).
(4) Overall, 100 µl of adjusted sample was aliquoted in the control tube as well as each sample tube and then 20 µl of each MoAb or its isotypic control was added.

(5) Before MoAb addition, samples were washed by 3 ml PBS, vortexed for 3–5 s, followed by centrifugation for 5 min at 300*g* at room temperature, and supernatant was removed by aspiration. IntraPrep cytoplasmic permeabilization kit (Coulter Electronics) was used according to manufacturer instructions. Overall, 10 µl from anti-ANXA1 antibody was added to samples and control tubes followed by 15-min incubation in the dark at room temperature followed by washing by PBS. Then 10 µl from the FITC-labeled secondary antibody was added to samples and control tubes followed by 15-min incubation in the dark at room temperature followed by washing by PBS.
(6) The control and test tubes were incubated for 15 min at room temperature, protected from light.
(7) After incubation, 1–2 ml of ammonium chloride-based erythrocyte lysing solution was added to every tube [8.29 g (0.15 M) NH_4Cl, 1 g (10 mmol/l) $KHCO_3$, 0.037 g (0.1 mmol/l) EDTA, 1 l distilled water, adjusted to pH 7.3]. Tubes were vortexed then analyzed using Coulter Epics XL flow cytometer (Coulter Electronics).

Results were expressed as percentage of blast cells expressing the tested MoAb, where expression was considered positive if more than or equal to 20%.

Ethical considerations

An approval for the study were obtained from the Ethical Committee in the Faculty of Medicine in Sohag University and Ain Shams University.

Any data taken from the patients, either from the history, the examination, or the investigations were dealt with in a confidential manner.

Written informed consent form was obtained from the patient.

Statistical analysis

Data were analyzed using STATA, version 14.2 (Stata Statistical Software: Release 14.2; Stata Corp LP, College Station, Texas, USA). Quantitative data were represented as mean, SD, median, and range. Data were analyzed using Student *t* test to compare means of two groups and analysis of variance for comparison of the means of three groups or more. When the data were not normally distributed, Kruskal–Wallis test was used for comparison of three or more groups, and Mann–Whitney test was used to compare two groups. Qualitative data were presented as number and percentage and compared using either χ^2 test or Fisher exact test. Spearman's rank correlation analysis was used to detect the correlation between Annexin and different parameters. Survival analysis was carried out using the Kaplan–Meier test, and comparison of survival between groups was made with the log-rank test. Graphs were produced by using Excel or STATA program. *P* value was considered significant if it was less than 0.05.

Results

The current prospective study included 60 adult patients with newly diagnosed AML, with a mean age of 36.08±11.2 years. Among controls, the mean age was 36.6±11.7 years. The study included 34 (56.7%) female patients and 26 (43.3%) males, and whereas the control group included 12 (60%) females and eight (40%) males (Table 1).

Clinical, laboratory, and radiological characteristics of patients with AML are shown in Table 2.

Annexin A1 expression in patients with acute myeloid leukemia and control group

Patients with AML had statistically higher mean ANXA1 expression (57.1±28.0) as compared with mean ANXA1 expression in control group (6.28 ±5.4) at D0, with *P* value less than 0.0001 (Table 1). Highest ANXA1 level was seen in AML-M0 [French American British (FAB) subtypes] followed by M3, M4, M2, M5, and M1 subtype.

Table 1 Characteristics of controls and patients

Variables	patients with AML (*N*=60)	Control (*N*=20)	*P* value
Age (years)			
Mean±SD	36.08±11.2	36.3±11.7	0.86
Range	18–59	18–58	
Sex [n (%)]			
Male	26 (43.3)	8 (40.0)	0.60
Female	34 (56.7)	12 (60.0)	
ANXA1 expression			
Mean±SD	57.1±28.0	6.28±5.4	<0.0001**
Range	3.35–90.6	1.33–18.5	

AML, acute myeloid leukemia; ANXA1, Annexin A1. **P value less than 0.01, highly significant.

