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Prognostic Impact of *WT-1* and *Survivin* Gene Expression in Acute Myeloid Leukemia Patients

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ABSTRACT

Background: Wilms Tumor gene 1 (*WT1*) product and Survivin are important leukemia associated antigens (LAAs) and their expression has been anticipated as a predictor of AML prognosis.

Objectives: The aim of this study is to evaluate the gene expression pattern of *Survivin* and *WT1* in AML, and to correlate the gene expression profile with the different clinical and survival data.

Patients and Methods: We investigated expression levels of *WT*1 and *Survivin* by real time polymerase chain reaction (RT-PCR) in 61 newly diagnosed AML patients in correlation with clinical characters and outcome.

Results: *WT1* over-expression was found in 45 patients (73.8%). It was associated with higher BM blasts (p=0.017), lower incidence of cytogenetics associated with favourable prognosis [t (8;21) and inv (16)] (p=0.035) and higher incidence of *FLT3-ITD* mutations (p=0.026). *Survivin* over-expression was found in 17 patients (27.9%) and was associated with higher white blood cell (WBCs) count (p=0.049). Patients with over-expression of either gene showed worse Day 28 complete remission (CR) rates and poor survival rates. Combined expression of both genes enhanced its prognostic value; patients with over-expression of both genes showed higher WBCs (p=0.035) and higher BM blasts (p=0.029) while the double negative group showed higher incidence of favourable cytogenetics (p=0.021), better D28 CR rates and better survival rates.

Conclusion: Our findings demonstrated the bad prognostic impact of *WT1* and *Survivin* genes in AML patients especially the over-expression of both genes. Detection and monitoring of these LAAs genes have an important role to risk-stratify AML patients, understand AML immunobiology and develop better immunotherapeutic options.

Key Words: *WT1 – Survivin – LAAs – Cytogenetic – AML.*

INTRODUCTION

Acute myeloid leukemia (AML) represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and uncontrolled proliferation of the stem cell compartment result in accumulation of non-functional myeloblasts, impaired hematopoiesis and cytopenias; however, its etiology remains largely unknown **[1]**.

Discovering the molecular abnormalities involved in carcinogenesis and progression of acute leukemia is an important strategy for detection and treatment of the disease. Molecular alterations in AML are divided into 2 groups. The first group includes mutations that activate the signal transduction pathway, while the second group comprises mutations that affect the cell cycle components **[2]**.

Both leukemogenesis and resistance to chemotherapy can be attributed to block of apoptosis. Inhibitors of apoptosis proteins (IAPs) are originally identified in malignant cells and during fetal development **[3]**. In many instances IAP family proteins can suppress apoptosis across species barriers **[4]** implying that these proteins evidently target a common mechanism. Six human IAPs have been described so far: *NAIP, CIAP1, CIAP2, XIAP, Survivin* and *Apollon* **[5-10]**. Although there is some evidence that IAPs play an important role in the chemo-resistance of leukemia cell lines, little is known about their influence on this phenomenon in primary acute leukemia cells.

Survivin is an anti-apoptotic gene, which is over-expressed in most human tumors and involved in mitotic checkpoint control. High levels of *Survivin* expression have been associated with cancer progression, drug resistance, poor prognosis, and short survival **[11,12]**. Recently, silencing of Survivin gene by small interfering RNAs provided novel approaches for treatment of androgen-independent prostate cancer **[13]**, childhood osteogenic sarcoma as well as pancreatic

cancer. To date, several approaches have been taken to target and eliminate IAP function in an attempt to re-establish sensitivity, reduce toxicity, and improve efficacy of cancer treatment.

Many genetic products that modulate immune effector cells function influence the microenvironment of AML. *Wilms'* tumor gene 1 (*WT1*) hinders cell differentiation of both normal hematopoietic progenitor cells and leukemic blasts **[14]**. *WT1* gene product has been demonstrated to perform both transcriptional repression, activation as well as both oncogenic and tumor suppressor properties **[15]**. *WT1* is frequently expressed in AML patients where it carries unsatisfactory impact on the outcome. In addition, *WT1* expression qualifies as an independent prognostic parameter prior to bone marrow transplantation **[16]**.

