Original Research Article

Study of CD 47 Expression in Patients with Acute Myeloid Leukemia

Abstract

Background: Acute Myeloid Leukemia (AML) is the most common acute leukemia affecting adults, and its frequency increases with age. CD47 may not only be a valuable biomarker to recognize [write LSCs in full) (LSCs) but also may denote a clinically relevant pathogenetic factor of disease. The aim of this work was to study the expression of CD47 in patients with acute myeloid leukemia and to identify its role as a prognostic marker.

Methods: The present study was carried out on 40 newly diagnosed untreated AML patients. AML patients were divided into two groups according to status of expression: Negative Group including patients expressing CD47 on less than 20% of their blast cells (28 patients) and positive Group including patients expressing CD47 on 20 % or more of their blast cells (12 patients). All patients were subjected to laboratory investigation for detection of CD47 expression m bone marrow aspirate and venous blood samples of AML patients by flowcytometry.

Results: Complete remission was found in 2 patients (16.67%), 6 patients relapsed (50%) and 4 patients died (33.33%) of the 12 cases that had exhibited CD47 positive expression. Kaplan-Meier Survival curve showed significant higher overall survival and disease-free survival in negative CD47 expression group than in positive CD47 expression group.

Conclusions: Positive CD47 expression levels are associated with a poor outcome in AML patients and its expression can be easily determined in routine flow cytometric analysis. Therefore, it should be regularly investigated as a bad prognostic factor for the assessment of AML patients.

Keywords: CD 47, Expression, Acute Myeloid Leukemia.

Introduction:

Acute myeloid leukemia (AML) is a cancer of the myeloid line of blood cells, which is characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. AML is the most common acute leukemia affecting adults, and its frequency increases with age [1].

AML has many subtypes; treatment and prognosis AML differ among subtypes. AML is cured in 35-40% of people under 60 years old and 5-15% over of 60 years old. Older people who are not able to withstand intensive chemotherapy have an average survival of 5-10 months [2].

CD47 is a transmembrane glycoprotein, which is widely expressed in several human tissues and functions as a ligand for many receptors, including signal regulatory protein alpha (SIRPa).

CD47- SIRPa signaling on macrophages or dendritic cells results in inhibition of phagocytosis by immunoreceptor tyrosine- based inhibition motif (ITIM) mediated recruitment of protein tyrosine phosphatase Src homology region 2 damain-containing phosphatase 1\2 (SHP-1\2) [3].

CD47 inhibits nitric oxide (NO) \cyclic guanine monophosphate (cGMP) signaling pathway and restricts NO-mediated vasodilatation and decrease the inhibition of platelet aggregation [4].

Upregulation of CD47 expression is a physiological mechanism: administration of cyclophosphamide granulocyte colony stimulating factor (G-CSF) and lipopolysaccharide, respectively stimulate mobilization of hematopoietic stem cells (HSCs), resulting m extensively elevated CD47 expression on circulating HSCs in comparison to their bone marrow (BM)-resident counterparts. This CD47 up regulation apparently protects mobilized HSC from subsequent macrophage mediated phagocytosis [5].

Evasion of macrophage mediated phagocytosis by CD47-SIRPa signaling resulted in decreased blast clearance by the innate immune system and conferred a survival advantage to the leukemic stem cells (LSCs) as compared with the normal HSC counterparts [6].

The residual leukemic stem cells fraction of cells following conventional chemotherapies can lead to relapse of the disease. CD47 has been demonstrated to be differentially expressed on AML LSC compared with normal HSC [7].

So, CD47may not only be a valuable biomarker to recognize LSCs but also it may denote a clinically relevant pathogenetic factor of disease [6].

The aim of this work was to study the expression of CD47 in patients with acute myeloid leukemia and to identify its role as a prognostic marker.

Patients and Methods:

The present study was carried out on 40 newly diagnosed untreated AML patients, 26 male and 14 female, whose ages had ranged between 29 years and 71 years at the hematology /oncology units, Internal Medicine Department, Tanta University Hospital. The study was done after approval from the Ethical Committee Tanta University Hospitals. An informed written consent was obtained from the patients.

