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Research Article

CD47 Expression as a Possible Prognostic Tool in Egyptian AML Patients

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Abstract

Background: Acute myeloid leukemia (AML) is organized as a cellular hierarchy initiated and maintained by a subset of self-renewing leukemia stem cells (LSC). Increased CD47 expression on human AML leukemic cells contributes to its pathogenesis by inhibiting their phagocytosis through binding of CD47 to signal regulatory protein alpha (SIRPα), an inhibitory receptor that negatively regulates phagocytosis. In AML, CD47 is over-expressed on peripheral blasts, leukemia stem cells and inversely correlates with survival.

Purpose: Estimation of CD47 gene expression in Egyptian AML patients for evaluation of its role in the pathogenesis of the disease and as a prognostic marker related to overall survival (OS).

Methods: The study included 45 AML patients in addition to 20 healthy volunteers as controls. Blood samples of patients and controls were subjected to positive magnetic selection of CD34+ leukemic cells, and then CD47 expression on these cells was estimated by flowcytometry. Quantitative assessment of CD47 gene expression was performed by real-time polymerase chain reaction (qRT-PCR).

Results: Statistically significant difference (P-value < 0.001) between low and high CD47 gene expression was found, where 27 (60%) patients showed high CD47 gene expression levels above the cutoff value with a median of 1.34 (range 1.03 - 1.93), while18 (40 %) patients showed lower CD47 gene expression levels with median level of 0.48 (range 0.08 - 0.97). No significant correlation between CD47 gene expression by RT-qPCR and CD47 surface marker by flowcytometry was found. Also, there was inverse correlation between CD47 gene expression levels and OS where increased expression was also associated with a worse OS (P = 0.0001).

Conclusion: In conclusion, the results obtained by the current study provide additional evidence of the role played by CD47 gene as predictive factor impact on OS and prognosis.

Keywords: AML; CD47; SIRPa; qRT- PCR

Introduction

Acute myeloid leukemia (AML) is a clonal hematopoietic stem cell malignancy with high heterogeneity [1]. AML is the most frequent hematological malignancy in adults, with an estimated worldwide annual incidence of three to four cases per 100,000 people. Despite intensive research for new therapies and prognostic markers, it remains a disease with a highly variable prognosis among patients and a high mortality rate [2]. In Egypt, the National Cancer Institute (NCI) data showed that during the year 2002, out of a total of 19405 new cancer cases, 169 patients (1.8%) were diagnosed as AML [3]. Increased expression of CD47 on human AML leukemic cells contributes to pathogenesis by inhibiting phagocytosis of these cells through the interaction of CD47 with SIRPa [4]. CD47 also known as integrin-associated protein (IAP), is a widely expressed transmembrane protein characterized by its physical and functional association with multiple binding partners. Thrombospondin-1 (TSP1); signal-regulatory protein alpha (SIRPa) the integrin family members avb3, aIIbb3; and several beta1 and beta2 integrins are among them [5]. As a result, CD47 has been shown to participate in cellular processes such as apoptosis, proliferation, adhesion, and migration. In particular, CD47 functions as a marker of ''self'' by inhibiting phagocytosis of autologous cells through interaction with SIRPa expressed by professional phagocytes, such as macrophages [6]. SIRPa engagement by CD47 initiates a signaling cascade through an immunoreceptor tyrosine-based inhibitory motif (ITIM) present on the cytoplasmic portion of the SIRPa molecule that results in the inhibition of phagocytosis. Inversely, loss of CD47 expression, for example, by aged or damaged cells, removes the inhibitory signal and leads to clearance of these cells by phagocytes. Consequently, blocking the interaction of CD47 with SIRPa with anti- CD47 antibodies removes the inhibitory signal and promotes phagocytosis. It has been shown that one of the mechanisms of cancer cell survival is the evasion of the immune surveillance by phagocytic cells through overexpression of CD47 [7]. The previous observations raise the possibility that increased expression of CD47 might have a role in the pathogenesis of AML; hence in the present study CD47 gene and protein expression were performed in AML patients to evaluate their importance and relate it to overall survival.

Materials and Methods

Study Population: The present study included 45 AML patients. Patients were recruited from the outpatient clinic and the inpatient wards of Kasr Al Ainy clinical oncology department, faculty of medicine, Cairo University. Twenty age and sex matched healthy volunteers were included in the study as a control group. The study was approved by the Research Ethical Committee of clinical oncology department, Faculty of Medicine, Cairo University and informed consents were obtained from all participants prior to enrollment in the study.

