RESEARCH ARTICLE

Clinical Impact of Overexpression of FOXP3 and WT1 on Disease Outcome in Egyptian Acute Myeloid Leukemia Patients

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Abstract

Background: In the last decade, it has become clear that change of gene expression may alter the hematopoietic cell quiescent state and consequently play a major role in leukemogenesis. WT1 is known to be a player in acute myeloid leukemia (AML) and FOXP3 has a crucial role in regulating the immune response. **Objectives:** To evaluate the impact of overexpression of WT1and FOXP3 genes on clinical course in adult and pediatric AML patients in Egypt. Patients and methods: Bone marrow and peripheral blood samples were obtained from 97 de novo non M3 AML patients (63 adult and 34 pediatric). Real-time quantitative PCR was used to detect overexpression WT1 and FOXP3 genes. Patient follow up ranged from 0.2 to 39.0 months with a median of 5 months. **Results:** In the pediatric group; WT1 was significantly expressed with a high total leukocyte count median 50X109/L (p=0.018). In the adult group, WT1 had an adverse impact on complete remission induction, disease-free survival and overall survival (p=0.02, p=0.035, p=0.019 respectively). FOXP3 overexpression was associated with FAB subtypes AML M0 +M1 vs. M2, M4+M5 (p =0.039) and the presence of hepatomegaly (p=0.005). **Conclusions:** WT1 and FOXP3 overexpression has an adverse impact on clinical presentation, treatment response and survival of pediatric and adult Egyptian AML patients.

Key words: AML - WT1 - FOXP3

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Introduction

Acute myeloid leukemia (AML) is the most prevalent acute leukemia in adults and is relatively infrequent in the pediatric population (15% - 20% of acute leukemia) (Clavel et al., 2004). AML patients sustain fair overall survival (OS); the 5-year OS is 40%-50% in younger AML patients and deteriorates dramatically with age (Kantarjian et al., 2008).

Many genetic products that modulate immune effector cells function influence the microenvironment of AML. The Wilms' tumor gene 1 (WT1) hinders cell differentiation of both normal hematopoietic progenitor cells and leukemic blasts (Gu et al., 2005 ; Simpson et al., 2006).WT1 gene product has been demonstrated to perform both transcriptional repression, activation as well as, both oncogenic and tumor suppressor properties (Morrison et al., 2008). Further, a myriad of alterations affecting the WT1 gene has been described in association with leukemia, including aberrant expression, loss-of-function mutations, and deregulated splicing. WT1 is frequently expressed in AML patients that carry unsatisfactory impact on the outcome. In addition, WT1 expression qualifies as an independent prognostic parameter prior to bone marrow transplantation (Caroline et al., 2015).

Regulatory T cells (Tregs) have been recognized as a contributing factor and may be recruited and exploited by leukemic cells to evade immune surveillance. Tregs frequency in both bone marrow and blood is greater in patients with AML than in control (Celalettin et al., 2011; Yang and xu, 2013; Hamed et al., 2015; Rooney 2014). The Forkhead box Protein 3 (FOXP3) gene is a member of the forkhead winged helix family transcription factors and is responsible for the development and function of Tregs (Fontenot and Rudensky, 2005).

Aim of study, to determine the influence of over expression of WT1 and FOXP3 genes on pediatric and adult AML patients.

Materials and Methods

Patient Cohort

This study was approved by the Institutional Review Board at national cancer institute (NCI), Cairo University (CU) and all participants provided written informed consent. All patients were presented to the outpatient clinic at the national cancer institute. Between June 2010 and December 2012. Ninety-seven newly diagnosed (De novo) AML patients (63 adults and 34 pediatrics) with a median age of 37(range 18-68 years) and 11.5 (range

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1.2–17 years) respectively in additions to 15 healthy donors were for normalization with normal blood picture. The normal healthy donors were 8 - 72 years old with median age 30. Patients and normal donors were recruited for WT1and FOXP3 expression profiling by qRT-PCR. All patients were diagnosed and classified according to the consensus guidelines for the immunologic diagnosis of acute leukemia and the 2008 Revision of WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues and were treated according to standard protocols.

Treatments

All adult patients received the 3 and 7 protocols which consisted of adriamycin 30mg/m2 for 3 days and ARAC 100mg/m2 by continuous infusion for 7 days. Further treatment of AML patients was according to their risk group. Patients with favorable risk group were treated with high dose ARAC containing usually HAM protocol

(ARAC 1 gm/m2 over 3 hours infusion every 12 hours day 1 to 3 and mitoxantrone 12mg/m2 short infusion days 3 to 5. If relapse occurred to these patients a second induction by HAM or AVVV protocol was given. Then if the donor was available, an allogeneic BM transplantation was carried out. For unfavorable risk group, allogeneic BMT was carried out if a suitable donor was available, but if not HD-ARAC containing regimen was given for 3 cycles then autologous BMT was done. If relapse occurred for this group of patients they were treated with palliative care only.