Exclusion criteria were secondary, mixed or paediatric AML, other malignancies or treated AML.

AML patients were divided into two groups according to status of expression: Negative Group including patients whose tumours had expressed CD47 on less than 20% of their blast cells (28 patients) and positive Group including patients whose tumours had expression of CD47 on 20 % or more of their blast cells (12 patients).

All patients were subjected to: Detailed history, careful clinical examination to the presence and extent of leukemia involvement including pallor, purpuric eruptions, size of liver and spleen, lymphadenopathy and CNS involvement. Also, routine laboratory investigations

including complete blood count (CBC), erythrocyte sedimentation rate (ESR), serum lactate dehydrogenase (LDH), liver function tests, bone marrow aspiration, immunophenotyping of blast cell in BM aspirate samples using Becton Dickinson (BD FACS calibre) flow cytometer.

The panel for acute leukemia was fluorescent isothiocyanate (FITC/phycoerythrin PE), Conjugated monoclonal antibodies (MoABs) which were used to diagnose AML and included common progenitor marker (CD34, HLA-DR), myeloid markers (CD13, CD33, myeloperoxidase (MPO)), Monocytic marker (CD14, CD64), Erythroid marker (Glycophorin A), megakaryoblastic markers (CD61, CD41), lymphoid markers (B cell markers: CD10, CD 19 and T cell markers: CD2, CD7). Specific laboratory investigations were performed for the detection of CD47 expression m bone marrow aspirate and venous blood samples of AML patients by flowcytometry.

After being fully investigated, all of the patients, received chemotherapy and were observed for 12 months with regard to clinical and laboratory findings of remission and relapse taking care to estimate the date of first complete remission (CR), date of first relapse and date of death or last seen alive. A patient was relapsed when the bone marrow blasts >5%, reappearance of blasts in the blood, or development of extramedullary disease [8].

The patient was followed up for one year then the time at which the patient achieved remission, relapsed, died or last seen alive were **recorded** for calculation of overall survival (OS) and the **disease-free** survival (DFS) [8].

TWO values were used in evaluating the prognosis of the patients: overall survival (OS) which was measured from the date of diagnosis of the patient to the date of death or date last seen and disease-free survival alive (DFS) which was measured from the date of occurrence of disease-free state to disease recurrence or death [8].

Peripheral blood and bone marrow aspiration samples were taken. Bone marrow aspiration was performed under complete aseptic technique and the samples were collected in sterile covered EDTA tube and labelled for immunophenotyping and detection of CD47 expression. Smears were stained with leishman's stain and cytochemical SBB stain for morphological diagnosis.

Flowcytometric immunophenotyping: Immunophenotyping is the term applied to the identification of cells using antibodies to antigens expressed by these cells. Flowcytometry is a process of passing cells singly in a fluid stream (isotonic sheath fluid) through a beam of light, the light source is a laser. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labelled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths.

The use of multiple fluorochromes each with similar excitation wavelengths and different emission wavelengths (or "colours) allows several cell properties to be measured simultaneously. Commonly used dyes include propodeum iodide, phycoerythrin, and fluorescein, although many other dyes are available. Tandem dyes with internal fluorescence resonance energy transfer can create even longer wavelengths and more colors [9]. Detection of CD47 expression was done by clone B6H12 (CD47 monoclonal antibody)

Flowcytometric analysis:

FACS calibre flowcytometry from Becton Dickinson (BD) was used, where 10.000 events (cells) at least were acquired. Analysis was done using automated CELL QUEST Pro software, the percentage positive CD47 blasts were calculated by gating on blast population. The instrument setting was set by using calibrated beads provided the manufacture. Isotopic quality control was used to exclude non-specific binding and auto- fluorescence. Lightscatter histogram, forward light scatter versus logside scatter, was used to delineate cell populations of interest by bitmap drawing (gating). Gating fluorescence histogram is evaluated for positive cells by using cursor position from histograms for isotopic controls, so that 98% of positive are defined. In routine diagnostic, the universal accepted cut off for positivity is 20%

in AML. As regard CD47, a case with positive expression was defined if 20% of the gated cell or more expressed the marker.

