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SALL4 Oncogene in Acute Myeloid Leukemia: Expression Levels and Prognostic Impact

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ABSTRACT

Background: SALL4, a zinc finger transcription factor, is an embryonic stem cell regulator that controls self-renewal and pluripotency. Recently, SALL4 overexpression has been observed in malignant tumors such as lung cancer and hepatocellular carcinoma. **Objective:** We aimed at evaluating the expression pattern of the SALL4 gene in individuals with acute myeloid leukaemia (AML) and determine its prognostic impact. **Patients and Methods:** This study was a prospective, single center study conducted on 35 adult Egyptian patients with AML, recruited from the Hematology Unit of the Internal Medicine Department at Ain Shams University Hospitals during the period from January 2021 to April 2021 whose ages ranged between 18 and 73 years old. Real-time quantitative PCR (RT-PCR) was used to assess the expression level of SALL4 mRNA in bone marrow (BM) mononuclear cells from 35 AML patients, and 15 patients who were candidates for BM aspiration for causes other than hematological or solid malignancies as controls. **Results:** The expression of SALL4 was significantly higher in cases of acute myeloid leukemia compared to controls (p value= 0.001). Strong association was also found between SALL4 expression levels and failure to achieve complete remission (CR) revealed by post-induction BM blast percentage and minimal residual disease (MRD) detection by immunophenotyping (r values of 0.68 and 0.62, respectively, p values= 0.001 in both cases). **Conclusion:** SALL4 is significant prognostic factor in de-novo AML and could be strong target for novel types of therapy.

Keywords: Acute myeloid leukemia, SALL4 gene, Prognosis, RT-PCR.

INTRODUCTION

About 80% of all occurrences of leukemia in adults are acute myeloid leukemia (AML), making it the most prevalent kind. It is defined by the clonal proliferation of blast cells in the BM and peripheral circulation, which leads to inefficient erythropoiesis and, ultimately, BM failure [1]. It is a very diverse illness with a mixed prognosis that may be brought on by chromosomal translocations, genetic abnormalities, or modifications to the molecular structure. Transcription factor fusions, the NPM1 gene, tumor suppressor genes, signaling genes, DNA methylation-related genes, chromatin-modifying genes, myeloid transcription-factor genes, cohesin complex genes, and spliceosome complex genes are the nine functional categories into which mutated genes were divided [2].

Spalt-like transcription factors (SALLs) have an impact on the development, course, and outcome of a number of malignancies, including renal and cervical cancer [3]. While SALL4 is substantially expressed in malignancies, SALL1, SALL2, and SALL 3 are often downregulated [4]. One zinc finger transcription factor that regulates self-renewal and pluripotency in embryonic stem cells is oncofetal SALL4 [5,6]. It is found on chromosome 20.q13.2. It is translated into two isoforms, SALL4A and SALL4B, as a result of distinct internal splicing patterns in exon 2 [7]. In SALL4B transgenic mice, it has been discovered that both isoforms bind to the β-catenin protein. This results in the synergistic stimulation of the Wnt/β-catenin pathway, which is crucial in regulating the self-renewal of leukemia stem cells (LSCs) [8].

With the exception of germ cells, most adult tissues usually quiet SALL4 [9]. SALL4 was discovered to be overexpressed in a number of solid tumors, including hepatocellular carcinoma [10-13], endometrial cancer [12], gastric cancer [10], lung cancer [11], and endometrial cancer [12]. Thus, knowing SALL4's roles and processes might help us develop new ideas about how to target SALL4 in tumor treatments. SALL4 may be a target for cancer treatments and a clinically identified unique biomarker in several tumor types, according to mounting data. Numerous studies have revealed that SALL4 affects pro- and anti-apoptotic pathways in leukemic and normal cells differently [14]. During normal hematopoiesis, SALL4 is primarily expressed in human CD34+ hematopoietic stem/progenitors (HS/PCs), and during hematopoiesis differentiation, it is down-regulated in CD34- cells [15].

Additionally, the expression of the SALL4 gene has been connected to the course of the illness in human chronic myeloid leukemia and to the outcome of treatment in individuals with AML [16]**.** However, more research is required to demonstrate SALL4 expression levels and understand their diagnostic and prognostic significance in acute leukemias.

The purpose of this study was to analyse the expression pattern of the SALL4 gene in patients with AML and assess its prognostic significance.

PATIENTS AND METHODS

This study was a prospective, single center study conducted on 35 adult Egyptian patients with AML, recruited from the Hematology Unit of the Internal Medicine Department at Ain Shams University Hospitals during the period from January 2021 to April 2021 whose ages ranged between 18 and 73 years old

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and we followed up the patients for one year after first cycle of chemotherapy.

The diagnosis of AML was based on morphologic, cytochemical evaluation, immunophenotyping and complementary cytogenetics according to updated WHO 2016 diagnostic criteria[1]. AML patients were subtyped into patient AML M0[10], patients AML M1-M2[6], patients AML M2[13], patients AML[3], patients AML M4[2] and patients AML M5. All patient received 3+7 protocol (Adriamycin + Ara C) except AML M3 patients received PETHEMA protocol. Only de novo AML patients were included in our study including AML with balanced translocations and AML not otherwise specified. Relapsed AML patients were excluded from the study. Another fifteen subjects, who were candidates for BM aspiration for causes other than hematological or solid malignancies, were enrolled in our study (Control group).