Pediatrics protocol consisted of two induction courses of ADE protocol (doxorubicin 50mg/m2 D 1, 3, 5, ARAC 3.3 MG/KG-D 1 to 10 and etoposide 100mg/m2 D 1 to D 5). Intensification course was done by 4 cycles of MIDAC (ARAC 1gm/m2 every 12 hours for 6 doses and mitoxantrone 8mg/m2 D1 to D3). For special subgroups of AML, special treatment was used. Patients with AML M5 have a high risk of central nervous system disease, so they were given triple intrathecal prophylaxis (methotrexate 15mg, ARAC 40mg, and dexamethasone 4mg) every 8 weeks for a total 6 injections with induction treatment. If CNS disease was diagnosed at presentation, triple intrathecal injections were given until CSF is free then craniospinal irradiation was given to be followed by intrathecal injection of ARAC and dexamethasone every 8 weeks for 7 doses. Patients with AML M3, induction treatment was consisted of ATRA 45mg/m2 oral daily in divided doses every 12 hours till complete remission or for maximum 90 days, and adriamycin 30mg/m2 for 3 days for every month for 3 courses. These patients received maintenance treatment after complete remission with ATRA (45mg/m2 oral daily for two weeks every 2 years) and 6 mercaptopurine and methotrexate for 2 years.

RNA isolation and qRT-PCR

Total RNA was isolated from EDTA anticoagulated bone marrow for WT1, peripheral blood for FOXP3 and healthy donors samples by using QIAamp RNA blood Mini Kit (QIAGEN, Hilden, Germany), (Cat no. 52304) according to the standard protocol. The concentration of RNA was measured using Nano- Drop ND1,000 spectrophotometer (Thermo Scientific, USA).then 1 μg of mRNA was reverse transcribed according to the manufacturer's instructions using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) on a PCR thermocycler Gene (Applied Biosystems, USA).

The analysis of FOXP3 and WT1 gene expressions was constructed using reverse transcriptase quantitative real-time PCR (qRT-PCR) Which performed using TaqMan real-time PCR methods and A housekeeping genes GAPDH and ACTB respectively were used as internal controls for calibration of possible variations caused by the variable efficiencies of RNA extraction, RT-PCR, and operation) on the Step One Real-Time PCR (Applied Biosystems, USA).

The quantification of all genes performed according to the manufacturer's instructions. The assay IDs were: FOXP3 (Hs_FOXP3_QF_1 QuantiFast probe assay, NM_001114377), WT1 (Hs_WT1_QF_1 QuantiFast probe assay, NM_000378, NM_024424), GAPDH (Hs_GAPDH_2_MAX, NM_014009 cat. no. QF 00202986) and ACTB (Hs_ACTB_2_MAX, NM_024425, NM_024426, cat.no. QF 00338345).

The CT values were obtained for FOXP3 and WT1 then normalized to GAPDH and ACTB respectively, and then the fold changes were calculated using 2–ΔΔCT method. All of CT values were in the linear range of detection. We consider the mean of the controls is a cut off value above this value considers over expression and under this value is down expression (1.1 for WT1 and 1.6 for FOXP3).

Statistical Methods

Data was analyzed using IBM SPSS advanced statistics version 22 (SPSS Inc., Chicago, IL). Numerical data were expressed as a mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For not normally distributed quantitative data, the comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Survival analysis was done using Kaplan-Meier method and comparison between two survival curves was done using log-rank test. Kappa test was used to evaluate agreement between two diagnostic methods. Multivariate analysis was done using logistic regression when the outcome variable was binary qualitative one while Cox-regression analysis was used when the outcome variable was survival. This multivariate analysis was done for the significant factors affecting outcome variable on univariate analysis. Odds ratio (OR) or Hazard ratio (HR) with it 95% confidence interval (CI) were used for risk estimation. All tests were two-tailed. A p-value <0.05 was considered significant.

Results

Ninety-seven Egyptian patients (63 adults and 34 pediatrics) with De novo acute myeloid leukemia AML from a period June 2010 to December 2012 were enrolled in the study.

We assessed the (a) overexpression of WT1 in 97

Table 1. Clinicolaboratory Characteristics of Studied Groups

Variables	Adults	Pediatrics
		Median (Range)
Age	37 (18-68)	11.5 (1.2-17)
n (%)	≤37 34(54.0%)	≤11.5 17(50.0%)
	>37 29(46.0%)	>11.5 17(50.0%)
Hb(g/dL)	7.7 (3.3-12.5)	7.5 (3.6-10.8)
n (%)	≤7.7 34(54.0%)	≤7.5 17(50.0%)
	>7.7 29(46.0%)	>7.5 17(50.0%)
TLC X109/L	38.6(0.7-289.0)	18.4 (0.4-256.5)
n (%)	≤38.6 32(50.8%)	≤18.4 17(50.0%)
	>38.6 31(49.2%)	>18.4 17(50.0%)
PLTs X109/L	37.0(5.0-258.0)	28.2 (4.0-572.0)
n (%)	≤37.0 32(50.8%)	≤28.2 17(50.0%)
	>37.0 31(49.2%)	>28.2 17(50.0%)
BM blasts%	69.0(5.0-93.0)	67.0 (2.0 - 94.0)
n (%)	≤69.0 31(50.0%)	≤67.0 17(50.0%)
	>69.0 31(50.0%)	>67.0 17(50.0%)
		Number (%)
	63 (100.0%)	34 (100.0%)
Gender		
Males	25 (39.7)	23 (67.6)
Females	38 (60.3)	11 (32.4)
FAB Classification		
M0 + M1	19 (31.7)	15 (46.9)
M2	28 (46.7)	14 (43.8)
M4 + M5	13 (21.7)	3 (9.4)
M7		1 (2.9)
Undiffereniated	3 (4.8)	1 (2.9)
Splenomegaly		
No	28 (45.9)	16 (48.5)
Yes	33 (54.1)	17 (51.5)
Hepatomegaly		
No	27 (44.3)	13 (39.4)
Yes	34 (55.7)	20(60.6)
CD34		
Negative	28 (44.4)	13 (38.2)
Positive	35 (55.6)	21 (61.8)
CD117		
Negative	18 (31.0)	11 (37.9)
Positive	40 (69.0)	18 (62.1)
Cytogenetics		
Normal Karyotype	13 (68.4)	15 (68.2)
Favorable	6 (31.6)	7 (31.8)
WT1		
Down expression	25 (39.7)	20 (58.8)
Over expression	38 (60.3)	14 (41.2)
FOXP3		
Down expression	22 (56.4)	9 (56.3)
Over expression	17 (43.6)	7 (43.7)
FLT3		
W/W	44 (75.9)	24 (88.9)
W/ITD	14 (24.1)	3 (11.1)
Combined Markers		
3 -ve/1+ve	19 (54.3)	8 (66.7)
2 or 3 +ve	16 (45.7)	4 (33.3)