Statistical analysis

Statistical analysis was done by SPSS v26 (IBM Inc., Chicago, IL, USA). Quantitative variables were presented as mean and standard deviation (SD) and compared between the two groups utilizing unpaired Student's t- test. Qualitative variables were presented as frequency and percentage (%) and were analysed utilizing the Chi-square test or Fisher's exact test when appropriate. A two tailed P value ≤ 0.05 was considered statistically significant.

Results:

Table 1 shows gender data, hepatosplenomegaly, lymphadenopathy, pallor, laboratory data, FAB classification and distribution of the studied AML cases according to CD 47 expression % of studied AML cases. **Table 1**

Table 1: Gender data, hepatosplenomegaly, lymphadenopathy, pallor, laboratory data, FAB classification, distribution of the studied AML cases according to CD 47 expression % and Outcome in studied AML cases (n=40)

Sex	**Male**	26 (65 %)
	Female	14 (35 %)
HSM		18 (45%)
LN		14 (35 %)
Pallor		29 (72.5 %)
Laboratory data	**Hb (gm/dl)**	7.953 ± 1.421
	WBC (x $l0^9$/L)	43.093 ± 26.088
	Platelets (x l09/L)	74.800 ± 22.758
	Blasts (%)	27.600 ± 12.993
	ESR (1^{st} hr.)	75.350 ± 20.252
	LDH (IU/L)	933.850 ± 349.519
	BM blast (%)	62.900 ± 16.086
FAB subtypes	**M1**	4 (10 %)
	M2	11 (27.5 %)
	M3	10 (25 %)
	M4	7 (17.5 %)
	M5	8 (20 %)
CD47 (%)		12 (30 %)
Outcome	**Complete remission**	25 (62.5 %)
	Relapse	9 (22.5 %)
	Death	6 (15 %)

HSM: Hepatosplenomegaly, LN: Lymphadenopathy, Hb: hemoglobin, WBC: White blood cells, ESR: Erythrocyte sedimentation rate, BM: Bone marrow, M1: Acute myeloblastic leukemia with minimal maturation,

M2: Acute myeloblastic leukemia with maturation, M3: Acute promyelocytic leukemia, M4: Acute myelomonocytic leukemia, M5: Acute monocytic leukemia, CD47: Cluster of differentiation 47.

There was no significant relation between Sex, HSM, LN, laboratory data at diagnosis, FAB classification and CD47 expression. **Table 2**

Table 2: Relation between Sex, HSM, LN, laboratory data at diagnosis and FAB classification according to CD47expression

		CD47 (%)		P-value
		Negative	Positive	
Sex	**Male**	17 (60.71%)	9 (75%)	0.385
	Female	11 (39.29%)	3 (25%)	
HSM	**Present**	13 (46.43%)	5 (41.67%)	0.781
	Absent	15 (53.57%)	7 (58.33%)	
LN	**Present**	9 (32.14%)	5 (41.67%)	0.563
	Absent	19 (67.86%)	7 (58.33%)	
Laboratory data at diagnosis	**Hb (gm/dl)**	8.004 ± 1.380	7.833 ± 1.569	0.733
	WBC (x 10^9/L)	43.254 ± 27.009	42.717 ± 24.944	0.953
	Platelets (x 10^9/L)	76.929 ± 22.264	69.833 ± 24.105	0.373
	Blasts (%)	27.500 ± 13.585	27.833 ± 12.059	0.942
	ESR (1^{st} hr.)	76.429 ± 19.101	72.833 ± 23.424	0.613
	LDH (IU/L)	951.929 ± 353.904	891.667 ± 350.606	0.624
	BM blast (%)	62.500 ± 15.999	63.833 ± 16.964	0.814
FAB subtypes	**M1**	1 (3.57%)	3 (25%)	0.259
	M2	9 (32.14%)	2 (16.67%)	
	M3	8 (28.57%)	2 (16.67%)	
	M4	5 (17.86%)	2 (16.67%)	
	M5	5 (17.86%)	3 (25%)	

HSM: Hepatosplenomegaly, LN: Lymphadenopathy, Hb: hemoglobin, WBC: White blood cells, ESR: Erythrocyte sedimentation rate, BM. Bone marrow, M1: Acute myeloblastic leukemia with minimal maturation, M2: Acute myeloblastic leukemia with maturation, M3: Acute promyelocytic leukemia, M4: Acute myelomonocytic leukemia, M5: Acute monocytic leukemia, CD47: Cluster of differentiation 47.