A thorough medical history was obtained from both groups together with comprehensive clinical examination, with particular attention to investigations to detect organomegaly. Complete blood count (CBC) using peripheral venous blood samples was examined by XN-1000 [Sysmex, Japan]. Leishman-stained peripheral blood films were examined. Both the patient and control groups underwent bone marrow (BM) aspiration and Leishman-stained BM smear examinations. Flow-cytometric immuno-phenotyping was carried on BM or peripheral blood samples (for patients group only) using an extended panel of monoclonal antibodies (MoAbs) on NAVIOS 2 Laser 6 Color FCM [Beckman Coulter, USA]. All monoclonal antibodies were purchased from Beckman-Coulter (Marcillia, France). Cells were stained with different antibody combinations using either fluorescein isothiocyanate (FITC), phycoerythrin (PE), PC5 or PC7 conjugated MoAbs for diagnosis and subclassifications of AML. Cytogenetic analysis was also carried on BM or peripheral blood samples (for patients group only) using FISH or karyotyping techniques. FISH was performed by scanning at least 100 interphase nuclei in every case under the chromo-scan. Low-power objective lens was used, the signals were then captured using an oil-immersion objective lens and detected using the CytoVision automated cytogenetics platform [Leica Biosystems Richmond, USA]. Positive findings were diagnosed with a cut-off value of more than 10% for single fusion probes and more than 3% for double fusion probes. The used probes were Vysis RUNX1/RUNX1T1 double fusion probe for t(8;21), Vysis PML/RARA single fusion probe for t(15;17), Vysis CBFB break apart probe for inv [16], Vysis LSI MLL Dual Color break apart rearrangement probe for 11q23 rearrangement and Vysis LSI BCR/ABL single fusion probe for t (9;22).

AML patients were re-assessed on the day 28 following induction therapy. BM samples were collected to detect BM blast percentage together with minimal residual disease (MRD) quantification using six color multiparameter flowcytometry with 0.01% cut off. Every AML case's leukemia-associated immunophenotype was determined upon diagnosis using different combinations of monoclonal antibodies directed against nuclear, cytoplasmic, or surface leukocyte antigens. The second method was represented by "different from normal (DFN)" analysis, which uses the assessment of antigenic pattern expressions to identify immunophenotypic changes in leukemic blasts relative to a normal counterpart population (either hematopoietic progenitors of similar lineage or maturational stage).

Döhner *et al.* [17] defined complete remission (CR) as follows: baseline neutrophil count (ABC) < 5%; absence of blasts with Auer rods; absence of extramedullary illness; platelet count >100 x 109/L; and independence from red cell transfusions. Together with accordingly, our patients were classified into responders (patients who achieved CR) and non-responders (patients who couldn't achieve CR) and SALL4 gene expression was examined in both groups.

Sampling:

For CBC and Leishman-stained smears, two mL of whole venous blood was collected on K2-EDTA (1.2 mg/ml) (Greiner). Fresh BM samples were collected on EDTA in the following amounts: 0.5 mL for blood smear preparation and cytochemical staining for preliminary leukemic lineage determination, 1 mL for IPT by FCM, and 1.5 mL for RT-PCR. For cytogenetic analysis, one mL was collected in a sterile tube coated with lithium heparin.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of SALL4 gene expression in hematopoietic tissues (using TaqMan probe technique).

ThermoFisher Analysis provided the PCR primers for amplification of the SALL4 gene Hs01010838 g1 Catalog no.: 4448892 and the GAPDH Housekeeping gene Catalog no.: 402869.

Sample preparation:

- **RNA Extraction** (GeneJet RNA Purification kit by ThermoScientific, cat no. # K0731): 1.5 mL human whole blood (typically 4000-7000 leukocytes/µL) was collected on an anticoagulant preferably k2-EDTA. Blood cells were obtained by centrifuging 0.5 mL of whole blood at 400 g for 5 minutes at 4ºC. The pellet was then removed and resuspended in 600 µL of lysis buffer supplemented with reducing agent dithiothreitol, 450 µL of ethano l (96-100%) was then added and mixed by pipetting. 700 mL of lysate was transferred to a collecting tube containing the GeneJET RNA Purification Column. The purification column passed through several steps of buffer wash and centrifugation before eluting the RNA with nuclease-

free water. The samples were kept at -80°C until they were ready to be processed.

- **Strand cDNA Synthesis:** (RevertAid First Strand cDNA Synthesis Kit by ThermoScientific, cat no. #K1622) cDNA was obtained according to manufacturer-provided instructions.
- **Master Mixing and rt-PCR:** (TaqMan Gene Expression Master Mix by Applied biosystems, cat no. 4370048), four µL of the cDNA template (1 to 100 ng) was added to 5 µL RNase-free water, which in turn were added to 10 µL of TaqMan Gene Expression Master Mix and 1 µL of TaqMan Gene expression assay forming final PCR reaction mix of total volume 20 µL. Next, 10 µL of PCR reaction mix was transferred to each well of the supplied optical reaction plate and then sealed with optical adhesive film and centrifuged briefly to collect the contents at the bottom of the plate.
- The Applied Biosystems StepOne™/ StepOnePlus™ Real-Time PCR System was programmed as follows:

  Annealing/extension was performed at 50 ºC for 2 minutes, 95 ºC for 10 minutes, and 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. The signal from the RT-PCR product was normalized to that of the internal control (GAPDH), which was amplified using a different set of primers.