Another important aspect of both *WT1* and *Survivin* is that both belongs to leukemia associated antigens (LAAs) that are recognized by specific cytotoxic T-lymphocytes **[17,18]** and characterization of expression profiles of these molecules is important to develop targeting immunotherapeutic approaches **[19,20]**.

We aimed to evaluate the pattern of *Survivin* and *WT1* gene expression in AML, and to correlate the gene expression profile with the different clinical and survival data of our AML patients.

PATIENTS AND METHODS

This study was conducted in the National Cancer Institute (NCI), Cairo University and included 61 patients with de novo AML who were referred to Medical Oncology Department in the period between June 2014 and December 2016 in addition to 15 healthy donors with normal blood picture. The patients included 30 males and 31 females with an age range of 18 to 67 with a median of 37 years; the controls included 7 males and 8 females with an age range of 24 to 60 with a median of 35 years.

Eligible patients were those with age ≥18 years, with confirmed diagnosis of AML, and had no contraindications to induction chemotherapy. Patients with AML-M3 subtype were excluded. All patients were subjected to clinical, morphological, cytochemical, flow cytometric and cytogenetic analysis to establish the diagnosis and to assess risk stratification before starting the induction chemotherapy.

Plain chest X-ray and/or CT chest, abdominal and pelvic ultrasonography as well as echocardiography were routinely done before induction treatment. Induction treatment was in the form of standard (7+3) regimen according to local NCI guidelines **[21]**.

The study was approved by the Institutional Review Board (IRB) of the NCI, Cairo University and was conducted according to the rules of Helsinki declaration for human studies. A Written informed consent was obtained from all study subjects.

Sample collection and RNA extraction:

Total RNA was isolated from EDTA anticoagulated bone marrow samples of AML patients and peripheral blood of healthy donors' using QIAamp RNA blood Mini Kit (QIAGEN, Hilden, Germany, Cat no. 52304) according to manufacturer's instruction. The concentration of RNA was measured using Nano- Drop ND1 spectrophotometer (Thermo Scientific, USA), (samples ratio ranged from 1.8-2). One µg of RNA was reverse transcribed according to the manufacturer's instructions using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) on Gene thermocycler (Applied Biosystems, USA).

Molecular detection of Survivin and WT1 gene:

The expression levels of *Survivin* and *WT1* gene and the house keeping gene *glyceraldehydes-3-phosphate dehydrogenase (GAPDH)* were measured by two step quantitative RT-PCR with duplex detection (Quantifast probe assay (QIAGEN) using an ABI PRISM 7500 Detector system (Applied Biosystems, Foster City, CA).

RT-PCR assays were performed for each sample in a final reaction volume of 25µl. *GAPDH*, *Survivin* and *WT1* gene were amplified using 5µl cDNA, 12.5µl universal master mix, 1.25µl Survivin and WT1 gene readymade primer and probe, 1.25µl *GAPDH* gene readymade primer and probe, together with 4.5µl distilled water and 0.5µl ROX dye solution.

Amplification was carried out at 95°C for 5 minutes as PCR initial activation step and Hot Start Taq Plus DNA Polymerase activation, followed by 40cycles at 95°C for 30 seconds (denaturation), and 60°C for 30 seconds (combined annealing/extension).

The cycle threshold (Ct) values were obtained for *Survivin* and *WT1*. The expression level of the target genes was calculated by normalizing it to the house keeping gene *GAPDH*. The relative gene expression (fold changes) levels were calculated by the $2^{-\Delta\Delta Ct}$ method using calibrator **[22]**. All of Ct values were in the linear range of detection. We used the mean of the controls as the cut off value for the expression level this value considers over expression and under this value is under expression (1.2 for *Survivin* and 1.1 for *WT1*).

Statistical analysis:

Data was analyzed using Graph Pad Prism statistical package (version 6.0f). For qualitative variables, Chi-square test and Fisher's exact test were used. For normally distributed quantitative data, ordinary *t*-test was used to compare means of two groups and ordinary two-way ANOVA test was used when number of groups was more than two. Kaplan and Meier analysis was used to estimate overall survival (OS) using two-sided log rank tests. OS was calculated from diagnosis to date of death and live patients were considered as censored. A *p*-value ≤0.05 was considered significant and all reported *p*-values are two-sided.