Complete remission was in 2 patients (16.67%), 6 patients relapsed (50%) and 4 patients died (33.33%) of 12 cases CD47 positive expression. There was a significant value between two groups regarding overall survival and disease-free survival (P value<0.001). **Table 3**

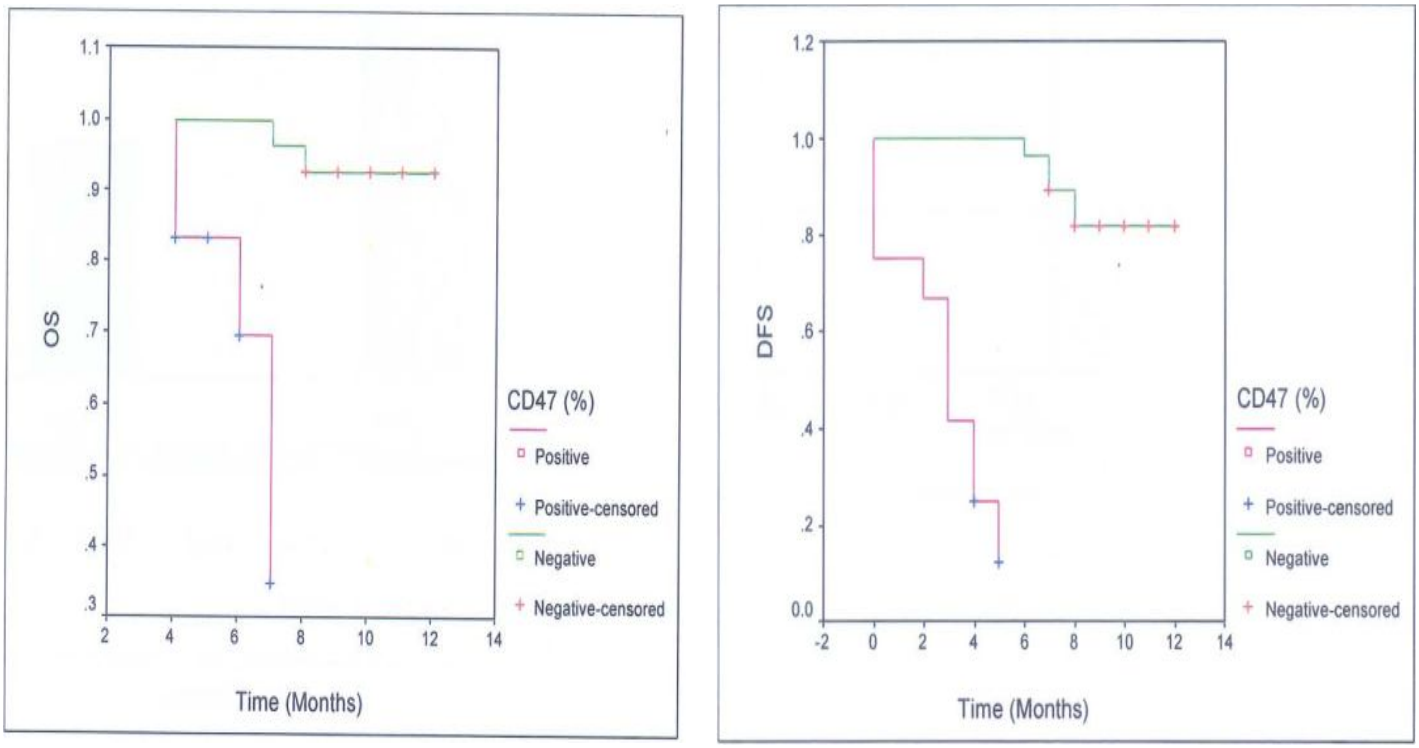
Table 3: Relation between outcome and CD47 expression and comparison between overall survival and disease-free survival with CD47 expression positive and negative groups.