CR, Complete Remission; FAB, French American British classification; Hb, Hemoglobin; PLTs, platelets; TLC, Total Leukocytes Count; BM, Bone Marrow blasts%; WT1 , Wilms Tumor gene1; combined markers (WT1, FOXP3, and FLT3)

patients (b) FLT3-ITD mutation in 85 patients (C) FOXP3 overexpression in 55 patients. Full history, clinical examination, bone marrow aspiration, cytochemistry, immunophenotyping, cytogenetic and molecular studies were applied (Table1).

The relation between the overexpression of WT1, FOXP3, another laboratory, and clinical parameters

We assessed the clinical significance of overexpression of WT1and FOXP3 with clinical and laboratory parameters in pediatric patients (Table 2) and adult (Tables 3, 4).

In pediatric patients

We assessed the genes in 34 Pediatric AML Patients 14 patients had overexpression of WT1 and 20 without overexpression of WT1. Patients with overexpression of WT1 had higher Total leukocytes count (TLC) median 50 X109/L (range 0.4-256.5) than those without WT1 over expression had TLC median 10.2 X109/L (range1.5-241.0). This was of statistical significance (P =0.018). No significant statistical associations were encountered between WT1 with age, gender, organomegaly, Hemoglobin (Hb), Platelets (PLTs) count and bone marrow blasts percentage (BM %) at diagnosis, French America British FAB classification, CD117, CD34, cytogenetic, FLT3 as well as FOXP3.

Mutation of FLT3-ITD was found in 3/27 (11.1%); while Overexpression of FOXP3 was found in 7/16 (43.7%) patients and 9/16 (56.3%) patients with standard expression. No statistically significant association between overexpression of FOXP3 gene with age, gender, Hb, TLC, PLTs count and BM blasts % at diagnosis, FAB classification, hepatomegaly, splenomegaly, CD117, CD34, FLT3 as well as WT1.

Among Thirty-four pediatric patients that underwent the conventional intensive induction chemotherapy 21 (61.8 %) achieved a complete remission induction (CR) and 13 (38.2 %) patients did not achieve a CR. No statistically significant association between achievement of primary CR with Age, gender, Hb, TLC,PLTs count, BM blasts % at diagnosis, CD117, CD34, FAB classification, FLT3, WT1and FOXP3 except for cytogenetic whereby all favorable Cytogenetic achieved CR (p =0.051).

No significant statistical association between single nor combined overexpression of Foxp3 and WT1 with

mutation of FLT3-ITD with any parameter of the analyzed clinical, laboratory, achievement CR and survival data.

In Adult patients

Overexpressions of WT1, FOXP3, as well as mutation of FLT3-ITD, were studied in 63, 39 and 58 adult patients respectively. Among 38 patients with WT1overexpressin 22 (57.9%), patients did not achieve CR while 16 (42.1%) achieved CR. out of 25 patients who had down expression of WT1, 7(28.0%) patients didn't achieve CR and 18 (72.0%) achieved CR (P=0.020). BM blasts %, age and TLC influence CR achievement (p=0.029, P=0.014 and p=0.042 respectively). No statistical association between CR achievement and gender, Hb, PLTs count, organomegaly, CD117, CD34, FAB classification, Cytogenetic, FLT3 and FOXP3 (Table 5).

Most of the adult AML patients (68.4%) who presented with hepatomegaly showed FOXP3 overexpression while most of those (77.8%) that did not present with

Table 2. The Relation between Expression of WT1 in **Pediatrics** Patients, Other Parameters, and Their Statistical Significance