For both patients and controls, 4 ml EDTA blood samples either peripheral blood or bone marrow (*for patients only*) were collected under complete aseptic conditions for routine workup, flowcytometry and molecular studies.

Treatment regimen and response to therapy: All patients were treated according to the adopted protocol of the Clinical Oncology Department, Cairo University. AML patients (with the exception of M3) were subjected to 7 - 3 protocol for induction of remission. Treatment of AML depends on the fitness of the patient. Fit patients (< 60 - 65 years, select patients up to age 75 years) receive intensive therapy. Treatment includes induction and post remission therapy (consolidation). Less fit patients (70 - 75 years and older, or younger patients with significant co-morbidities) received low-intensity therapy. For induction therapy, combination of cytarabine and anthracycline or anthracene Dione is recommended (cytarabine 100 - 200 mg/m^2 continuous IV infusion for 7 days plus idarubicin 12 mg/m^2/day for 3 days or daunorubicin 60 - 90 mg/m^2/day for 3 days). Follow-up performing bone marrow examination to assess response was done 7 - 14 days after completion of induction chemotherapy. For post remission therapy, all patients should be assessed for risk of relapse. Specific drug regimens are recommended based on the patient's risk of relapse high-dose cytarabine 3 g/m^2 IV over 3h every 12h on days 1, 3 and 5 for 4 cycles.

On the other hand, AML-M3 patients were treated by ATRA at a dose of 45mg/m^2/day given orally in two divided doses for 45-90 days. They were then given 7 - 3 protocol once. After achieving complete remission, M3 patients were consolidated by giving the 7 - 3 protocol twice. Maintenance therapy using intermittent ATRA (45 mg/m^2/day for15 days every 3 months), alternating with low-dose chemotherapy (6 mercaptopurine90 mg/m^2 + methotrexate 15 mg/m^2 weekly) for two years.

Endpoints: After a median duration of follow up for 9 months, the overall survival: defined from the date of the first visit till the last follow up was assessed.

Isolation of AML leukemic cells (CD34$^+$cells): Peripheral blood or bone marrow mononuclear cells (MNCs) were isolated by density gradient centrifugation, using Ficoll Hypaque. *All samples contained at least 60% leukemic cells as determined morphologically*. Cells were washed once with buffer and resuspended in a final volume of 300 µL of buffer for up to 10^8 cells. These Cells were then magnetically labeled with CD34 Microbeads (Miltenyi-Germany). Afterwards, the cell suspension was loaded onto a MACS (magnetic activated cell sorting) Column which was placed in the magnetic field of a Mini MACS Separator. The magnetically labeled CD34$^+$ cells were retained within the column. After removing the column from the magnetic field, the magnetically retained CD34+ cells were eluted as the positively selected cell fraction.

Immunophenotyping (IPT) for surface CD47: Flowcytometry was done on the positively selected CD34$^+$ cells by surface monoclonal antibodies directed against human CD47 (FACS Calibur, BD). Ten µl of monoclonal anti-CD47 antibodies together with 100 µl positively selected CD34$^+$ cells were added in falcon tubes. These tubes were left for 15 min. in darkness, and then 0.5ml cell wash was added. The target population was CD34$^+$ - CD47$^+$ cells which was analyzed on Cell Quest program.

CD47 gene expression:

RNA isolation and cDNA synthesis: Extraction of total RNA from positively selected CD34+ cells were performed by QIAamp RNA Blood Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Total RNA was reverse transcribed using random primers with a high capacity cDNA archive kit (Applied Biosystems, Foster city, CA, USA).

PCR amplification: cDNA specific CD47 Taqman primer and probe sets were developed using primer express software. The nucleotide numbering throughout this study is based on the published sequence available from EMBL/GenBank/DDBJ under accession number (NM97565_D2): The forward primer of CD47: 5 CCGATTTGGAGAGTAGTAAGACGTG-3, the reverse primer of CD47: 5 TCTCCCCAACAGTGAATCATCA-3, and the probe ([6-FAM]-AAGGAATACACTTCTGTTTAAGCACCATGGCC-[TAMRA-6-FAM]). For CD47 mRNA; the concentrations were 15 pmol for forward and reverse primers and 3.75 pmol for the probe. Commercially available primers and probe for reference GAPDH gene were used for normalization (Applied Biosystems). The probe was labeled with VIC dye and to avoid competition in the PCR reaction tube, the concentrations of primers were adjusted. All PCR reactions were performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the fluorescent Taqman methodology. The PCR cycle at which the fluorescence arises above the background signal is called the Cycle threshold (Ct) and it is inversely proportional to the log of the initial copy number. The total volume of reaction was 25 µl as follows: Forward primer 1.25 µl, Reverse primer 1.25 µl, Probe 1 µl, GAPDH 1.25 µl, Master Mix 12.5µl, Nuclease-free H2O 2.75 µl, Sample c-DNA 5 µl. The thermal cycling conditions comprised 10 min at 95º C, 15 secs at 95º C (~45 cycles of 15 sec denaturation at 95º C) and 60 sec annealing at 60º C.