		CD47 (%)		P-value
		Negative	Positive	
Outcome	**Complete remission**	23 (82.14 %)	2 (16.67 %)	<0.001 *
	Relapse	3 (10.71 %)	6 (50 %)	
	Death	2 (7.14 %)	4 (33.33%)	

OS (Months)	9.929 ± 1.464	5.417 ± 1.084	<0.001 *
DFS (Months)	9.214 ± 1.729	2.750 ± 1.865	<0.001 *

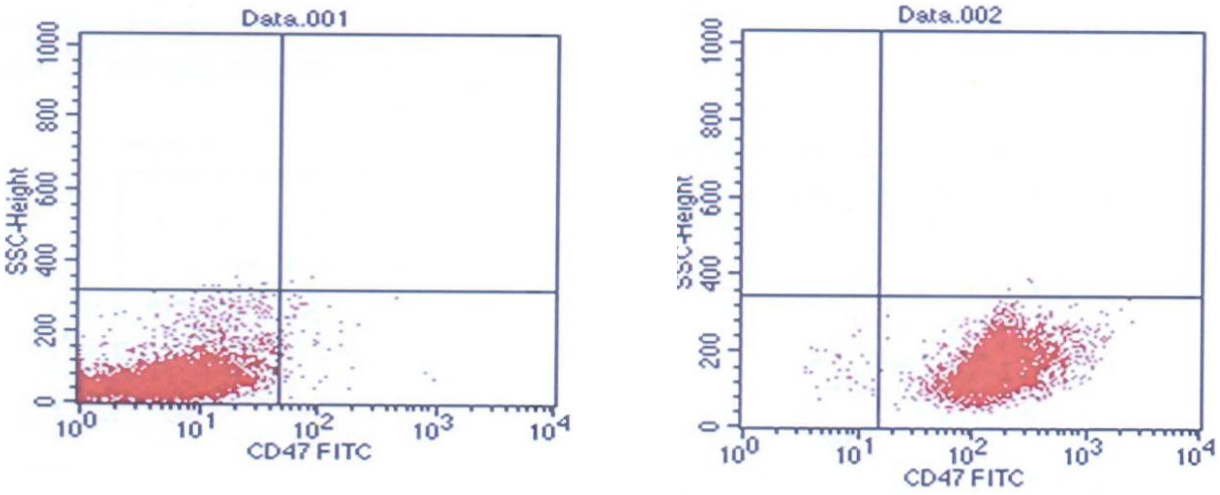
* significant as P value ≤ 0.05. HSM: Overall survival, DFS: Disease-free survival, CD47: Cluster of differentiation 47.

Kaplan-Meier Survival curve shows significant higher overall survival and disease-free survival in negative CD47 expression group than in positive CD47 expression group. **Figure 1**



(A) **(B)**

Figure 1: Kaplan - Meier survival curve (A) overall survival with CD47 Expression positive and negative groups (B) disease free survival with CD47 expression positive and negative groups



(A) (B)

Figure 2: Flowcytometric analysis represents: (A) dot plot showing negative control for CD47 expression (B) dot plot showing positive CD 47 expression

Discussion

Acute myelogenous leukemia (AML) is a clonal, malignant disease of hematopoietic tissues that is characterized by accumulation of abnormal (leukemic) blast cells, principally in the marrow, and impaired production of normal blood cells. Thus, the leukemic cell infiltration in marrow is accompanied, nearly invariably, by anemia and thrombocytopenia. The absolute neutrophil count may be low or normal, depending on the total white cell count [2].

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 [10].

This Study stated that AML can occur in any age, but in general the incidence of AML increases with age also, Deschler and Lubbert [11] stated that incidence of AML increases through adulthood period; during with 70% to 80% of acute leukemias are AML, with marked rise in incidence in elderly. Much of this increase is attributed to AML with a

myelodysplastic related change, which become more with age, while the incidence of de novo AML remains approximately constant across all adult age groups.