RESULTS

Patients' characteristics:

Sixty-One De Novo AML patients were enrolled in the study in addition to 15 ages and gender matched healthy control subjects. The clinico-pathological characteristics of the patients are shown in Table (1). AMLM4 was the most frequent FAB subtype representing 37.7% of the patients followed by M2 and M1 representing 29.5% and 24.5% respectively.

Favorable cytogenetics including t(8;21) and inv(16) were encountered in 7 and 2 patients respectively while unfavorable like FLT3 and c-kit were detected in 12 and 4 patients respectively. Fifty-two patients received full induction chemotherapy with 3&7 regimen. At day 28, 28 patients (53.8%) achieved CR, 17 (32.7%) died during induction period while 7 (13.5%) patients were refractory to treatment.

Table (1): Clinico-pathological characteristics of 61 adult acute myeloid leukemia patients.

Parameter	Findings	Parameter	Findings
Age: years*	37.8±13.36 37 (18-67)	CD34 expression: No (%) (n=58)	35 (60.34%)
Gender:		Favorable cytogenetic (n=56)	
Males	30 (49.2%)	t(8;21)	7 (12.5%)
Females	31 (50.8%)	inv(16)	2 (3.6%)
Hemoglobin: gm/dl*	7.47±2.19	Unfavorable genetics: No (%)	
		FLT3-ITD	12/56 (21.4%)
		C-Kit	4/40 (10%)
TLC: $x10^9$/L*	59.61±59.54 33.45 (0.8-231)	Gene expression: No (%) WT Survivin Double positive Double negative	 45 (73.8%) 17 (27.87%) 15 (24.6%) 14 (22.9%)
Platelets: x 10^9/L*	61.94±87.06 25 (5-458)	D14 CR (n=39)** D28 CR (n=35)**	24/39 (61.5%) 28/35 (80%)
*Blast: %**		Response at D28 (n=52)**	
PB	58.8±22.4	CR**	28 (53.8%)
BM	66.4±16.9	Refractory	7 (13.5%)
		Early death	17 (32.7%)
LN	12/51 (23.5%)	Relapse***	6/29 (20.7%)
HSM	15/42 (35.7%)		
Morphology (FAB types):			
M0	2	Median survival time (months)****	6
M1	15		
M2	18		
M4	23		
M5	3		

* Mean ± SD, Median (range).
* Mean ± SD.
** CR percentage was calculated who achieved complete response.
*** Relapse percentage was calculated excluding refractory patients.
**** Survival data were available for 54 patients.

Expression of WT1 and Survivin in newly diagnosed AML patients:

Out of 61 patients assessed, *WT1* over-expression was found in 45 patients (73.8%) while *Survivin* over-expression was found in 17 patients (27.9%). Patients with *WT1* over-expression showed higher mean WBCs counts (67.2±9.58 vs. 36.9±12.27, *p*=0.09) and significantly higher BM blast percentages (69.4±2.31 vs 57.8±4.59, *p*=0.017). Also, *Survivin* over-expression was associated with a significantly higher WBCs count (83.2±17.35 vs vs 49.3±8.2, *p*=0.049) and higher PB and BM blast percentages (Table 2, Fig. 1).

Significant associations were found between AML-FAB subtypes and over-expression of *WT1* (*p*=0.018) and *Survivin* (*p*=0.029) (Fig. 1, Table 2). The frequency of *WT1* over-expression was significantly higher in M1 (n=13/15) and M4 (n=20/23) (87%), while over-expression of *Survivin* was significantly higher in M1 (n=7/15, 47%) (In contrast to *Survivin* for which no significant association with either favourable or unfavourable cytogenetics could be demonstrated, *WT1* over-expression was associated with significantly lower incidence of favourable cytogenetics [t(8;21) and inv (16)] (9.5% vs. 35.7%, *p*=0.035) and higher incidence of *FLT3-ITD* mutations (28.6% vs. 0%, *p*=0.026).

Table (2): Clinical and hematological findings of acute myeloid leukaemia patients according to WT1 and survivin expression.