Table 2 Clinical, laboratory, and radiological characteristics of patients with acute myeloid leukemia

Variables	*n* (%)	Mean±SD
Manifestations of anemia	57 (95)	
Bleeding manifestations	28 (46.7)	
Infections	19 (31.7)	
Respiratory	13 (21.7)	
Gastrointestinal	5 (8.3)	
Genitourinary	3 (5)	
Skin	1 (1.7)	
CNS	1 (1.7)	
Constitutional symptoms		
Fever	28 (46.7)	
Weight loss	25 (41.7)	
Night sweats	19 (31.7)	
Bone pain	5 (8.3)	
Splenomegaly	22 (36.7)	
Spleen size (cm)		
Mean±SD		12.8±2.9
Range		8.5–18.6
Hepatomegaly	32 (53.3)	
Liver size (cm)		
Mean±SD		16.4±2.4
Range		13–22
Enlarged LNs	12 (20)	
WBCs		
Mean±SD		22.3±25.6
$>11\times10^9$/l	30 (50)	
$4–11\times10^9$/l	18 (30)	
$<4\times10^9$/l	12 (20)	
Hemoglobin (g/dl)		
Mean±SD		7.6±2.0
MCV (fl)		
<80	7 (11.7)	
80–100	49 (81.7)	
<100	4 (6.7)	
Anemia	60 (100)	
Platelets		
Mean±SD		49.3±51.2
$>150\times10^9$/l	3 (5)	
$100–150\times10^9$/l	4 (6.7)	
$<100\times10^9$/l	53 (88.3)	
Peripheral blast (%)		
Mean±SD		47.1±29.9
Range		3–97
ESR 1st h		
Mean±SD		98.1±29.3

CNS, central nervous system; ESR, erythrocyte sedimentation rate; MCV, mean corpuscular volume; WBC, white blood cell.

However, there was no statistically significant difference in mean ANXA1 expression among different FAB subtypes (P=0.08) (Table 3).

Among the 34 patients with unfavorable cytogenetics, 12 (85.7%) were negative for ANXA1 expression and 22 (47.9%) were positive for ANXA1 expression. Among the 26 patients with favorable cytogenetics, two (14.3%) were negative for ANXA1 expression and 24 (52.1%) were positive for ANXA1 expression (Table 4).

Table 3 Comparison among patients with different French American British subtypes regarding Annexin A1 expression at the time of diagnosis

FAB subtype	ANXA1 expression		*P* value
	Mean±SD	Median (range)	
M0	80.6±8.7	76.4 (76.9–90.6)	0.08*
M1	41.4±31.02	39.6 (3.4–83.4)	
M2	60.7±26.9	74.3 (12.5–88.8)	
M3	68.8±11.9	62.4 (60.3–90)	
M4	67.5±21.6	76.5 (35.3–81.5)	
M5	54.3±32.7	74.3 (18.2–80.7)	

ANXA1, Annexin A1; FAB, French American British classification.
*P value less than 0.05, significant.

The mean ANXA1 level in the favorable cytogenetics group was 67.7±22.9, whereas the mean ANXA1 level in the unfavorable cytogenetics group was 48.9±29.1. When compared with the mean ANXA1 level at diagnosis (D0) according to cytogenetics risk analysis, the difference was highly statistically significant (P=0.009) (Table 6).

Comparison between ANXA1-positive and ANXA1-negative patients with AML is shown in Table 4.

Annexin A1 expression in relation to response to therapy

A high statistically significant difference was detected between mean ANXA1 expression before and after 28 days of induction chemotherapy (P<0.001). Moreover, there was a highly statistically significant difference between mean ANXA1 expression before and after 6 months of induction chemotherapy (P=0.001) (Table 5).

With respect to response to induction chemotherapy, a high statistically significant difference (P<0.001) was found when comparing mean ANXA1 expression at time of diagnosis (D0) in patients who had favorable response [complete remission (CR)], failed to achieve response, and who died (mean ±SD=72.9±21.1, 44.6±29.6, and 45.01±26.0, respectively) (Table 6).