Data Analysis: Quantitative data were presented as minimum, maximum, mean, median and standard deviation (SD) values. Data showed a non-parametric distribution and so Mann- Whitney U test was used for comparisons between two groups. This test is the nonparametric alternative to Student's t-test. Qualitative data were presented as frequencies and percentages. Chi-square (x2) test was used for studying the comparisons and associations between different qualitative variables. Spearman's correlation coefficient was used to determine significant correlations between CD47 expression and different variables. Kaplan-Meier survival curve was constructed for survival analysis. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

Results

Demographic data of AML patients

Among the 45 AML patients (Table 1), 24 (53%) were females and 21 (46%) were males with a female to male ratio 1.1: 1. Their ages ranged from 5 to 65 years with mean ± SD of 34.64 ± 16.27 years.

Characteristics	No = 45
Gender Male (no., %) Female (no., %)	 21 (47%) 24 (53%)
Age (years) Median (range)	 32(5 – 65)
Hb (g/dl) Mean ± SD	 7.78 ± 2.1.
WBCs x 10^3/cmm Median (range)	 43 (1.7-588)
Platelets x 10^3/cmm Median (range)	 45 (5-117.4)
P.B. blasts (%) Median (range)	 76 (10-95)
B.M. blasts (%) Median (range)	 90 (22-98)

Table 1: Demographic Data for 45 AML Patients.

Results of morphological examination, cytochemical staining and immunophenotyping showed that the most common subtype of AML in our study population according to the FAB classification was as follow: 15(33.3%) cases were M2, 9(20%) cases were M1 and 9 (20%) cases were M3.

Among these 9 M3 cases, 3 (33. 3%) cases were *M3 variant (hypogranular)* subtype with negative PML- RARA (bcr exon 1 and exon 3) by RT-PCR. Six cases (13.3%) were M4, 3 cases (6.6%) were M0, one case (2.2%) was M5b, one case (2.2%) was M5a, and one case (2.2%) was M7.

Immunophenotyping (IPT) for sCD47 in AML patients:

Surface CD47 values in the positively selected CD34+ cells ranged between 83% and 99% of these cells with a mean value of 92.9% ± 4.1%. No significant correlation between CD47 gene expressions by RT-qPCR and CD47 surface marker by flowcytometry was found (table 5).

Immunophenotyping (IPT) for sCD47 in AML patients:

ΔΔCt Method: To quantify the relative expression of CD47, the Ct (threshold cycle) values were normalized for endogenous reference ($\Delta Ct = Ct_{sample} - Ct_{GAPDH}$) and compared with a calibrator, using the 'delta-delta Ct method' ($\Delta\Delta Ct = \Delta Ct_{Sample} - Ct_{control}$) [8] as calibrator we used the average CD47 Ct value in the 20 P.B samples of healthy volunteers. Using the ΔΔCt value, the relative expression was calculated ($2^{-\Delta\Delta Ct}$). The mean ratio of CD47 mRNA in the controls was 1.046 (considered as cutoff value) using the ΔΔ Ct method. The results obtained using this method showed that the majority of patients (60%) had higher CD47 expression levels, values were above the cutoff with median level of 1.34 (range 1.03 - 1.93) while (40 %) showed lower levels with median of 0.56 (range 0.08-0.97) (Table 2).

CD47 Subgroups	No. of Cases	Mean ± SD	Median	Minimum	Maximum	*P*-value
Low expression < 1.046	18	0.56 ± 0.33	0.48	0.08	0.97	< 0.001*
High expression > 1.046	27	1.36 ± 0.21	1.34	1.03	1.93	

Table 2: CD47 Gene Expression Classification of AML Patients (N = 45).

*: Significant at $P \leq 0.05$

CD47 expression according to gender showed no statistical significant difference between males and females. But showed statistical significant difference (P = 0.008) as regard age between different subgroups (Table 3) where higher expression was seen in older age. No statistically significant difference was found between different FAB subtypes in the two groups, the majority of M4 cases showed high expression. Regarding cytogenetics by (FISH) there was no statistically significant difference between t (8; 21) and t (15; 17) distributions in cases with high and low CD47 expression.