Interpretation of the results:

- The relative expression of SALL-4 was measured using the delta cycle threshold (CT) method. A comparative (CT) was used to determine the gene expression relative to a normal control (calibrator) and used for comparison between patients and controls, and relative SALL-4 expression for every patient was calculated using $\mathbf{2^{-\Delta\Delta CT}}$ formula, where **ΔΔCT = ΔCT sample-ΔCT calibrator**.

Ethical approval:

The study was carried out in accordance with Declaration of Helsinki's ethical principles and the rules for human testing, as approved by the Ain Shams University Ethics Committee locally. All participants were given the opportunity to provide written informed consent after being fully told about the purpose, nature, and necessary interventions and investigations.

Statistical analysis

Using IBM SPSS V. 23.0, the gathered data were coded, tabulated, and statistically examined. The analysis of numerical values for descriptive data involved the use of arithmetic mean (±SD), median, and IQR, whereas frequency and percentage were employed for qualitative data, which were compared by the chi square test. Regarding quantitative non-parametric data, the Mann Whitney was utilized to compare the differences between two groups. When comparing non-parametric variables between more than two groups, Kruskal-Wallis was used to find differences. Spearman's correlation coefficient was computed to assess the degree of relationship between two numerical variables. Using a ROC curve graphic plot, the right cut-off value for the prognostic marker under inquiry was determined. Lastly, the p value was used to calculate the power of significance. P-values < 0.05 were deemed significant.

RESULTS

The age of the control group was significantly older than the AML patients. Regarding gender, hepatomegaly, and splenomegaly, the difference between the two groups was insignificant **(Table 1).**

Table (1): Demographic and clinical data among AML patients and control groups

Paramater		AML patients	Controls	P-value
Age (years)	Mean±SD	41.77±14.13	55.73±13.36	0.002
Gender	Male N (%)	19 (54.3%)	6 (40%)	0.355
	Female N (%)	16 (45.7%)	9 (60%)	
Hepatomegaly	Yes N (%)	19 (54.3%)	8 (53.3%)	0.951
	No N (%)	16 (45.7%)	7 (46.7%)	
Splenomegaly	Yes N (%)	18 (51.4%)	6 (40%)	0.459
	No N (%)	17 (48.6%)	9 (60%)	

Laboratory data among AML patients:

The median value of TLC and platelets was 33.8 x10^9/L (IQR= 5.6-61) and 49 x 10^9/L (IQR= 23-95); respectively, whereas the mean hemoglobin level was 8.23 ± 1.67 g/dL. The mean percentage of peripheral blasts and BM blasts were 71.17±17.62 and 70.37±17.12; respectively. AML patients were further subdivided according to the results of IPT into: M0 (1/35; 2.5%), M1-M2 (10/35; 28.6%), M2 (6/35, 17.1%), M3 (13/35; 37.1%), M4 (3/35; 8.6%), M5 (2/35; 5.7%). It was observed that 4 cases showed co-aberrant expression of lymphocytic marker CD7 and 2 cases co-aberrantly expressing CD56 **(Table 2)**

Table (2): Laboratory data among AML patients

Variable		AML patients (n=35)
TLC (x 10^9/L)	Median (IQR) Range	33.8 (5.6 - 61) 0.6 - 340.4
Hb (g/dl)	Mean±SD	8.23±1.67
PLT (x 10^9/L)	Median (IQR) Range	49 (23 - 95) 1-195
Peripheral blasts %	Mean±SD	71.17±17.62
BM blasts %	Mean±SD	70.37±17.12
IPT markers		
CD7	N (%)	4 (11.4%)
CD56	N (%)	2 (5.7%)
CD33	N (%)	34 (97.1%)
CD13	N (%)	34 (97.1%)
CD14	N (%)	2 (5.7%)
CD117	N (%)	31 (88.6%)
CD64	N (%)	23 (65.7%)
Myeloperoxidase	N (%)	35 (100%)
CD34	N (%)	22 (62.9%)
HLA-DR	N (%)	21 (60%)

Median, IQR and range: Non-paramtric test.

Cytogenetic (FISH) analysis was done on the available BM or peripheral blood samples of AML patients at the time of diagnosis to detect common cytogenetic abnormalities, where t(15;17) PML/RARA was carried out on samples of 13 AML patients who had double negativity of CD34 and HLA-DR. Only 4 out of 13 cases revealed positive t(15;17) while the other cases were lacking the classic t(15;17) by routine cytogenetic analysis but they were further confirmed by expressing of PML/RARA transcript using PCR technique. 16 AML patients who were diagnosed as M1-M2 or M2 were investigated for presence of t(8;21) RUNX1/RUNX1T1, however, none of them was positive. Analysis of t(9;22) BCR/ABL was carried out to three AML patients, and only one of them was positive for t(9;22). Three AML cases that were diagnosed by IPT as M4 were analyzed to detect inv.16; but none of them was positive. 2 AML cases that were diagnosed by IPT as M5 were analyzed by LSI Break apart probe to detect MLL (11q23) and it was negative for that abnormality.

According to response to chemotherapy by MRD quantification, complete response (CR) was achieved in 22 cases out of 34 (64.7%), while 12 out of 34 (35.3%) exhibited partial response (PR). Unfortunately, one case died before day 28. Interestingly, this patient was positive for t(9,22).