		WT1 gene expression		
Parameter		Down-expression	Overexpression	P-value
Age (years)	N	20.0	14.0	0.796
	Median(range)	11.5 (1.3-17.0)	12.5 (1.2-17.0)	
Gender	Male	14.0 (60.9%)	9.0 (39.1%)	0.726
	Female	6.0 (54.5%)	5.0 (45.5%)	
FAB Classification	M0+M1	6.0 (40.0%)	9.0 (60.0%)	*
	M2	10.0 (71.4%)	4.0 (28.6%)	
	M4+M5	2.0 (66.7%)	1.0 (33.3%)	
Hb (g/dL)	N	20.0	13.0	0.080
	Median(range)	7.6 (4.4-10.8)	6.9 (3.6-9.3)	
TLC($x10^9$/L)	N	20.0	13.0	0.018
	Median(range)	10.2 (1.5-241.0)	50.0 (0.4-256.5)	
PLTs($x10^9$/L)	N	20.0	13.0	0.957
	Median(range)	29.1 (7.0-572.0)	28.0 (4.0-111.0)	
Bone Marrow blast(BM)%	N	20.0	14.0	0.436
	Median(range)	65.5 (2.0-93.0)	70.5 (4.0-94.0)	
	n(%)	n (%)		
Splenomegaly				
	No	10.0 (62.5%)	6.0 (37.5%)	0.829
	Yes	10.0 (58.8%)	7.0 (41.2%)	
Hepatomegaly				
	No	7.0 (53.8%)	6.0 (46.2%)	0.522
	Yes	13.0 (65.0%)	7.0 (35.0%)	
CD34	Negative	7.0 (53.8%)	6.0 (46.2%)	0.643
	Positive	13.0 (61.9%)	8.0 (38.1%)	
CD117	Negative	8.0 (72.7%)	3.0 (27.3%)	0.228
	Positive	9.0 (50.0%)	9.0 (50.0%)	
Cytogenetic	Favorable	5.0 (71.4%)	2.0 (28.6%)	0.648
	Normal	8.0 (53.3%)	7.0 (46.7%)	
CR	Not Achieved	7.0 (53.8%)	6.0 (46.2%)	0.643
	Achieved	13.0 (61.9%)	8.0 (38.1%)	
FOXP3	Down expression	4.0 (44.4%)	5.0 (55.6%)	*
	Over expression	0.0 (0.0%)	7.0 (100.0%)	
FLT3	W/W	15.0 (62.5%)	9.0 (37.5%)	*
	W/ITD	2.0 (66.7%)	1.0 (33.3%)	

CR, complete remission; FAB, french american british classification; HB, hemoglobin; PLTS, platelets; TLC, total leucocytes count; (*), mean no p- value because a small number of cases within subgroups; p-Value, considered significant <0.05.

hepatomegaly showed FOXP3 down expression. The difference was statistically significant associated (P=0.005).

Most AML patients with FAB subtype M2, M4+M5 had down expression of FOXP3 while most patients with M0+M1 had overexpression (P = 0.039).

No statistically significant association between FOXP3 gene expression with age, gender, Hb, TLC, PLTs count and BM blasts % at diagnosis, splenomegaly, CD117, CD34, cytogenetic, FLT3 as well as WT1.

When combination between the three markers (WT1, FOXP3, and FLT3) was done a highly statistically significant association was found only in Adults with hepatomegaly in a univariate analysis. Patients who presented with overexpression of at least 2 or 3 markers had hepatomegaly (P=0.009). No statistically significant association between combined markers with an otherwise parameter.

In whole group patients

Univariate analysis of FOXP3 and BM blasts % in the pediatrics was not significant but in adults, there was a trend (p =0.087).When combined together in the whole group this association became significant (p = 0.042).

In M2+M4+M5 FAB-subtype, FOXP3 expression was mostly down-regulated (74.2%) while M0+M1 (68.2%) FOXP3 was overexpressed the different was statistically highly significant (p = 0.002), when using Pearson chi-square method.

Most of the M2+M4+M5 (67.2%) entered CR while

Table 3. The Relation between Expression of WT1 in **Adult** Patients and Other Parameters and Their Statistical Significance

		WT1 gene expression		
Parameter		Down-expression	Overexpression	P-value
Age (years)	n	25.0	38.0	0.532
	Median (range)	40.0 (21.0-60.0)	34.0 (18.0-68.0)	
Gender	Male	10.0 (40.0%)	15.0 (60.0%)	0.967
	Female	15.0 (39.5%)	23.0 (60.5%)	
FAB Classification	M0+M1	8.0 (42.1%)	11.0 (57.9%)	0.803
	M2	11.0 (39.3%)	17.0 (60.7%)	
	M4+M5	4.0 (30.8%)	9.0 (69.2%)	
Hb (g/dL)	n	25.0	38.0	0.983
	Median (range)	7.1 (3.3-12.5)	7.8 (3.6-11.3)	
TLC(x109/L)	n	25.0	38.0	0.407
	Median (range)	24.6 (0.8-289.0)	57.9 (0.7-250.0)	
PLTs(x109/L)	n	25.0	38.0	0.950
	Median (range)	38.0 (5.0-136.0)	36.5 (7.0-258.0)	
Bone marrow blast(BM)%	n	24.0	38.0	0.914
	Median (range)	71.5 (10.0-93.0)	65.0 (5.0-92.0)	
	n(%)	n(%)		
Splenomegaly	No	9.0 (32.1%)	19.0 (67.9%)	0.196
	Yes	16.0 (48.5%)	17.(51.5%)	
Hepatomegaly	No	12.0 (44.4%)	15.0 (55.6%)	0.624
	Yes	13.0 (38.2%)	21.0 (61.8%)	
CD34	Negative	14.0 (40.0%)	21.0 (60.0%)	0.954
	Positive	11.0 (39.3%)	17.0 (60.7%)	
CD117	Negative	6.0 (33.3%)	12.0 (66.7%)	0.509
	Positive	17.0 (42.5%)	23.0 (57.5%)	
Cytogenetic	Favorable	3.0 (50.0%)	3.0 (50.0%)	0.320
	Normal	3.0 (23.1%)	10.0 (76.9%)	
CR	Not Achieved	7.0 (24.1%)	22.0 (75.9%)	0.020
	achieved	18.0 (52.9%)	16.0 (47.1%)	
FOXP3	Down expression	3.0 (13.6%)	19.0 (86.4%)	*
	Over expression	1.0 (5.9%)	16.0 (94.1%)	
FLT3	W/W	20.0 (45.5%)	24.0 (54.5%)	0.522
	W/ITD	5.0 (35.7%)	9.0 (64.3%)	

CR, complete remission; FAB, french american british classification; HB, hemoglobin; PLTS, platelets; TLC, total leucocytes count; (*), mean (no p-value because of a small number of cases within subgroups); p-value, considered significant < 0.05.

most M0+M1 (61.8%) did not achieve CR. This was statistically significant (p = 0.007) in univariate analysis.