Correlation analysis

Correlation studies between ANXA1 levels at the time of diagnosis and flow cytometry of BM showed statistically significant positive correlation between level of ANXA1 and level of myeloperoxidase (MPO) and CD64 in patients with AML (r=0.35, P=0.01, and r=0.28, P=0.03, respectively) (Table 7).

Table 4 Comparison between Annexin A1-positive and Annexin A1-negative patients with acute myeloid leukemia

Characteristic	*n* (%)	ANXA1 positive (*N*=46) (mean±SD)	ANXA1 negative (*N*=14) (mean±SD)	*P* value
Age (years)		36.30±11.2	35.36±11.3	0.74
WBCs (×10^3/μl)		22.30±26.03	22.61±24.90	0.97
Hb (g/dl)		7.54±1.90	7.89±1.90	0.55
PLTs (×10^3/μl)		51.59±48.80	42.07±59.91	0.54
BM blast (%)		67.28±23.99	70.79±14.19	0.61
Peripheral blast (%)		45.46±31.14	52.64±25.84	0.44
ESR (1st h)		101.50±28.84	87.00±29.05	0.11
Flow cytometry				
CD34		60.70±36.15	67.88±28.9	0.50
CD13		84.17±12.3	79.8±6.7	0.11
CD33		89.77±11.78	88.05±21.76	0.64
CD117		75.94±23.36	87.19±7.45	0.08
MPO		72.89±21.61	58.14±30.22	0.03*
HLADR		73.89±29.99	89.77±7.46	0.48
CD14		2.35±2.34	3.95±3.60	0.05
CD15		8.87±8.00	7.26±8.37	0.52
CD64		27.09±27.62	14.76±22.92	0.13
CD5		3.33±2.55	2.32±2.33	0.19
CD7		12.19±11.65	9.49±8.38	0.42
CD19		7.02±12.62	15.37±30.22	0.14
AML subtype				
M0	3 (5.0)	3 (6.5)	0	0.14
M1	17 (28.3)	10 (21.7)	7 (50.0)	
M2	24 (40.0)	19 (41.3)	5 (35.7)	
M3	7 (11.3)	7 (15.2)	0	
M4	4 (6.7)	4 (8.7)	0	
M5	5 (8.3)	5 (10.9)	2 (14.3)	
Cytogenetic abnormalities				
Normal karyotyping	31 (51.7)	21(45.7)	10 (71.43)	0.55
t (8,21)	16 (26.7)	14 (30.4)	2 (14.9)	
t (15,17)	7 (11.7)	7 (15.22)	0	
Y	3 (5.0)	0	1 (7.14)	
Trisomy 21	1 (1.7)	1 (2.17)	0	
Trisomy 8	1 (1.7)	0	1 (7.14)	
t (16,16)	1 (1.7)	3 (6.52)	0	
Cytogenetic risk				
Favorable	26 (43.3)	24 (52.1)	2 (14.3)	0.012*
Unfavorable	34 (56.7)	22 (47.9)	12 (85.7)	

AML, acute myeloid leukemia; ANXA1, Annexin A1; BM, bone marrow; CD, cluster of differentiation; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; MPO, myeloperoxidase; PLT, platelet; WBC, white blood cell. **P* value less than 0.05, significant.

A statistically significant negative correlation was detected between ANXA1 level in patients with AML at diagnosis and level of CD14 (r=–0.32, P=0.01).

Annexin A1 expression and patient survival

The difference in mean overall survival (OS) between patients with AML with positive ANXA1 expression (mean±SD, 8.68±1.20 months) and patients with AML with negative ANXA1 expression (mean±SD, 4.16±1.53 months) was statistically significant (log rank, 4.90; P=0.03) (Fig. 1 and Table 8).

Discussion

AML is a malignant disorder of hemopoietic stem cells with variable outcomes and characterized by clonal proliferation of myeloid precursors and accumulation of leukemic blasts in BM, ultimately resulting in various cytopenias owing to BM failure [1].