During 1 year after first cycle of chemotherapy, 9 patients developed relapse (25%), 2 patients after 4,6 and 9 months and one patient after 5 and 12 months.3 patients died (8,6%) due to relapse related complications. All these patients achieved PR after first cycle and achieved CR on the second line chemotherapy protocol and expressed higher levels of SALL4.

SALL4 gene expression levels in both AML and control groups:

SALL4 gene level was highly expressed among AML patients with median value 18.46 (IQR= 11.52-83.09) compared to low expression among control group with median value 1.01 (IQR= 0.92-1.05) (**Figure 1)** and this difference was statistically highly significant (p <0.001).

In a trial to depict the best cut off value for SALL4 gene expression level that could predict diagnosis of AML, ROC curve was drawn and a cutoff value of >1.61 turned out to be the best value that could discriminate between AML cases and controls, as evidenced by area under curve (AUC) of 0.996. At this value, the specificity was 100% and sensitivity was 97.14% (**Figure 2).**

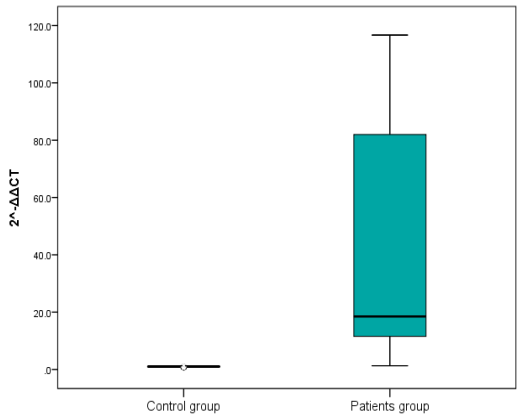

Figure (1): SALL4 gene expression levels among AMLcases and control groups

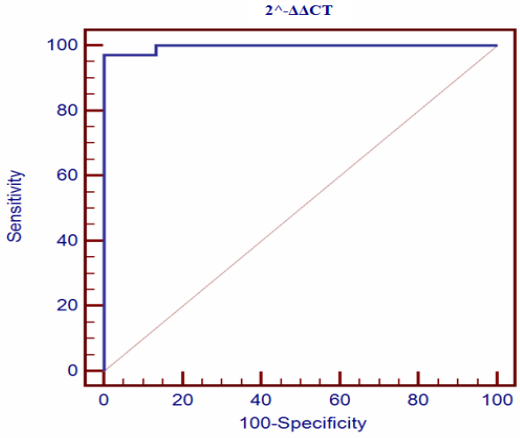

Figure (2): ROC curve for SALL 4 gene expression to discriminate between AML cases and controls.

Correlation between SALL4 gene expression levels and both demographic and clinical data:

Understanding the significance of age in predicting prognosis of AML disease, we classified our patients into two groups based on their age. It was observed that SALL4 was highly expressed among patients above 60 years than those below 60 years,

however, this difference was statistically insignificant, this may be attributed to small sample size.

SALL4 gene was also highly expressed in patients with hepatomegaly and splenomegaly (median: 26.85 and 22.4; respectively) compared to patients without organomegaly, however, this difference was statistically insignificant **(Table 3)**

Table (3): Relation between SALL4 gene expression levels and clinical data

		SALL4 gene expression level		P-value
		Median	IQR	
Age	<60 years	16.53	(11.37–83.09)	0.276
	≥60 years	56.94	(25.51–92.64)	
Gender	Male	17.96	(11.37 – 70.85)	0.778
	Female	20.29	(12.2 – 97.13)	
Hepato-megaly	Present	26.85	(11.85 – 80.82)	0.843
	Absent	16.68	(11.45 – 107.81)	
Spleno-megaly	Present	22.4	(11.52 – 70.85)	0.564
	Absent	18.46	(11.52 – 111.17)	

Correlation between SALL4 gene expression and laboratory parameters in AML group:

No significant correlation was observed between SALL4 gene level and each of TLC and platelet count. Although negative correlation was found between Hb level and SALL 4 gene expression, yet it was of no statistical significance. AML patients were classified into two groups based on their TLC according to prognostic criteria adopted by American Cancer Society; 2021 to assess the prognostic performance of SALL4. Although elevated SALL4 levels were noticed among AML patients whose TLC exceeded $100x10^9$ /L (median value= 24.61) than patients whose TLC was below $100x10^9$ /L (median value= 18.46), yet this difference was statistically insignificant (p= 0.641). Positive correlation was noticed between SALL4 gene expression levels and peripheral and BM blast percentages at time of diagnosis in AML group; however, this correlation was also statistically insignificant **(Table 4)**.

Table (4): Correlation between SALL4 and laboratory parameters in AML patients

	SALL4 gene expression	
	r	p
TLC (x 10^9/L)	0.04	0.819
Hb (g/dl)	- 0.234	0.177
PLT (x 10^9/L)	0.099	0.57
Peripheral blasts %	0.113	0.518
BM blasts %	0.016	0.927

Immunophenotyping and SALL4 gene expression levels

It was observed that SALL4 gene level was highly expressed in AML patients with positive CD34 compared to those patients lacking the above-mentioned marker (median value= 22.66 vs. 14.89), however, it was of no significance. It was also noticed that AML cases co-aberrantly expressing either CD7 or CD56 revealed higher median expression levels of SALL4 gene (47.31 and 44.66; respectively) compared to cases neither expressing CD7 nor CD56 (18.46 and 17.96; respectively). However, these findings were of no statistical significance.