Parameter	WT1-pos (n=45)	WT1-neg (n=16)	*p*	Survivin-pos (n=17)	Survivin-neg (n=44)	*p*
Age: years*	38.5±2.06	35.8±3.03	Ns	40.47±3.32	36.7±1.99	Ns
Gender: Males:Female	23:22	7:9 (1:1.3)	Ns	9:8	21:23 (1:1.1)	Ns
Hemoglobin (gm/dl)*	7.6±0.32	7.1±0.7	Ns	7.0±0.48	7.7±0.36	Ns
TLC: $x10^9$/L*	67.2±9.58	36.9±12.27	0.09	83.2±17.35	49.3±8.2	0.049
Platelets: $x10^9$/L*	50.4±9.14	96.6±37.1	0.08	46.4±18.49	68.7±14.65	Ns
*Blasts: %**						
PB	60.5±4.18	58.17±5.39	Ns	61.5±8.19	59.2±3.45	Ns
BM	69.4±2.31	57.8±4.59	0.017	72.4±3.3	64.1±2.67	Ns
LN: No (%)	9/37 (24.3%)	3/14 (21.4%)	Ns	4/14 (28.6%)	8/37 (21.6%)	Ns
HSM: No (%)	20/38 (52.6%)	5/14 (35.7%)	Ns	7/15 (46.7%)	18/37 (48.6%)	Ns
FAB subtypes:						
M0 (2)	2	0		2	0	
M1 (15)	13	2		7	8	
M2 (18)	9	9	0.018	2	16	0.029
M4 (23)	20	3		5	18	
M5 (3)	1	2		1	2	
CD34 expression: No (%)	26/42 (61.9%)	9/16 (56.3%)	Ns	10/17 (58.8%)	25/41 (60.9%)	Ns
Favorable cytogenetics t(8;21) and inv16	4/42 (9.5%)	5/14 (35.7%)	0.035	2/16 (12.5%)	7/40 (17.5%)	Ns
FLT3-ITD	12/42 (28.5%)	0/14 (0%)	0.026	4/16 (25%)	8/40 (20%)	Ns
C-Kit e 17	3/30 (10%)	1/10 (10%)	Ns	1/10 (10%)	3/30 (10%)	Ns
D14 CR (n=39)**	15/29 (51.7%)	9/10 (90%)	0.05	4/10 (40%)	20/29 (69%)	Ns
D28 CR (n=35)**	18/25 (72%)	10/10 (100%)	0.08	6/7 (85.7%)	22/28 (78.6%)	Ns
Response at D28 (n=52):						
CR**	18 (46.2%)	10 (76.9%)	Ns	6 (35.3%)	22 (62.9%)	*p*=0.018
Refractory	7 (17.9%)	0 (0%)		1 (5.9%)	6 (17.1%)	
Early death	14 (35.9%)	3 (13.1%)		10 (58.8%)	7 (20%)	
Relapse***	3/16 (18.8%)	3/13 (23.1%)	Ns	1/7(14.3%)	5/22 (22.7%)	Ns
Median survival time/OS (months)****	2.5	5	*p*=0.35	1	5	*p*=0.37

* Mean ± SD.
** CR percentage was calculated who achieved complete response.
*** Relapse percentage was calculated excluding refractory patients.
**** Survival data were available for 54 patients.

WT1 and Survivin Expression and CR rates:

Patients with *WT1* over-expression showed worse CR rates at D14 and D28 compared to those without expression (52% and 72% versus 90% and 100% respectively). Also, patients with *WT1* over-expression showed higher incidence of early death during induction compared to those without over expression (36% versus 13%; Table 2).

Similarly, patients with *Survivin* over-expression showed worse CR rates at D14 compared to patients without (40% versus 69%). At Day 28, *Survivin* over-expression group also showed significantly more early deaths (59% versus 20%; p=0.018) (Table 2).

Correlation of survival rates with gene expression:

The median follow-up time was 6.0 months (range 0.2 to 41.0 months). Patients with *WT1* over-expression showed decreased median OS compared to patients without expression (2.5 versus 5 months, p=0.35) (Fig. 1E). Similarly, *Survivin* over-expression was associated with lower median OS (1 versus 5 months; p=0.37) (Fig. 1F).