ANXA1 is a member of the Annexin family and belongs to calcium-dependent phospholipid-binding protein. It was previously found that ANXA1 was an intracellular inflammation-related factor that regulates the anti-inflammatory effects of glucocorticoids, and it has also been shown to be involved in the regulation of cell proliferation, differentiation, and apoptosis, as well as development or progression of tumors [3].

The role of ANXA1 in tumors is paradoxical as ANXA1 appears to behave either as a tumor

Table 5 Annexin A1 expression in relation to response to therapy of patients with acute myeloid leukemia

Variables	Results
ANXA1 before treatment	
Mean±SD	57.01±28.0
Median (range)	68.2 (3.4–90.7)
ANXA1 after 28 days	
Mean±SD	6.2±6.8
Median (range)	2.5 (0.9–26.3)
ANXA1 after 6 months	
Mean±SD	5.2±8.1
Median (range)	1.8 (0.9–26.5)

ANXA1, Annexin A1. *P* value compared before and after 28 days less than 0.0001. *P* value compared before and after 6 months=0.0001. *P* value compared after 28 days and after 6 months=0.59.

Table 6 Comparisons between patients with acute myeloid leukemia regarding Annexin A1 expression according to sex, cytogenetic risk, age, and postinduction chemotherapy

Characteristic	ANXA1 expression (mean±SD)	*P* value
Sex		
Males	59.04±26.69	0.63
Females	55.55±29.33	
Age (years)		
ANXA1 positive (*N*=46)	36.30±11.2	0.74
ANXA1 negative (*N*=14)	35.36±11.3	
Cytogenetic risk		
Favorable	67.7±22.9	0.009**
Unfavorable	48.9±29.1	
Postinduction response		
CR (*N*=26)	72.9±21.1	0.001**
Failure to achieve CR (*N*=11)	44.6±29.6	
Death (*N*=23)	45.01±26.0	

ANXA1, Annexin A1; CR, complete remission. ***P* value less than 0.01, highly significant.

Table 7 Correlation between Annexin A1 level at the time of diagnosis and age, complete blood count, bone marrow, peripheral blast, spleen, liver size, and flow cytometry

Characteristic	*r*	*P* value
Age (years)	–0.146	0.26
WBCs	–0.22	0.87
Hb	–0.15	0.26
PLTs	0.07	0.66
BM blast	–0.105	0.43
Peripheral blast	–0.145	0.27
ESR	0.07	0.59
Spleen size	–0.18	0.26
Liver size	–0.14	0.27
Flow cytometry		
CD34	0.03	0.83
CD13	0.01	0.92
CD33	–0.027	0.84
CD117	–0.09	0.52
MPO	0.35	0.01*
HLADR	–0.148	0.26
CD14	–0.32	0.01*
CD15	0.04	0.76
CD64	0.28	0.03*
CD5	0.06	0.68
CD7	0.22	0.09
CD19	–0.65	0.62

BM, bone marrow; CD, cluster of differentiation; D0, day 0; ESR, erythrocyte sedimentation rate; Hb, hemoglobin; HLADR, human leukocyte antigen – dr isotype; MPO, myeloperoxidase; PLT, platelet; WBC, white blood cell. **P* value less than 0.5, significant.

suppressor or an oncogenic gene. As the mechanism of ANXA1 in cancer progression has not been still completely clarified, more studies are required to investigate the detailed action mechanisms of this protein in tumors [4].