The highest expression of SALL4 gene level was encountered among patients diagnosed as AML M4 (median value= 54.07), while the least expression was noticed among patients diagnosed as AML M3 (median value= 14.89). However, no statistically significant difference was found **(Table 5)**.

Table (5): Relation between SALL4 gene expression and FAB subtypes

FAB class (n=35)	SALL4 gene expression		P value
	Median	(IQR)	
M0 (n=1)	674.03	(-)	0.709
M1-M2 (n=10)	21.69	(8.73-61.68)	
M2 (n=6)	51.47	(8.5-196.27)	
M3 (n=13)	14.89	(11.85-70.85)	
M4 (n=3)	54.07	(11.52-83.09)	
M5 (n=2)	22.07	(12.88-31.27)	

Regarding relation between SALL4 gene expression level and cytogenetics, no statistically significant association was observed between SALL4 gene level and either patients revealing or lacking recurrent translocations, which may be due to small sample size. It should be noted that SALL4 was strikingly elevated in the patient with t(9;22) (RQ= 19710.36), however this finding requires further analysis on larger scale of patients.

SALL4 gene expression and response to induction therapy

At day 28 post-induction chemotherapy, a highly significant positive correlation was noticed between SALL4 gene expression level and both BM blast percentage morphologically examined in BM smears and MRD IPT percentage (r= 0.68, 0.62; respectively with p< 0.001) **(Figures 3, 4).**

It was also noticed that SALL4 was highly expressed among non-responders than responders **(Table 6).**

Table (6): SALL4 gene expression and response to treatment

	SALL4 gene expression levels		P value
	Median (IQR)	Range	
Outcome Survived (n=34) Deceased (n= 1)	18.21 (11.52 – 80.82) 19710.36 (--)	1.27 – 1906.44 (--)	0.092
Response PR (n=12) (non-responders) CR (n=22) (responders)	107.81 (75.84 – 156.48) 12.36 (8.5 – 17.96)	33.05 – 1906.44 1.27 – 54.07	<0.0001

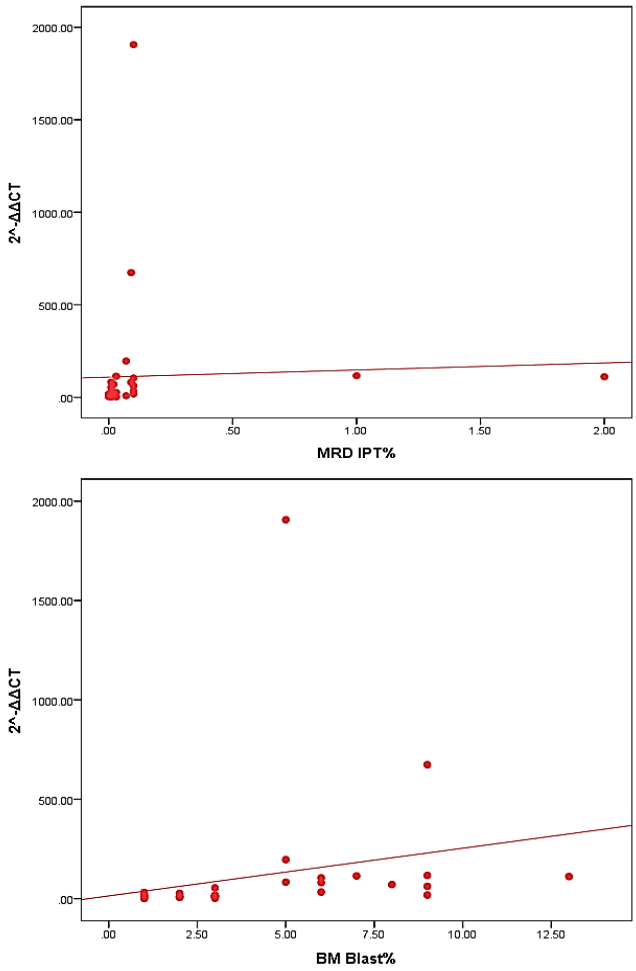

Figure (3) and (4): Scatter plot of post inductuion BM blasts%, MRD IPT% and SALL4 RQ

It is worth mentioning that the only patient who died during the follow up period of our study exhibited t(9;22) and had the highest expression level for SALL4 (19710.36).

To figure out a cutoff value for SALL4 gene expression that could predict poor response to chemotherapy and hence poor prognosis, ROC curve was drawn and **31.27** was found to be the best value above which poor prognosis could be predicted with AUC of 0.996, sensitivity 100% and specificity of 95.45% **(Figure 5).**

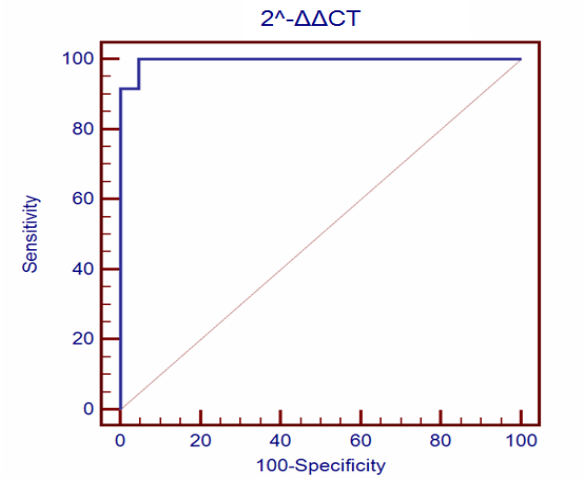

Figure (5): ROC curve for SALL4 gene expression level discriminating between CR and partial remission (PR)

DISCUSSION

One of the most common types of leukemias, AML, is typified by clonal growth originating from primitive hematopoietic stem cells or progenitor cells. Reduced differentiated red blood cells, platelets, and white blood cells as well as an increased concentration of immature malignant cells are the outcomes of abnormal myeloid cell differentiation [18].