In disease-free survival analysis, patients with down expression had longer disease-free survival than those with overexpression (p = 0.046) **Figure (3B)???????.**

Effect of different parameters on patients' survival

We studied the prognostic value of various clinical and laboratory parameters on disease-free survival (DFS) as well as overall survival (OS). These parameters include age, gender, FAB classification, and the presence of organomegaly, Hb, TLC, PLTs, BM blast %, the presence of CD34 or CD117 and Cytogenetic. In addition, we focused on the overexpression of WT1, FOXP3, and FLT -ITD mutation.

The median follow-up time was 5.0 months (ranging from 0.2 to 39.0 months).

Considering the OS, our study revealed that WT1 overexpression had a negative impact on OS.

In pediatric patients

relation of OS for pediatric AML patients and

Table 4. The Relation between Expression of FOXP3 in Adult Patients and Other Parameters and Their Statistical Significance

		FOXP3 gene expression		
Character		Down-expression	Overexpression	P- value
Age (years)	N	22.0	17.0	0.377
	Median (range)	40.5 (21.0-55.0)	34.0 (20.0-68.0)	
Gender	Male	9.0 (56.3%)	7.0 (43.8%)	0.987
	Female	13.0 (56.5%)	10.0 (43.5%)	
FAB Classification	M0+M1	4.0 (30.8%)	9.0 (69.2%)	0.039
	M2	13.0 (76.5%)	4.0 (23.5%)	
	M4+M5	5.0 (62.5%)	3.0 (37.5%)	
Hb (g/dL)	N	22.0	17.0	0.221
	Median (range)	7.1 (3.6-9.8)	7.7(4.7-11.3)	
TLC(x109/L)	N	22.0	17.0	0.232
	Median (range)	30.3 (2.0-289.0)	72.0 (0.7-236.0)	
PLTs(x109/L)	N	22.0	17.0	0.812
	Median (range)	42.5(11.0-193.0)	38.0 (17.0-258.0)	
Bone marrow blast(BM)%	N	22.0	17.0	0.087
	Median (range)	64.5 (28.0-92.0)	79.0 (5.0-93.0)	
	n(%)	n(%)		
Splenomegaly	No	11.0 (55.0%)	9.0 (45.0%)	0.900
	Yes	9.0 (52.9%)	8.0 (47.1%)	
Hepatomegaly	No	14.0 (77.8%)	4.0 (22.2%)	0.005
	Yes	6.0 (31.6%)	13.0 (68.4%)	
CD34	Negative	11.0 (55.0%)	9.0 (45.0%)	0.789
	Positive	9.0 (52.5%)	8.0 (47.1%)	
CD117	Negative	7.0 (58.3%)	5.0 (41.7%)	0.923
	Positive	15.0 (60.0%)	10.0 (40.0%)	
Cytogenetic	Favorable	3.0 (100.0%)	0.0 (0.0%)	*
	Normal	9.0 (75.0%)	3.0 (25.0%)	
CR	Not Achieved	12.0 (50.0%)	12.0 (50.0%)	0.307
	Achieved	10.0 (66.7%)	5.0 (33.3%)	
WT1	Down expression	3.0 (75.0%)	1.0 (25.0%)	*
	Over expression	19.0 (54.3%)	16.0 (45.7%)	
FLT3	W/W	17.0 (65.4%)	9.0 (34.6%)	0.269
	W/ITD	4.0 (44.4%)	5.0 (55.6%)	

CR, complete remission; FAB, french american british classification; HB, hemoglobin; PLTS, platelets; TLC, total leukocytes count; (*), mean (no p- value because of a small number of cases within subgroups); p-Value, considered significant <0.05.

clinicolaboratory data is shown in (Table 6), patients have bone marrow blasts ≤ 67% have a higher OS than those with >67% (p =0.027) and patients with favorable cytogenetic have a higher OS than those with normal karyotype (p=0.017). There is no statistically significant association between AML patient OS and clinicolaboratory data of studied group.

And in pediatric patients with hemoglobin Hb >7.5 have also better disease-free survival than with Hb ≤7.5 (p =0.017). Other parameters did not have an influence on DFS in our study.