Parameters		*Low CD47 expression* < 1.046	*High CD47 expression* > 1.046	P-value
Gender	Male	8 (44.4%)	13 (48.1%)	0.807
	Female	10 (55.6%)	14 (51.9%)	
Age (Mean ± SD)		27.1 ± 11.6	39.7 ± 17.1	**0.008***
TLC x 10^3/cmm (Mean ± SD)		44.47 ± 49.07	94.15 ± 133.49	0.215
Hb g/dl (Mean ± SD)		8.1 ± 2.8	7.6 ± 1.4	0.711
PLT x 10^3/cmm (Mean ± SD)		64.9 ± 61.1	91.7 ± 220.3	0.694
P.B. blast (%)(Mean ± SD)		64.1 ± 25	64.7 ± 25.1	0.935
B.M. blast (%) (Mean ± SD)		75.9 ± 22.5	70.3 ± 26.2	0.523
CD13(%)(Mean ± SD)		68.8 ± 15.1	53.5 ± 26.3	**0.054**
CD33(%)(Mean ± SD)		70.8 ± 22.7	76.8 ± 20.8	0.430
CD34(%)(Mean ± SD)		28.8 ± 32.8	21.6 ± 27.9	0.635
HLA-DR (%)(Mean ± SD)		59.3 ± 31.4	52.2 ± 28.9	0.297
MPO (%)(Mean ± SD)		59.3 ± 26.1	52.1 ± 23.6	0.302
CD7 (%)(Mean ± SD)		33.1 ± 29.6	22.4 ± 28.5	0.132
CD19 (%)(Mean ± SD)		10.1 ± 18.5	6.7 ± 11.9	**0.057**
CD117 (%)(Mean ± SD)		50.8 ± 28.6	35.8 ± 29.6	0.112
CD47 (%)(Mean ± SD)		93.7 ± 3.9	92.9 ± 4.2	0.522
FAB subtypes				
M0		1 (5.6%)	2 (7.4%)	0.742
M1		5 (27.8%)	4 (14.8%)	
M3		3 (16.7%)	6 (22.2%)	
M4		1 (5.6%)	5 (18.5%)	
M5		1 (5.6%)	1 (3.7%)	
M7		0 (0)	1 (3.7%)	
FISH Analysis				
t (8;21)(No. = 45) Negative	Positive	1 (5.6)	1 (3.7)	0.768
	17 (94.4)	26 (96.3)		
t (15;17)(No. = 45) Negative	Positive	3 (16.7)	5 (18.5)	0.874
	15 (83.3)	22 (81.5)		

Table 3: Comparison between Demographic Data in the Two Groups.

*: Significant at P ≤ 0.05

CD47 gene expression and response to therapy:

After a median duration of follow up of 9 months, the Overall Survival (defined from the date of entry till the date the patient died, or was last seen) was 12.6 ± 1.5 months with median 9 months for patients with low CD47 expression while for the patients with high CD47 was 2.3 ± 0.3 months with median 1 month. Eight cases (44.4%) in low expression group died before reaching the follow up period of 9 months, while all cases with high expression died before reaching this period. Kaplan-Meier survival analysis showed an estimated mean survival time of 13.2 months for low CD47 expression and 2.3 months for high CD47 expression groups. There was a statistically significant difference between estimated overall survivals in the two groups: Low CD47 expression group showed higher estimated median survival time than high CD47 expression group (Table 4 and Figures 1).

Figure 1: Kaplan-Meier Survival Curve for Low and High CD47 Expression Groups.

CD47 subgroups	OS (ms)		
	Mean ± SD	**Median**	**P* value**
Low expression	12.6 ± 1.5	9	*P* < 0.0001*
High expression	2.3 ± 0.3	1	

Table 4: CD47 Gene Expression and OS of our Study Population.
*: Significant at P ≤ 0.05

Correlation between CD47 gene expression and different parameters

A statistically significant positive correlation was found between CD47 gene expression and age (P-value = 0.009) where older ages at diagnosis were associated with increased CD47 expression and vice versa. Also, there was a statistically significant negative correlation between CD47 expression and CD117 (P-value = 0.027) (Table 5 - Figure 2).

Figure 2: Scatter Diagram Showing Positive Correlation between Age and CD47 Expression.