It is a molecularly diverse disease with a bad prognosis in most cases. Based on cytogenetic and molecular abnormalities, AML patients are divided into risk categories for risk-adjusted treatment [19]. The prognosis is usually poor in patients with complicated karyotype anomalies or adverse molecular features. However, not all AML patients have cytogenetic abnormalities, necessitating the development of new genomic techniques to improve risk assessment [20].

A DNA-binding protein with many C2H2 zinc finger motifs is encoded by the SALL4 gene. Human and mouse embryonic stem cells need the SALL4 protein to maintain pluripotency [21], and its overexpression or lack thereof can alter the cells' "stemness" [22].

In this study we aimed to assess SALL 4 gene expression in 35 AML patients versus 15 subjects free from either solid or hematological malignancies as control group and to elucidate its prognostic influence on AML disease course.

We found that SALL4 gene level was highly expressed among AML group with median value 18.46 (IQR=11.52-83.09) compared to low expression among control group with median value 1.01 (IQR=0.92-1.05) (p <0.001). A previous studies supported our results and showed a significant higher expression of SALL4 among AML patients compared to the control group (p= 0.001) [23]. Moreover, the results of another study concluded that none of the studied controls expressed SALL4 > 1.0 RQ while the cases showed a significant higher expression (p< 0.001) [24]. Consistently, a study conducted by **Shen *et al.*** [25] studied 24 newly diagnosed Chinese AML

patients from the Institute of Hematology in China and found that SALL4 expression was higher in AML patients (median: 1.051) compared to healthy individuals (median: 0.394) with p= 0.009, which was consistent with **Chen *et al.*** [26] ($p < 0.001$). In a study of 60 leukemic patients and 10 normal controls at Dalian Medical University in China, **Duan *et al.*** [27] discovered significantly higher expression of SALL4 in AML patients compared to controls ($p<0.05$). Meanwhile, **Farawela *et al.*** [28] observed that de novo AML patients had significant levels of SALL4 gene expression (median 5.180-fold change), however these levels did not approach statistical significance. Our results could be explained by the influence of SALL4 on proapoptotic and antiapoptotic pathways in leukemic and normal cells. It has been discovered that SALL4 is involved in tumor growth, invasion, and migration through the TNF family, Notch, PTEN/PI3K/AKT pathways, Wnt/β-catenin, and some caspase-related proteins.

In a trial to depict the best cut-off value for SALL4 gene expression level that could predict diagnosis of AML, ROC curve was applied and a value of >1.61 turned out to be the best value that could discriminate between AML cases and controls, as evidenced by area under curve (AUC) of 0.996. At this value, the specificity was 100% and sensitivity was 97.14%

In the present study, SALL4 gene expression in AML patients did not significantly correlate with age (r=0.1, p=0.447), hemoglobin levels (r=0.234, p=0.177), total leucocytic count (r=0.04, p=0.819), platelet counts (r=0.099, p=0.57), peripheral blood blast count (r=0.113, p=0.518), or BM blast count (r=0.016, p= 0.927). All other factors did not significantly affect SALL4 gene expression in AML patients. In line with these findings, **Farawela *et al.*** [28] reported that there was no significant correlation between the AML patients' age (r= 0.1, p= 0.532), hemoglobin levels (r= 0.103, p= 0.532), total leucocytic count (r= 0.13, p= 0.416), platelet counts (r= 0.079, p= 0.625), peripheral blood blast count (r = 0.29, p= 0.065), or BM blast count (r = 0.084, p= 0.601).

Regarding relation between SALL4 gene expression level and cytogenetics, no significant association was observed with either patients revealing or lacking recurrent translocations, which may be attributed to small sample size. It should be noted that SALL4 was strikingly elevated in a patient with t(9;22) (median = 19710.36), however this finding requires further analysis on larger scale of patients. These findings also agreed with **Chen *et al.*** [26] **and Farawela *et al.*** [28] who discovered no connection between chromosomal aberrations and SALL4 expression in AML patients.

On studying SALL4 gene expression in AML patients with different FAB subtypes, the highest expression of SALL4 gene level was encountered among patients diagnosed as AML M4 (median = 54.7), followed by AML M2 then M5 then AML M1 (median = 51.47, 22.07 and 21.69, respectively) while the least expression was noticed among patients diagnosed as AML M3 (median = 14.89), yet with no statistical significance (p = 0.7). Partially supporting our results, in their study, **Swelem *et al.*** [23] found that, in comparison to M1 and M2, SALL4 expression was significantly higher in M4 and M5. **Chen *et al.*** [26], on the other hand, discovered that SALL4 gene expression varied considerably with AML FAB subtypes. M2 (86.7%, 13/15) > M3 (75%, 6/8) > M1 (60%, 3/5) > M4 (14.3%, 1/7) (p = 0.008) had the highest frequency of SALL4 expression. When **Farawela *et al.*** [28] compared SALL4 expression in several FAB subtypes, they discovered that M1 and M2 had a median 5.180-fold change, whereas M4 and M5 had a median 3.085-fold change. These variations did, however, become statistically significant. **Shen *et al.*** [25] observed that the highest SALL4 expression level was among patients diagnosed as AML M5 (median = 1.465) followed by M2 (median = 0.974) and M3 (median = 0.799), however, none of them was statistically significant**.** More extensive research in this point with larger sample size is highly recommended.