In Adult patients

Relation of OS for adult AML patients and clinicolaboratory data are shown in (Table 7). Patients with down WT1 gene expression have a higher OS than those with overexpression (P =0.019); patients have bone marrow blasts ≤ 69% have a higher OS than those with >69% (p =0.030) and patients with total leukocytes count

Table 5. The Relation between **Adult** Patient's Characteristics and Response to Chemotherapy

Parameter	CR Not achieved n=29(46%)	CR Achieved n=34(54%)	P-value
Age(years) Median(range)	42.0 (20.0 - 68.0)	31.0 (18.0 - 59.0)	0.014
Gender			
Male	11.0 (44.0%)	14.0 (56.0%)	0.793
Female	18.0 (47.4%)	20.0 (52.6%)	
FAB classification			
M0 + M1	12.0 (63.2%)	7.0 (36.8%)	0.180
M2	10.0 (35.7%)	18.0 (64.3%)	
M4 + M5	6.0 (46.2%)	7.0(53.8%)	
Hb(g/dL) Median(range)	6.8 (5.4 – 11.3)	7.8 (3.3 – 12.5)	0.294
TLC(x109/L) Median(range)	71.0 (3.0 – 289.0)	22.8 (0.7 – 250.0)	0.042
PLTs(x109/L) Median(range)	38.0 (15.0 - 193.0)	34.0 (5.0 - 258.0)	0.294
BM Blast% Median(range)	75.0 (5.0 – 92.0)	65.0 (10.0 – 93.0)	0.029
CD34	29.0	34.0	0.337
Negative	18.0 (51.4%)	17.0 (48.6%)	
Positive	11.0 (39.3%)	17.0 (60.7%)	
CD117	277.0	31.0	0.724
Negative	9.0 (50.0%)	9.0 (50.0%)	
Positive	18.0 (45.0%)	22.0 (55.0%)	
Splenomegaly	27.0	34.0	0.178
No	15.0 (53.6%)	13.0 (46.4%)	
Yes	12.0 (36.4%)	21.0(63.6%)	
Hepatomegaly	27.0	34.0	0.586
No	14.0 (41.2%)	20.0 (58.8%)	
Yes	13.0 (48.1%)	14.0 (51.9%)	
Cytogenetic	6.0	13.0	1.000
Normal karyotype	4.0 (30.8%)	9.0 (69.2%)	
Favorable	2.0 (33.3%)	4.0 (66.7%)	
WT1	29.0	34.0	0.020
Down expression	7.0 (28.0%)	18.0 (72.0%)	
Over expression	22.0 (57.9%)	16.0 (42.1%)	
FOXP3	24.0	15.0	0.307
Down-expression	12.0 (54.5%)	10.0 (45.5%)	
Overexpression	12.0 (70.6%)	5.0 (29.4%)	
FLT3	26.0	32.0	0.287
W/W	18.0 (40.9%)	26.0 (59.1%)	
W/ITD	8.0 (57.1%)	6.0 (42.9%)	
combined_markers	21.0	14.0	0.332
3 -ve/1+ve	10.0 (52.6%)	9.0 (47.4%)	
2 or 3 +ve	11.0 (68.8%)	5.0 (31.3%)	

CR, complete remission; FAB, french american british classification; HB, hemoglobin; PLTS, platelets; TLC, total leukocytes count; BM, bone marrow; WT1,wilms tumor gene1; combined markers, (WT1, FOXP3 and FLT3); p- value , considered significant <0.05.

Table 6. Impact of Different Parameters on the Disease-Free Survival and Overall Survival for **Pediatric** Patients

Parameter	DFS				OS			
	n	Proportion surviving at 6 months	Median survival estimate (months)	p value	n	Proportion surviving at 6 months	Median survival estimate (months)	p value
All	21	70.8%	12	-	33	57.6%	12	-
Age (yrs)				0.928				0.699
≤11.5	12	64.8%	12		17	64.7%	12	
>11.5	9	77.8%	11		16	50.0%	6	
Gender				0.602				0.967
Male	15	66.7%	12		22	59.1%	12	
Female	6	83.3%	NR		11	54.5%	10	
FAB classification				*				*
M0+M1	6	33.3%	4		14	35.7%	4	
M2	11	80.8%	12		14	71.4%	12	
M4+M5	3	100.0%	NR		3	100.0%	NR	
Hb g/dL				0.017				0.320
≤7.5	11	45.5%	6		17	52.9%	7	
>7.5	10	100.0%	NR		16	62.5%	16	
TLC(x109/L)				0.336				0.578
≤18.4	10	80.0%	NR		17	52.9%	12	
>18.4	11	63.6%	11		16	62.5%	10	
PLTs (x109/L)				0.471				0.839
≤28.2	9	77.8%	NR		17	47.1%	6	
>28.2	12	64.8%	11		16	68.8%	12	
BM blasts%				0.677				0.027
≤ 67	14	70.7%	12		17	70.6%	16	
>67	7	71.4%	11		16	43.8%	4	
Splenomegaly				0.355				0.678
No	9	88.9%	NR		16	50.0%	4	
Yes	12	58.3%	9		17	64.7%	12	
Hepatomegaly				0.711				0.612
No	7	68.6%	12		13	53.8%	12	
Yes	14	71.4%	NR		20	60.0%	10	
CD34				0.951				0.489
Negative	9	64.8%	NR		13	69.2%	12	
Positive	12	75.0%	12		20	50.0%	6	
CD117				0.831				0.652
Negative	7	57.1%	NR		11	54.5%	12	
Positive	9	66.7%	12		17	47.1%	5	
Cytogenetics				*				0.017
Normal Karyotype	8	72.9%	9		15	46.7%	6	
Favorable	7	71.4%	NR		7	100.0%	NR	
WT1				0.945				0.963
Down expression	13	76.2%	12		20	55.0%	12	
Over expression	8	62.5%	9		13	61.5%	10	
FOXP3				*				0.835
Down expression	4	100.0%	NR		9	44.4%	4	
Over expression	5	40.0%	6		6	83.3%	10	
FLT3				*				*
W/W	17	69.7%	NR		24	58.3%	12	
W/ITD	1	0.0%	11		3	33.3%	2	
combined_markers				*				*
3 -ve/1+ve	4	100.0%	NR		8	50.0%	4	
2 or 3 +ve	3	33.3%	6		4	50.0%	4	