In the present Study, it was found that 26 patients (65%) were males, and 14 patients (35%) were females. This is in concordance with a study done by Jemal et al. [12] who reported that, the incidence of AML is higher in male than in females.

As regards the hepatosplenomegaly it was observed in 18/40 (45%) of the studied patients. In contrast Lichtman and Lieveld [13] who reported that hepatosplenomegaly occurs in one about one third of AML patients.

Lymphadenopathy was absent in 26 patients (65%). This is in concordance with Lichtman and Lieveld [13] who reported that Lymphadenopathy is an uncommon feature of AML.

As regards pallor, Out of the studied patients, 29 patients (72.5%) showed pallor and 11 patients (27.5%) had absence of pallor This is in concordance with Mathur et al. [14]who reported that Pallor was seen as a presenting symptom in 100% of their patients.

As regards FAB classification and its distribution among patients' groups with CD47 Expression (12 patients). Ml was in 3 patients (25%), M2 was in 2 patients (16.67%), M3 was in 2 patients (16.67%), M4 was in 2 (16.67%) and M5 was in 3 patients (25%) of l2 cases CD47 expressed. So, the present study showed no significant difference in CD47 expression in relation to FAB subgroups. This is in concordance with kassem et al [15]. It was also in agreement with previously reported studies Majeti [7].

With regard to CD47 Expression in relation to gender, Hb, TLC, platelets count, blast count, ESR, LDH, showed no statistically significant difference between the negative and positive groups. These findings were in Concordance with kassem et al [15].

IN this study by comparing between groups, it was found that patients with positive CD47 expression had a significant lower CR rate and a significant higher relapse and death rate.

Thus, there was a significant association between CD47 expression and poor outcome of patients ($p<0.001$). These findings were in concordance with Majeti et al [7].

Overall survival (OS) and disease-free survival (DFS) were evaluated in the negative CD47 expression group using Kaplan-Meier Survival curve. A Significant decrease in both OS ($p< 0.001$) and DFS ($p<0.001$) was found in the positive CD47 expression group. This is in accordance with Majeti et al [7]. Also, kassem et al [15] observed that there was inverse correlation between CD47 expression and Overall survival where increased expression was associated with worse overall survival.

Acute myelogenous leukemia (AML) is organized as a cellular hierarchy initiated and maintained by a subset of self-renewing leukemic stem cells (LSC). It was hypothesized that increased CD47 expression on human AML LSC contributes to pathogenesis by inhibiting their phagocytosis through the interaction of CD47 with an inhibitory receptor on phagocytes. It was found that CD47 was more highly expressed on AML LSC than their normal counterparts, and that increased CD47 expression predicted worse overall survival in AML patients [16].

By expressing CD47, AML cells trigger the "don't eat me" signal to macrophages via SIRPa engagement and can therefore escape the immune system by inhibiting phagocytosis [16].

Also, Sallman [17] reported that CD47 is a dominant macrophage checkpoint that is overexpressed on most cancer cells and increased CD47 expression has been associated with poorer prognosis.

Several anti-human CD47 monoclonal antibodies have been generated including some that are capable of blocking the CD47-SIRPa interaction (B6H12.2 and BRIC126) and others unable to do so [18]. So, blocking monoclonal antibodies directed against CD47 enabled phagocytosis of AML LSC [19].

Hu5F9-G4 (5F9), a first- in- class antibody targeting CD47, used as monotherapy or in combination with standard azacitidine was well tolerated and provided deep and durable response in patients with acute myeloid leukemia (AML) which is according to a study presented at the 2019 European Hematology Association (EHA) Congress.

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Conclusions:

Positive CD47 expression levels are associated with a poor outcome in AML patients and its expression can be easily determined in routine flow cytometric analysis. Therefore, it should be regularly investigated as a bad prognostic factor for the assessment of AML patients.

Conflict of interest ---make a statement

Source of funding --- make a statement

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