In the current study, we observed that SALL4 gene level was highly expressed in AML patients with positive CD34 compared to those patients lacking the above-mentioned marker (median: 22.66 vs. 14.89), however, it was of no significance. It was also noticed that AML cases co-aberrantly expressing either CD7 or CD56 revealed higher median expression levels of SALL4 gene (47.31 and 44.66; respectively) compared to cases neither expressing CD7 nor CD56 (18.46 and 17.96; respectively) However, these findings were of no statistical significance. **Abo-Elwafa *et al.*** [29] had a consistent result as they stated that there was no statistically significant difference between the expression level of CD markers and SALL4 mRNA in AML cases. Comparably, **Farawela *et al.*** [28] discovered that although SALL4 expression in AML patients with positive CD34 expression increased more than in those with negative CD34 expression (median 2.608-fold increment), the difference was not statistically significant. This might be explained by the way SALL4 keeps progenitor undifferentiated cells with a high potential for self-renewal stem-like characteristics. One important component needed for the development and upkeep of hematopoietic stem cells and pluripotent cells is SALL4. Overexpression of SALL4 in peripheral blood CD34+ cells that were mobilized increased the ex vivo expansion efficiency of CD34+/CD38− and CD34+/CD38+ cells by more than 10,000 times when the right cytokines were used [8]**.**

Researching SALL4's function in healthy hematopoiesis revealed that CD34 expression was lost in normal CD34 positive cells when SALL4 expression was downregulated. The stem/progenitor cell marker CD34 influences the expression of genes involved in differentiation and self-renewal [14]. These findings could imply that the kind of leukemia that favors the undifferentiated phenotype depends on the expression level of SALL4. When **Swelem *et al.*** [23] examined the

flowcytometric parameters, they discovered that case people who tested positive for CD34 had significantly higher levels of SALL4 expression than case persons who tested negative for CD34 ($p< 0.001$).

All our AML patients were re-assessed on the 28th day following induction therapy. BM samples were collected from patients on day 28 post-induction chemotherapy, to detect BM blast percentage together with MRD quantification using six color multiparameter flowcytometry with 0.01% cut off.

Highly significant positive correlation ($r= 0.68$, $p< 0.001$) was noticed between SALL4 gene expression level and BM blast percentage morphologically examined in postinduction BM smears of AML patients. Our patients were then classified into responders (patients who achieved CR) and non-responders (patients who couldn't achieve CR) according to MRD quantification and SALL4 gene expression was examined in both groups. It was noticed that SALL4 was highly expressed among non-responders than responders (median value=107.81 vs. 12.36), and this difference was found to be statistically highly significant ($p< 0.0001$).

It is worth mentioning that the only patient who died during the follow up period of our study exhibited t(9;22) and had the highest expression level for SALL4 (19710.36)

This is coinciding with **Swelem *et al.*** [23] whose findings showed a significant correlation ($p=0.02$) between SALL4 and patients who did not achieve CR or who even relapsed. **Farawela *et al.* [28]** found that patients with high SALL4 gene expression among their examined de novo AML patients showed shorter disease-free survival (DFS) rates (29.4% and 100%, respectively; $p = 0.022$) than those with lower SALL4 gene expression levels.

To figure out a cutoff value for SALL4 gene expression that could predict poor response to chemotherapy and hence poor prognosis, ROC curve was drawn and 31.27 was found to be the best value above which poor prognosis could be predicted with AUC of 0.996, sensitivity 100% and specificity of 95.45%. To our knowledge, no other studies showed its diagnostic or prognostic cut off values for SALL4 gene expression and hence more studies are highly recommended to verify these cut offs.

CONCLUSION

In de-novo AML patients, SALL4 gene expression is constitutive. Greater SALL4 gene expression at the time of diagnosis leads to partial remission and a worse prognosis. SALL4 gene expression at elevated levels necessitates a reinforced induction treatment regimen. According to this study, the RQ (Relative quantification) cutoff value for the SALL4 gene for AML diagnosis is 1.61, and an RQ cutoff value > 31.27 indicates a bad prognosis.