Parameters	*Correlation coefficient*	*P*-value
Age	0.386	**0.009***
HB	-0.043	0.780
TLC	0.153	0.316
Platelets count	-0.105	0.491
P.B. blasts (%)	0.009	0.954
B.M. blasts (%)	-0.195	0.199
CD34 (%)	-0.204	0.179
HLA-DR (%)	-0.247	0.101
MPO (%)	-0.128	0.403
CD13 (%)	-0.231	0.127
CD33 (%)	0.193	0.204
CD7 (%)	-0.277	0.065
CD19 (%)	-0.247	0.102
CD117 (%)	-0.330	**0.027***
CD47 (%)	-0.081	0.598

Table 5: Correlation between CD47 Gene Expression and Different Parameters.
*: Significant at P ≤ 0.05

Discussion

Acute myeloid leukemia (AML) represents a clinically and biologically heterogeneous malignancy with uncontrolled proliferation of hematopoietic precursors. Almost 80% of patients with AML will demonstrate chromosome abnormalities, usually a mutation resulting from a chromosomal translocation causing abnormal oncogene or tumor suppression gene expression, resulting in unregulated cellular proliferation [9]. Increased expression of CD47 on human AML cells contributes to pathogenesis by inhibiting phagocytosis of these cells through the interaction of CD47 with SIRPa [10] SIRPa engagement by CD47 initiates a signaling cascade through an immunoreceptor tyrosine-based inhibitory motif (ITIM) present on the cytoplasmic portion of the SIRPa molecule that results in the inhibition of phagocytosis. Consequently, blocking the interaction of CD47 with SIRPa, for example, with anti-CD47 antibodies, removes the inhibitory signal and promotes phagocytosis. It has been shown that one of the mechanisms of cancer cell survival is the evasion of the immunosurveillance by phagocytic cells through over expression of CD47 [7]. The aim of the current study was to investigate the CD47 gene expression in Egyptian AML patients for evaluation of its role in the pathogenesis of the disease and as a prognostic marker related to overall survival. To achieve this aim, blood samples from patients and controls were subjected to positive magnetic selection of CD34+ leukemic cells, then CD47 expression on these cells was estimated by flowcytometry and quantitative real-time polymerase chain reaction for patients and controls in relation to GAPDH gene expression as endogenous control for the relative quantitation of CD47 gene expression by "ΔΔCt method" using TaqMan technology. Our study population showed a statistically significant difference between low and high CD47 gene expression where the majority of patients 27/45(60%) had high CD47 gene expression levels while 18/45(40%) cases showed lower expression levels (P-value < 0.001). Irandoust M and colleagues reported CD47 higher expression on leukemic cells as well [10]. CD47 expression in relation to gender, TLC, Hb, blast count, platelets count, showed no statistically significant difference between the high and low expressers. On the other hand, the present study showed positive correlation between CD47 expression levels and age (P = 0.008) while no reported data related to age were found in the literature. There was no significant correlation between CD47 gene expression by RT-qPCR and CD47 surface marker by flowcytometry was found, which is in disagreement with Majeti M., *et al.* [11], this may be due to the difference in selection of leukemic cell population. In agreement with the previously reported data by Majeti R.in (2009), Irandoust M and colleagues (2013), the present study showed no significant difference in CD47 gene expression in relation to FAB subgroups [10,11]. Regarding CD47 gene expression in relation to cytogenetic subgroups especially t (8;21) our findings were in disagreement with Majeti, R.in (2009) who reported that in most cytogenetic subgroups, CD47 was expressed at similar levels, except for cases harboring t (8;21) a favorable risk group that had a statistically significant lower CD47 expression [11]. Finally,

there was inverse correlation between CD47 gene expression levels and overall survival where increased expression was also associated with a worse overall survival (P = 0.0001). This is in accordance with Jaiswal *et al.* and Majeti., *et al.* [4,11] who conducted their work on leukemic stem cells. Analysis of AML patients has revealed that higher levels of CD47 expression were associated with a poorer prognosis [12,13]. We conclude that CD47 gene expression could be used as a prognostic indicator in survival analysis for AML patients.

Conclusion and Future Directions

Using qRT- PCR, we were capable to define two main subgroups of AML patients with low and high CD47 m-RNA expression, suggesting the importance of CD47 gene expression as prognostic indicator. Further researches in this field are required for understanding the role of CD47 expression in the pathogenesis of AML that could be valuable as biological target, also defining pathways that control leukemic cells survival is crucial for a better understanding of underlying mechanisms and for the development of targeted therapies.

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Conflicts of interest

Authors declared no conflicts of interest

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