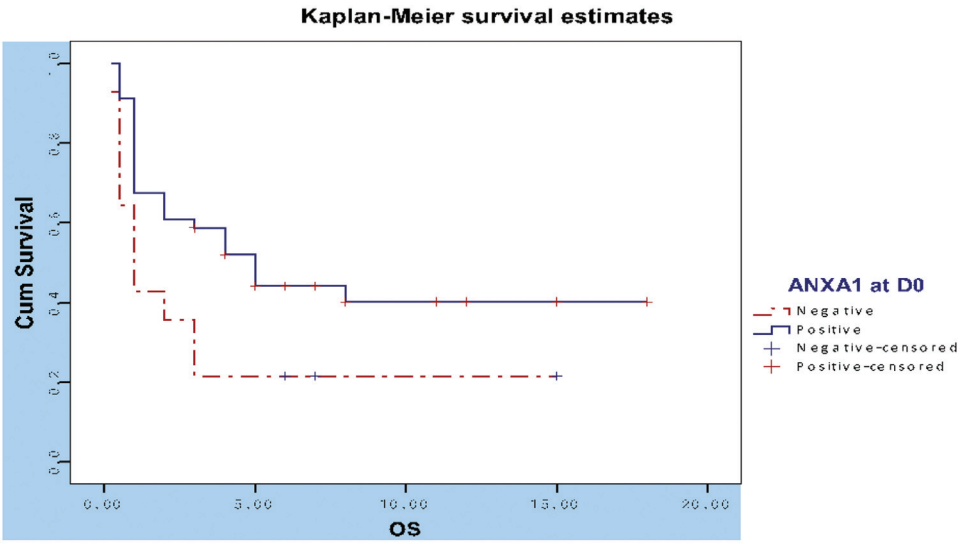
However, few studies have been conducted until now concerning the role of Annexins in AML. These studies have shown that ANXA1 expression analysis may help to understand the development of different phenotypes and the biological functions of ANXA1 in AML. The primary aim of the present study was to assess the expression level of ANXA1 in Egyptian adult patients with AML.

To the best of our knowledge, the present study is the first study aiming at assessment of ANXA1 expression in Egyptian adult patients with AML. In addition, we tried to identify the relations between ANXA1 and clinicopathological features and other prognostic markers to explore the usefulness of its expression level in predicting prognosis and outcome in adult patients with AML.

In our study, the mean age of patients with AML with positive ANXA1 expression (36.30±11.2) and the mean age of patients with AML with negative ANXA1 expression (35.36±11.3) when compared showed a statistically nonsignificant difference (*P*=0.74) and when compared the mean ANXA1 level in male patients with AML (59.04±26.69) with the mean ANXA1 level in female patients with AML (55.55±29.33), the result was statistically nonsignificant (*P*=0.22). This is in agreement with the data obtained from Cheng *et al.* [5] and Ydy *et al.* [6] who reported that the expression of ANXA1 did not correlate with the age or sex of the patients.

In the present study, a higher statistically significant difference in mean ANXA1 expression was found in AML (57.1±28.0) as compared with mean ANXA1 expression in control group (6.28±5.4) at the time of diagnosis, with *P* value less than 0.0001. ANXA1 was positive in 46 (76.7%) patients with AML and negative in 14 (23.3%) patients with AML.

Figure 1



Kaplan–Meier curve for overall survival of patients with AML with positive Annexin A1 (ANXA1) expression and patients with AML with negative ANXA1 expression. AML, acute myeloid leukemia.

Table 8 Comparison between the overall survival in patients with acute myeloid leukemia with positive Annexin A1 expression and in those with negative Annexin A1 expression

ANXA1 expression	Survival time (months) (mean±SD)	Log-rank	*P* value
ANXA1 positive	8.68±1.20	4.90	0.03*
ANXA1 negative	4.16±1.53		

ANXA1, Annexin A1. **P* value less than 0.5, significant.

In agreement with our findings, López-Pedrera *et al* [7] aimed to study the pattern of differential protein expression including ANXA1 expression of blasts from patients with AML in comparison with matched healthy individuals and declared that ANXA1 is significantly increased in AML BM blast cells when compared with controls in a study of 13 patients with AML versus 10 healthy controls.

Luczak *et al.* [8] as well, upon studying the protein profiles of PB and/or BM samples collected from 38 patients with AML and 17 healthy volunteers using two-dimensional electrophoresis and mass spectrometry, discovered that ANXA1 level was higher in patients with AML compared with healthy individuals, and they even suggested that ANXA1 can be used as a biomarker in AML.