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REFERENCES

1. **Bain B, Béné M (2019):** Morphological and immunophenotypic clues to the WHO categories of acute myeloid leukaemia. Acta Haematol., 141(4):232-244.
2. **Welch J, Ley T, Link D *et al.* (2012):** The origin and evolution of mutations in acute myeloid leukemia. Cell, 150(2): 264-278.
3. **Misawa K, Mochizuki D, Imai A *et al.* (2017):** Epigenetic silencing of SALL3 is an independent predictor of poor survival in head and neck cancer. Clin Epigenetics, 9: 64. doi: 10.1186/s13148-017-0363-1
4. **Ma T, Shi S, Jiang H *et al.* (2021):** A pan-cancer study of spalt-like transcription factors 1/2/3/4 as therapeutic targets. Arch Biochem Biophys., 711: 109016. doi: 10.1016/j.abb.2021.109016
5. **Diener J, Baggiolini A, Pernebrink M *et al.* (2021):** Epigenetic control of melanoma cell invasiveness by the stem cell factor SALL4. Nat Commun., 12: 5056. doi: 10.1038/s41467-021-25326-8
6. **Yang Y, Wang X, Liu Y *et al.* (2021):** Up-regulation of SALL4 is associated with survival and progression via putative WNT pathway in gastric cancer. Front Cell Dev Biol., 9: 600344. doi: 10.3389/fcell.2021.600344.
7. **Al-Baradie R, Yamada K, St Hilaire C *et al.* (2002):** Duane radial ray syndrome (Okihiro syndrome) maps to 20q13 and results from mutations in SALL4, a new member of the SAL family. Am J Hum Genet., 71: 1195–1199.
8. **Sun B, Xu L, Bi W *et al.* (2022):** SALL4 oncogenic function in cancers: Mechanisms and therapeutic relevance. Int J Mol Sci., 23(4):2053. doi: 10.3390/ijms23042053
9. **Kong N, Bassal M, Tan H *et al.* (2021):** Zinc finger protein SALL4 functions through an AT-rich motif to regulate gene Expression. Cell Rep., 34: 108574. doi: 10.1016/j.celrep.2020.108574.
10. **Shao M, Zhang J, Zhang J *et al.* (2020):** SALL4 promotes gastric cancer progression via hexokinase II mediated glycolysis. Cancer Cell Int., 20: 188. doi: 10.1186/s12935-020-01275-y.
11. **Du W, Ni L, Liu B *et al.* (2018):** Upregulation of SALL4 by EGFR activation regulates the stemness of CD44-positive lung cancer. Oncogenesis, 7: 36. doi: 10.1038/s41389-018-0045-7
12. **Li A, Jiao Y, Yong K *et al.* (2015):** SALL4 is a new target in endometrial cancer. Oncogene., 34: 63–72. doi: 10.1038/onc.2013.529
13. **Zeng S, Yamashita T, Kondo M *et al.* (2014):** The transcription factor SALL4 regulates stemness of EpCAM-positive hepatocellular carcinoma. J Hepatol., 60:127–134.
14. **Gao C, Kong N, Li A *et al.* (2013):** SALL4 is a key transcription regulator in normal human hematopoiesis. Transfusion, 53:1037-49.
15. **Ma Y, Cui W, Yang J *et al.* (2006):** SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leuke-mia (AML) and induces AML in transgenic mice. Blood, 108: 2726-35.
16. **Lu J, Ma Y, Kong N *et al.* (2011):** Dissecting the role of SALL4, a newly identified stem cell factor, in chronic myeloge-nous leukemia. Leukemia, 25:1211-3.

17. **Döhner H, Estey E, Amadori S *et al.* (2010):** European LeukemiaNet. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European Leukemia Net. Blood, 115(3):453-474.
18. **Khwaja A, Bjorkholm M, Gale R *et al.* (2016):** Acute myeloid leukaemia. Nat Rev Dis Primers, 2:16010. doi: 10.1038/nrdp.2016.10.
19. **Coombs C, Tallman M, Levine R (2016):** Molecular therapy for acute myeloid leukaemia. Nature Reviews Clinical Oncology, 13(5):305-318.
20. **Ma X (2020):** MicroRNA-181a-3p as a diagnostic and prognostic biomarker for acute myeloid leukemia. Mediterr J Hematol Infect Dis., 12: e2020012. doi: 10.4084/MJHID.2020.012
21. **Yang J, Gao C, Chai L *et al.* (2010):** A novel SALL4/OCT4 transcriptional feedback network for pluripotency of embryonic stem cells. PLoS One, 5:e10766. doi: 10.1371/journal.pone.0010766.
22. **Hou P, Li Y, Zhang X *et al.* (2013):** Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science, 341: 651-54.
23. **Swelem R, Elneely D, Shehata A (2019):** The study of SALL4 gene and BMI-1 gene expression in acute myeloid leukemia patients. Laboratory Medicine, 51(3):265–70.
24. **Ibraheem F, Badawy R, Ayoub M *et al.* (2019):** SALL4 gene expression in acute myeloid leukemia. Asian Pac J Cancer Prev., 20(10):3121–27.
25. **Shen Q, Liu S, Hu J *et al.* (2012):** The differential expression pattern of the BMI-1, SALL4 and ABCA3 genes in myeloid leukemia. Int J Cancer, 12: 124-32.
26. **Chen Q, Qian J, Lin J (2013):** Expression of SALL4 gene in patients with acute and chronic myeloid leukemia. J Exp Hematol., 21: 315-19.
27. **Duan M, Bi C, Chen H *et al.* (2013):** Detection of SALL4 mRNA expression in leukemia by real-time fluorescent quantitative PCR. J Exp Hematol., 21:1153–56.
28. **Farawela H, Zawam H, Al-Wakeel H *et al.* (2019):** Expression pattern and prognostic implication of SALL4 gene in myeloid leukemias: a case-control study. Scandinavian Journal of Clinical and Laboratory Investigation, 79(1–2):65–70.
29. **Abo-Elwafa H, Aziz S, Salah M *et al.* (2015):** The SALL4 gene in acute leukemias. Egypt J Haematol., 40(3): 121-29.