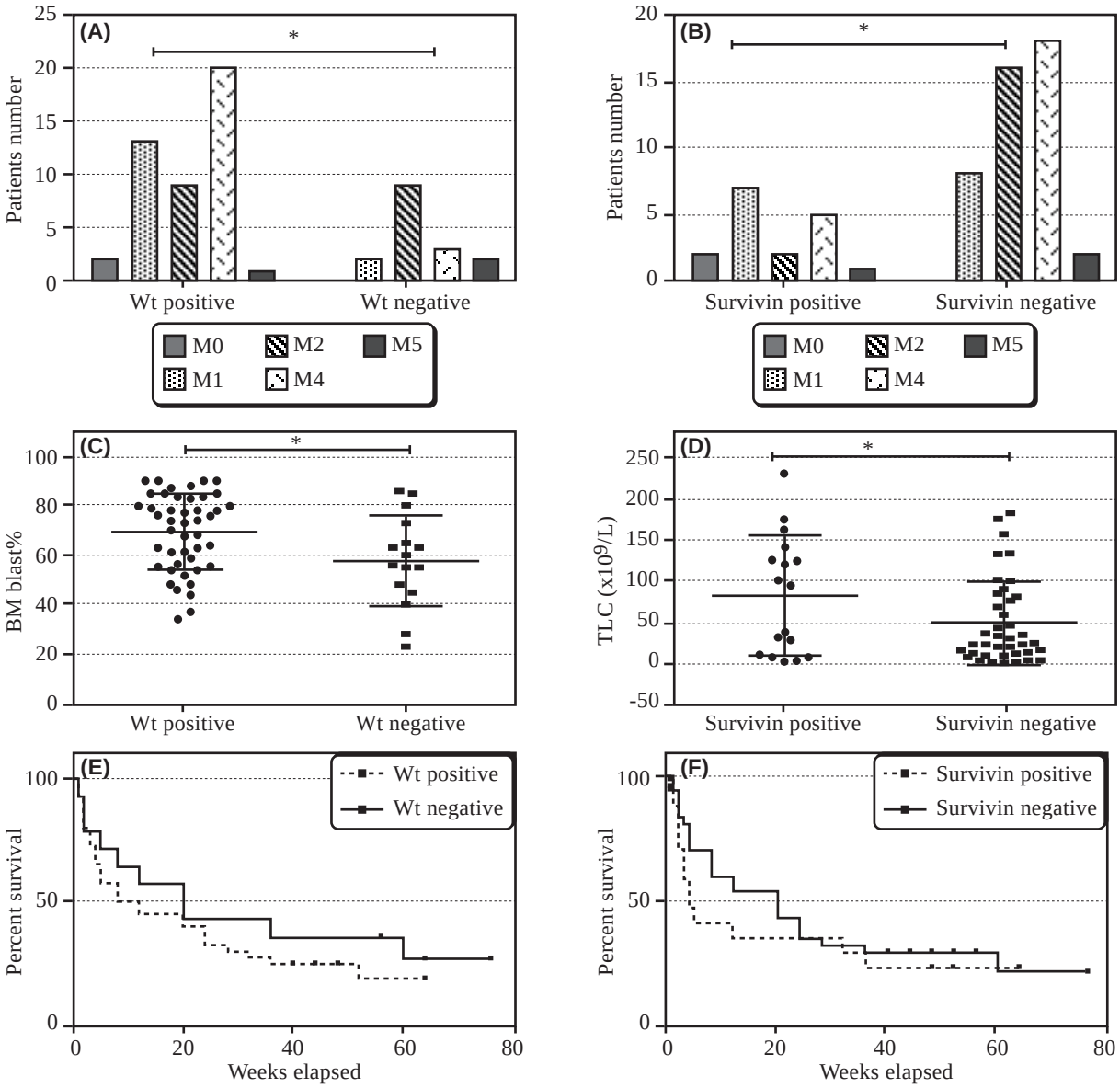

Fig. (1): (A) Comparison of FAB subtypes between WT1 positive and WT1 negative groups (B) Comparison of FAB subtypes between Survivin positive and negative groups. (C) Comparison of BM blast% between WT1 positive and WT1 negative groups. (D) Comparison of BM blast% between Survivin positive and negative groups. (E) Overall Survival curves of WT1 positive and negative groups and (F) Overall Survival curves of Survivin positive and negative groups.