Moreover, Sabran *et al.* [9] found in an experimental study on AML cell line (U937) that ANXA1 had a significantly higher ANXA1 concentration ($P \leq 0.05$) compared with the PB mononuclear cells obtained from healthy donors.

However, very few research studies were designed to investigate ANXA1 in other hematological malignancies like lymphoma [10] and reported that ANXA1 was commonly lost in B-cell lymphomas tissues and cell lines, compared with the positive expressions of ANXA1 mRNA and protein in normal B cells. Gene deletion was not the cause for ANXA1 loss, as the *ANXA1* gene was intact in B-cell lymphoma Raji and OMA-BL-1 cells. In fact, the methylation of the ANXA1 promoter could be a possible mechanism for its silencing, as the restored expression of ANXA1 occurred in B-cell lymphoma cells exposed with a demethylation agent (methylase inhibitor deoxycytidine).

Falini *et al* [11] showed that ANXA1 gene was upregulated and ANXA1 protein was strongly expressed on the cell membrane and occasionally in the cytoplasm of tumor cells in 62 of 64 (97%) samples from patients with hairy cell leukemia. Falini *et al* [11] concluded that ANXA1 protein expression is specific to hairy cell leukemia. Immunocytochemical detection of ANXA1 represents a simple, inexpensive, highly sensitive and specific (100%) assay for diagnosis of hairy cell leukemia.

Elevated levels of ANXA1 have been detected in many solid tumors such as lung cancer, colorectal cancer, hepatocellular carcinoma, and pancreatic cancer and in melanomas. Brichory *et al.* [12] reported that ANXA1 was overexpressed diffusely in neoplastic cells in lung tumor tissues by immunohistochemical analysis. Moreover, Biaoxue *et al.* [13] concluded that

upregulation of ANXA1 was seen in patients with lung cancer and associated with lymphatic metastasis. By proteomic and immunohistochemical analysis, Duncan *et al.* [14] proved over-expression of ANXA1 in colorectal cancer in comparison with normal colon. Moreover, Masaki *et al.* [15] and Lin *et al.* [16] by analysis of both ANXA1 mRNA and protein in liver tumors showed increased expression of ANXA1 in hepatocellular carcinoma, with ANXA1 almost being undetectable in normal liver.

Conversely, particular cancers are more prone to develop in downregulated states of ANXA1 expression including cancers such as prostate, esophageal, larynx [17], nasopharyngeal [18], and oral squamous cell carcinoma [19]. These studies indicated that ANXA1 might function as a tumor suppressor in tumor development.

Patton *et al* [20] declared that ANXA1 expression is significantly down-regulated in prostatic cancer when compared with the normal prostate. Patton *et al* [20] as well found that expression of ANXA1 inversely correlates with the increasing histological grade of prostatic adenocarcinoma and suggested that loss of ANXA1 may therefore serve as a biomarker for tumor cell proliferation and progression to late-stage cancer.

In esophageal squamous cell carcinoma, Xia *et al.* [21] investigated the protein profiles of tumors were compared with those of normal epithelia, where 11 proteins were found as dysregulated in tumors. Among them, three isoforms of ANXA1 were seen down-expressed in cancerous tissues.

In the current study, the results obtained from complete blood count, BM aspiration, other laboratory tests, and radiological assessment revealed no significant correlation between ANXA1 level and white blood cell, hemoglobin, platelet, PB blasts, BM blasts, ESR first hour, spleen size, and liver size, with *P* values of 0.87, 0.26, 0.66, 0.27, 0.43, 0.59, 0.26, and 0.27, respectively.

Comparison between the mean ANXA1 expression in patients with AML according to presence or absence of splenomegaly, hepatomegaly, and enlarged lymph nodes showed a statistically nonsignificant difference, with *P* values of 0.79, 0.07, and 0.21, respectively.

Comparison of the flow cytometry results with the results of ANXA1 expression showed that there was no statistically significant correlation between ANXA1 expression level and CD34, CD13, CD33, CD117, HLADR, CD15, CD5, CD7, and CD19 expression (*P*=0.83, 0.92, 0.84, 0.52, 0.26, 0.76, 0.68, 0.09, and 0.62, respectively).