HB, hemoglobin; PLTs, platelets; TLC, total leukocytes count; FAB, french american british classification; BM, bone marrow blast %; WT1, wilms tumor gene1; (*), no p-value because a small number of cases within subgroups; combined markers (WT1, FOXP3 and FLT3); NR, not reach overall survival; * p-value , considered significant <0.05

Table 7. Impact of Different Parameters on the Disease-Free Survival and Overall Survival for **Adult** Patients

Parameter	DFS				OS			
	n	Proportion surviving At 6 months	Median survival estimate (months)	p value	n	Proportion surviving At 6 months	Median survival estimate (months)	p value
All	34	80.8%	16	-	62	44.7%	3	-
Age (yrs)				0.181				0.086
≤37	22	81.8%	12		34	55.9%	15	
>37	12	80.0%	NR		28	30.8%	1	
Gender				0.240				0.393
Male	14	76.6%	12		25	39.6%	3	
Female	20	83.9%	NR		37	48.0%	4	
FAB Classification				0.510				0.236
M0+M1	7	83.3%	12		19	31.6%	2	
M2	18	87.8%	NR		27	55.3%	15	
M4+M5	7	53.6%	NR		13	35.9%	3	
Hb g/dL				0.282				0.102
≤7.7	15	70.7%	16		34	34.5%	2	
>7.7	19	88.5%	NR		28	56.9%	15	
TLC(x109/L)				0.158				0.008
≤38.6	21	85.4%	16		31	60.8%	15	
>38.6	13	71.6%	8		31	29.0%	2	
PLTs (x109/L)				0.857				0.874
≤37	19	77.0%	16		32	46.7%	3	
>37	15	85.7%	12		30	42.2%	3	
BM Blasts%				0.357				0.030
≤ 69	20	75.0%	12		30	55.9%	15	
>69	13	91.7%	NR		31	32.3%	2	
Splenomegaly				0.019				0.314
No	13	74.6%	9		28	37.2%	3	
Yes	21	84.7%	16		33	51.5%	15	
Hepatomegaly				0.307				0.893
No	14	77.4%	12		27	43.1%	4	
Yes	20	83.5%	16		34	47.1%	3	
CD34				0.694				0.438
Negative	17	80.4%	16		34	38.2%	3	
Positive	17	81.6%	NR		28	53.1%	15	
CD117				0.073				0.748
Negative	9	88.9%	NR		18	44.4%	1	
Positive	22	74.6%	12		39	45.2%	4	
Cytogenetics				*				0.288
Normal Karyotype	9	62.5%	12		13	52.7%	NR	
favorable	4	100.0%	NR		6	83.3%	NR	
WT1				0.035				0.019
Down expression	18	100.0%	16		25	64.0%	15	
Over expression	16	62.5%	12		37	31.5%	2	
FOXP3				0.371				0.269
Down expression	10	60.0%	NR		21	36.4%	3	
Over expression	5	75.0%	8		17	23.5%	1	
FLT3				0.816				0.123
W/W	26	83.2%	16		43	52.8%	9	
W/ITD	6	80.0%	NR		14	28.6%	1	
combined_markers				0.374				0.094
3 -ve/1+ve	9	62.5%	NR		18	41.9%	4	
2 or 3 +ve	5	60.0%	8		16	18.8%	1	

HB, hemoglobin; PLTS, platelets; TLC, total leukocytes count; FAB, french american british classification; BM, bone marrow blast %; WT1, wilms tumor gene 1; (*), no p-value because small number of cases within subgroups; combined markers (WT1, FOXP3 and FLT3); NR, not reach overall survival;* p-value ,considered significant <0.05.

less than ≤38.6 have higher OS than those with more than >38.6 (P =0.008). Patents with (WT1 overexpression, FOXP3 overexpression and FLT3-ITD) have lower OS while those with none or one marker positive have higher OS, on the other hand at the whole group (adults and pediatrics) the p-value near to significant (p =0.053).

Eighteen adult patients with down expression for WT1 have better disease-free survival than 16 patients with Over-expression (p =0.035) and patients with splenomegaly have better DFS than without it (p =0.019).

Relation of Markers with each other (agreement between markers)

Agreement between markers was done using a measure of agreement with Kappa value.

In adult AML, no agreement between the 3 markers studied FOXP3, WT1, and FLT3.

In pediatric AML, a significant agreement was found between FOXP3 and WT1 (p =0.042) which coincides with moderate agreement since Kappa value was 0.412.

No agreement was found in pediatric AML group neither between FOXP3 and FLT3 nor between FLT3 and WT1.

Multivariate Analysis

Multivariate analysis was carried out using Logistic Regression (Odd Ratio) for Response (complete remission). We found that the independent factors that significantly affect response in the adult AML group were, age (p =0.005), BM blasts% (p =0.036) and WT1 gene (p =0.012).

When Multivariate analysis was carried out using Cox Regression (Hazard ratio) for survival analysis (Hazard of death in OS or relapse in DFS). Splenomegaly was the only factor with borderline significance (p=0.055) HR 3.4 (95%CI 0.97 – 11.9) which affects the DFS and TLC was the only independent factor that significantly affects the OS in the adult AML group (p =0.024) HR 2.2(95%CI 1.1 – 4.5).

For pediatric AML group, cytogenetic was the only factor that affects OS (p =0.038) HR 4.9 (95%CI 1.1 – 22.5).