Combined over expression of WT1 and Survivin in AML patients (Table 3):

Newly diagnosed AML patients were divided into 3 groups based on *WT1* and *Survivin* expression profiles: Double positive group (n=15, 24.6%), Double negative group (n=14, 23%) and Single positive group (n=32, 52.5%) where 30 patients were positive only for *WT1* and 2 were positive for *Survivin* only (Table 3). Double positive patients showed significantly higher WBC count and BM blast percentage than double negative patients (p=0.035 and 0.029 respectively, Table 3, Fig. 2).

Regarding FAB subtypes, Double positive patient groups had a slight tendency toward the immature phenotype AML-M1 with minimal differentiation. On the other hand, double negative group showed a different pattern with dominance of differentiated phenotype AML-M2 while the single positive group showed strong association with monocytic differentiation AML-M4 (Fig. 2).

Table (3): Clinical and haematological findings of 61 acute myeloid leukaemia patients according to combined WT1 and Survivin expression.

Parameter	Double pos (n=15)	Double neg (n=14)	Single pos. (No: 32) Wt+/Sur-(n=30) Wt-/Sur+(n=2)	*p*-value
Age: years*	40±14.36	34.6±12.3	38.1±13.47	Ns
Gender: Males: Females	9:6 (1.5:1)	7:7 (1:1)	14:18 (1:1.3)	Ns
Hemoglobin: gm/dl*	7.3±1.97	7.4±2.7	7.6±2.15	Ns
TLC: $x10^9$/L*	84±74.62	30.2±42.41	59.2±52.75	0.06 0.035
Platelets: $x10^9$/L*	48.8±81.2	108±147.6	49.7±43.36	Ns
*Blasts: %**				
PB	62.3±32.25	59.4±18.68	58.3±20.59	Ns
BM	74.3±13.33	57.9±19.71	66.4±15.83	0.029 0.013
LN: No (%)	3/12 (25%)	2/12 (17%)	7/27 (26%)	Ns
HSM: No (%)	6/13 (46%)	4/12 (33%)	15/27 (56%)	Ns
FAB subtypes:				
M0	2	0	0	
M1	7	2	6	
M2	1	8	9	0.014
M4	5	3	15	0.02
M5	0	1	2	
CD34 expression No (%)	9/15 (61.9%)	8/14 (56.3%)	18/29 (56.3%)	Ns
Favorable genes t(8;21) and inv16	2/15 (13%)	5/12 (42%)	2/29 (6.9%)	p=0.021
FLT3-ITD	4/14 (29%)	0/12 (0%)	8/30 (27%)	Ns
C-Kit e 17	1/9 (11%)	1/9 (11%)	2/22 (9%)	Ns
D14 CR (n=39)**	3/9 (33.3%)	8/9 (88.9%)	13/22 (59.1%)	0.015 0.049
D28 CR (n=35)**	7/8 (87.9%)	9/9 (100%)	12/18 (66.7%)	Ns
Response at D28:				
CR**	6 (40%)	9 (81.8%)	14 (53.8%)	(p=0.05)
Refractory	1 (6.7%)	0 (0%)	6 (23.1%)	p=0.044
Early death	8 (53.3%)	2 (18.2%)	6 (23.1%)	
Relapse***	1/6 (16.7%)	3/9 (33.3%)	2/14 (14.3%)	Ns 0.23
Median survival time/OS (months)****	1 mon	7 mon	2 mon	

* Mean ± SD.
** CR percentage was calculated who achieved complete response.
*** Relapse percentage was calculated excluding refractory patients.
**** Survival data were available for 54 patients.

Patients in the double negative group had significantly higher incidence of favourable cytogenetics (p=0.021). They also showed significantly better CR rates at D14 (89% in comparison to 59% and 33% for single and double positive groups respectively. Again, double negative group showed significantly higher CR rates at D28 (81.8% versus 40%) and lower early deaths (18.2% versus 53.3%) compared to double positive group. Survival rates showed that Double negative group had superior median OS when compared with either single positive or double positive groups (2 months and 1 month) respectively (Fig. 2).