Correlation studies between ANXA1 levels at the time of diagnosis and flow cytometry of BM showed statistically significant positive correlation between level of ANXA1 and level of MPO and CD64 in patients with AML (r=0.35, *P*=0.01, and r=0.28, *P*=0.03, respectively).

MPO is normally expressed variably in cells of neutrophil, eosinophil, and monocyte lineage. MPO is widely considered a hallmark of myeloid lineage and can be detected by cytochemical staining, immunohistochemistry, or flow cytometry. Expression is typically weak in myeloblasts and intense in mature granulocytes and neoplastic promyelocytes. Normal monocytes contain a few scattered MPO granules, whereas neoplastic promonocytes and monoblasts may have a similar pattern of expression or may be negative. Megakaryoblasts and erythroblasts are always MPO negative. By definition, poorly differentiated AMLs (FAB AML-M0) are cytochemically MPO negative [22].

Several studies in adults have examined the prognostic significance of the proportion of MPO-positive AML cells. An ECOG study of 72 adults with FAB M1 AML showed a significantly lower CR rate in those with <50% MPO-positive blasts [23].

In a large study conducted by the Japan Adult Leukemia Study Group with 491 patients (excluding FAB M3), patients with more than 50% MPO-positive blasts had a significantly better CR and OS than those with lower MPO positivity [24].

Several mechanisms have been suggested by which MPO may play a role in treatment outcome. MPO has been shown to mediate apoptosis via H_2O_2 in the HL-60 cell line [25].

CD64 is a type of integral membrane glycoprotein known as Fc-gamma receptor 1. After binding immunoglobulin G, CD64 interacts with an accessory chain known as the common γ chain (γ chain), which possesses an ITAM motif that is necessary for triggering cellular activation [26].

CD64 is constitutively found on only macrophages and monocytes, but treatment of polymorphonuclear

leukocytes with cytokines like interferon γ and granulocyte-colony stimulating factor can induce CD64 expression on these cells [27].

A German retrospective study of flow cytometric analysis of more than 700 patients with AML with a median follow-up exceeding 4 years reported that high CD64 expression AML associated with high CR rate and had a significant influence on OS [28].

In our study, we found a statistically significant negative correlation between ANXA1 level in patients with AML at diagnosis and level of CD14 (r=–0.32, P=0.01).

CD14 is a glycophosphatidylinositol-linked protein expressed by myeloid cells (mCD14) and also circulates as a plasma protein (sCD14) lacking the glycophosphatidylinositol anchor. Both membrane CD14 and soluble CD14 function to enhance activation of cells by lipopolysaccharide, which we refer to as receptor function. CD14 is expressed mainly by macrophages and neutrophils. It is also expressed by dendritic cells. The soluble form of the receptor (sCD14) is secreted by the liver and monocytes [29].

Choi *et al.* [30] reported that of immunophenotype markers, CD14 positivity only showed prognostic implications at the univariate analyses in secondary AML: lower CR rate after induction chemotherapy (P=0.034) and shorter survivals (OS, P<0.001; RFS, P=0.078; and EFS, P<0.001).

We found that the highest ANXA1 level was seen in AML-M0 (FAB subtypes) followed by M3, M4, M2, M5, and M1 subtype, but there was no statistically significant difference in mean ANXA1 expression among different FAB subtypes (P=0.08) or different cytogenetic aberrations (P=0.09), but the difference was highly statistically significant between favorable risk group, which had higher mean ANXA1 expression, and unfavorable risk group (P=0.009). This is in agreement with the results reported by López-Pedrera *et al.* [7] that expression of ANXA1 was present in all AML FAB subtypes without significant difference.

Concerning the outcome of our studied patients, patients who achieved CR (favorable response) had higher mean ANXA1 expression level at diagnosis than those who did not achieve CR or died before or during induction chemotherapy (unfavorable response). The difference in the means of ANXA1 expression level between different treatment outcomes was statistically significant, with P value less than 0.001.