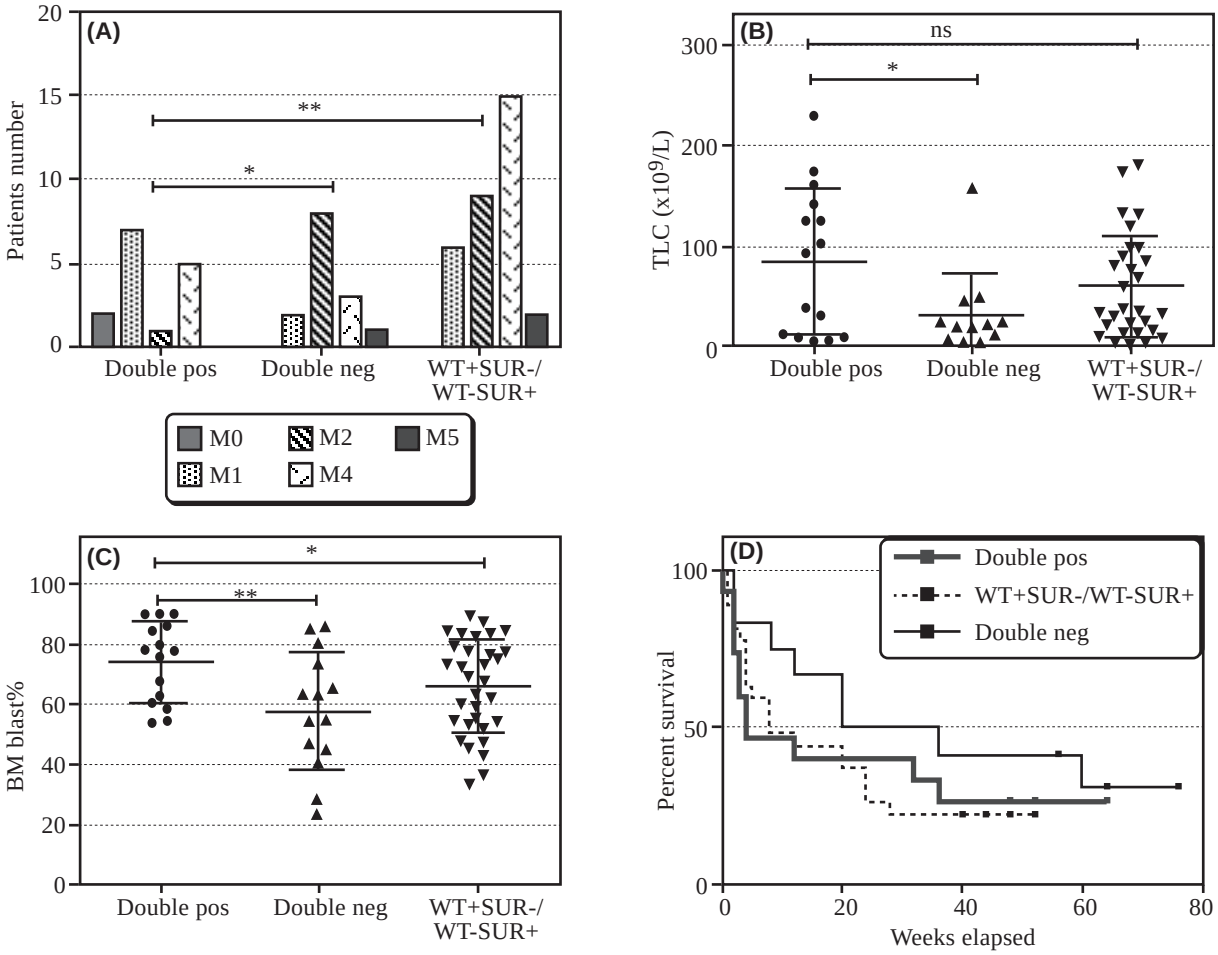

Fig. (2): Comparison between double positive, double negative and single positive groups (A) FAB subtypes. (B) Total leukocyte count. (C) Bone Marrow blast%. (D) Overall Survival.