These results are in accordance with the data reported by Luczak *et al.* [8], which showed that the mean ANXA1 expression level at diagnosis was significantly higher in patients who achieve CR than in patients who did not achieve CR. Moreover, Kaźmierczak *et al* [31] reported that on the basis of proteomic analysis of blood and BM samples from patients with AML before induction therapy who achieved CR or were resistant, they found four proteins, including ANXA1, significantly correlated with results of treatment. ANXA1 was present in both subgroups (patients who achieved CR and patients who did not); however, its concentrations were significantly higher (P<0.001) in the subgroup with CR.

In our study, the Kaplan–Meier survival analysis revealed that patients with positive ANXA1 expression had a significantly longer OS than those with negative ANXA1 expression, and the difference in mean OS between the two groups of patients with AML was statistically significant (log rank: 4.90, P=0.03).

As far as we know, no previous research has correlated survival rate to ANXA1 expression in patients with AML.Contrary to our results, ANXA1 expression had an adverse effect on survival in other solid malignancies. Cheng *et al.* [5] reported that high ANXA1 expression was associated with more serosal invasion, more peritoneal metastasis, and poorer OS in patients with gastric carcinoma. Moreover, Lin *et al.* [16] concluded that patients with high ANXA1 expression had a significantly poorer OS versus patients with low ANXA1 expression (P<0.001) by log-rank test.

In contrast, Wang *et al.* [32] reported that lack of ANXA1 expression was significantly associated with advanced disease stage and worse OS in breast cancer.

Based on the aforementioned results, high ANXA1 expression in patients with AML was associated with favorable outcome and longer OS, which may be attributed to proapoptotic activity and/or antiproliferative effect of ANXA1.

It has been reported that enhanced ANXA1 level influences caspase 3 and caspase 9 signaling, which is of importance for the apoptosis pathway [33,34]. Caspases are members of cysteine protease where their activation will induce apoptosis in diverse cell types,

including cancer cells [35]. Increase in ANXA1 level results in a decrease in the bcl-2 level, a protein that functions to promote cell survival and prevent apoptosis [33,34]. COX-2 over-expression, on the contrary, can lead to an increase in bcl-2 expression, preventing the mitochondria from releasing cytochrome C and subsequently inhibiting apoptosis [36]. COX-2 also decreases the caspase activation, preventing apoptosis. Previous studies have shown that ANXA1 was able to inhibit COX-2 activities [37].

An experimental study by Ferreira *et al* [38] showed that ANXA1 inhibited COX-2 activities in the J774 murine macrophage-like cell line. In addition, COX-2 has also been shown to play a role in inhibiting leukemic cell apoptosis [39].

Taken together, these previous findings suggested that it is possible for ANXA1 to induce apoptosis in leukemic cells by decreasing COX-2 expression, leading to a decrease in bcl-2 expression and activating apoptosis.

However, the exact mechanism by which ANXA1 induces apoptosis needs to be further explored.

Although there is an evidence indicating that high ANXA1 expression is a good prognostic factor in adult patients with de novo AML, several issues need to be addressed when interpreting our results. The sample size in our study was not large enough to fully explore the synergistic relationship between ANXA1 expression and different combinations of confounding factors that may affect its behavior. Response rate to therapy seems to be lower than expected, as because of economic reasons, we could not include some effective recent expensive drugs in the protocol of therapy. Adding to that, AML is a heterogeneous disease at the molecular levels, even within the same patient.

Conclusion

In conclusion, our findings documented that ANXA1 is significantly expressed in Egyptian patients with de novo AML and its expression is associated with favorable prognostic effect on clinical outcome in the form of high CR rate and longer OS.

We suggest that high expression level of ANXA1 may identify distinct group of Egyptian patients with AML with favorable prognosis. It may serve as a prognostic predictor of outcome and response to chemotherapy and a potential target for therapeutic intervention.

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Conflicts of interest

There are no conflicts of interest.

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