DISCUSSION

In the current study, we assessed the pattern of expression of *WT1* and *Survivin* genes in newly diagnosed AML patients to define its impact on prognosis and survival. Our data suggest that expression of these LAAs has a reliable prognostic effect on AML patients which was more apparent when combining both markers.

WT1 was over-expressed in about three quarters of the studied patients which is consistent with recent Egyptian **[3]** and international studies **[4-6]**. The prognostic value of *WT1* over-expression at diagnosis is still not clear owing to the contradictory results from the studies focusing on this point. Assem et al. **[3]** showed that over-expression of *WT1* at diagnosis was associated with lower CR rates with induction, poor disease free and overall survival in an Egyptian patient cohort. Although the level of *WT1* mRNA was not correlated with response to treatment, Bergmann et al., found that patients with high levels of *WT1* had significantly poor

OS which was more distinct in patients younger than 60 years old **[7]**.

Similar to our findings, Caroline et al. **[16]** found that *WT1* is frequently expressed in AML patients and this expression had a bad impact on patient's outcome. In addition, *WT1* expression was reported to bean independent prognostic parameter prior to bone marrow transplantation. Despite the encouraging findings, these results were not reproducible in other studies which failed to find this prognostic impact **[6,8,9]**.

In our study, we were able to show a significant association between *WT1* over-expression and high BM blast percentage but not with WBCs, which is consistent with some previous reports **[10]** but not with others **[8,11]**.

Rodrigues et al. **[12]** evaluated the prognostic value of *WT1* in 41 AML pediatric patients and concluded that high expression was associated with favorable cytogenetic subtypes and better overall survival. In contrast to these results, we found a significant association of *WT1* over-expression and absence of favorable cytogenetic subtypes in our adult AML patients. This may be explained by the difference in patient's population as we included adult patients while the previous study included pediatric AML. On the other hand, we demonstrated a significant association of *WT1* over-expression with *FLT3-ITD* mutations which is consistent with other series **[11,13]**. A reliable prognostic role of *WT1* over-expression in predicting CR or OS was not confirmed, though there was a marginal association with good response to chemotherapy at day 14, which is in agreement with previous studies **[6,8,9]**.

Survivin has been reported as a strong predictor of AML prognosis **[23-25]**. One of the large series by Carter et al. **[23]** assessed its prognostic value in 511 newly, diagnosed AML patients. They used a validated reverse-phase protein array and found that higher *survivin* levels significantly predicted OS and EFS in multivariate analysis.

In our study, patients with *Survivin* over-expression had inferior OS compared with those without but the difference did not reach significance. This may be attributed, at least partly, to the small number of patients included in our study. Over-expression of *Survivin* was associated with significantly higher initial WBCs. This comes in contrast with previous reports showing no association between Survivin and WBCs **[11,24]**.

In contrast to a previous study by Kim et al. **[9]** including 151 patients with newly diagnosed AML patients that reported higher expression of Survivin in unfavorable cytogenetic subtypes, we could not demonstrate significant association of *Survivin* over-expression with any specific cytogenetic group. Again, our results may be limited by the number of patients included. Similar to Kim et al. **[9]** other studies **[12-14]** showed that *Survivin* over-expression was significantly associated with poor response at day 28 and adverse impact on achieving CR. These results are consistent with our findings indicating the bad prognostic impact of *Survivin* in AML.

Combination of *WT1* and *Survivin* expression profiles adds more power to the prognostic value of these genes. Over-expression of *WT1* and *Survivin* showed strong association with adverse prognostic variables, absence of favorable cytogenetics and poor CR achievement and poor day 28 responses. Although insignificant, there was an association between over-expression of both genes and poor overall survival rates, which indicates the better prognostic value of *WT1* and *Survivin* combination and agrees with previous studies **[9]**.

In conclusion, *WT1* and *Survivin* expression in AML patients at diagnosis showed bad prognostic effect, which becomes more evident when there is over-expression of both genes. Detection and monitoring of these LAAs genes at diagnosis and during treatment is important to risk-stratify AML patients, understand AML immunobiology and develop better immunotherapeutic options.

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