Discussion

WT1 is implicated in the prognosis of AML. In our study, the percentage of the WT1 overexpression was 60.3% for adults and this percentage varies among various studies ranging from 48% to 100% (Inoue et al., 1994; Patmasiriwat et al., 1996; Schmid et al., 1997; Karakas et al., 2002; Barragan et al., 2004; Mossallam et al., 2012). This variability could be due to either different primers (in fact many different isoforms exists for WT1) used, different housekeeping genes used or different ethnic gr oups.

In adults, WT1expression did not significantly affect neither the TLC nor BM blasts% (Table 3) our results agree with other Egyptian adult AML cohorts (16-18). In our adult cohort, OS is negatively affected by BM blasts (P = 0.03), TLC (p = 0.008) in the univariate analysis when multivariate analysis was done TLC was the only independent factor that significantly affects OS (p =0.024), this finding does not agree with (Mossallam, et al., 2012 and Ibrahim et al., 2015). The negative impact which WT1 overexpression had in our adult AML on CR induction, DFS and OS was not applied to the Assuit cohort who had a smaller sample size (Ibrahim et al., 2015). Also, it did apply to Mossallam et al., (2012) cohort where had different primers and probes, method, and shorter overall survival monitoring.

In the pediatric cohort, however, WT1over expression was significantly (p =0.018) associated with higher TLC while the BM blast cells % was not affected significantly (Table 2). While BM blasts (p =0.027) had a statistically negative impact on OS (Table 6). In fact, TLC affects OS in the whole group (p =0.001).

In the present study, at diagnosis WT1 overexpression was detected in 41.2% in the pediatric patients, 14/34 this value is slightly less than that of four other studies (Schmid et al.,1997; Bergmann et al.,1997; Ostergaard et al., 2004; Lapillonne et al., 2006) with respectively, over-expression in 72%, 72%, 74% and 73%. Among their 92 pediatric AML cases the quantified WT1transcript was significantly higher (p =0.001) in the favorable cytogenetic group and lower (p <0.001) in M5- FAB subtype (Lapillonne et al., 2006). We did not have any M5 - FAB subtype in our pediatric cases. Our favorable cytogenetic pediatric group 7/22 a relatively smaller number with no statistically significant association between WT1expression. However and in line with our results Lapillonne et al., (2006) in France found that WT1 overexpression at diagnosis did not affect OS nor EFS in their 92 pediatric AML cases studied. In our pediatric group cytogenetic was the only parameter with significantly better OS in both univariate (P =0.017) and multivariate (p =0.038). Favorable cytogenetic also significantly (p =0.051) affects CR induction only in our pediatric cohort. Neither expression of WT1 nor FOXP3 had a significant influence on CR induction, DFSand OS in our pediatric group, however, there was a significant moderate Kappa agreement value 0.412, (p =0.042) between the two markers in the pediatric group only. Ho et al. (2014) demonstrated that the presence of SNP rs 16754 was associated with significantly higher median diagnostic WT1 mRNA expression which is prognostically relevant. This might explain why our pediatric cohort OS was not negatively impacted upon by increased expression of WT1. SNP rs 16754 represents as A>G substitution at the third position of WT1 codon 352(CGA>CGG), resulting in a silent transversion (both codons encoding arginine). In their study twenty- eight percent of children were SNP +. The prevalence of this SNP is currently under investigation in our pediatric population.

FOXP3 expression had no significant influence on TLC in neither the pediatric, adult nor whole cohort. Because FOXP3 was done on a smaller number 55 patients, and because there was a trend (p =0.087) in a univariate analysis that increased BM blasts % in adults was associated with higher FOXP3 expression, when multivariate analysis was done (p =0.022, OR=1.1, 95% CI=1.0-1.1). When we grouped the whole cohort together, FOXP3 overexpression was significantly (p =0.042) higher with median BM blasts 80% and down expressed with median BM blasts 65%. Hamed et

al. (2015) demonstrated a significantly higher Tregs percentage (CD4/CD25/FOXP3) in adult AML compare to control ($p < 0.001$). In their 25 adult AML cases total CD4 lymphocytes were significantly lower than the control ($p < 0.002$). This was reflected on their complete remission induction, whereby those who did not achieve CR had significantly higher Tregs ($p < 0.016$). Although FOXP3 expression in our study did not influence significantly CR induction yet, There was a significant association between FOXP3 expression and BM blasts% in the whole group whereby those with FOXP3 overexpression had a significantly higher BM blasts 80% VS 65% in those down expression ($p = 0.042$).

In the present study, concordance between FOXP3 and WT1 expression in the whole group was 30/50 (both over-expressed in 23 and both down expressed 7) (Tables 2 and 3). All overexpressed FOXP3 except one in adult (Table 3) could be attributed to WT1 overexpression this is not the case with FLT3-ITD mutated cases, where only 5/11 mutant ones were able to mount FOXP3 overexpression. In fact, all of these five cases were and showed high expression of WT1 and the 2 pediatric cases which were mutant of FLT3 could not mount a Treg response, thus, one can safely conclude that Tregs response in this study can be attributed to overexpression of WT1 in all cases studied except one and not due to FLT3-ITD. An important question is raised here why is there a significant agreement between FOXP3 andWT1 expression in the present study only in the pediatric group although a lesser number (16 pediatrics and 39 adults).

One explanation could be attributed to immunologic derangement of costimulatory molecules CD80 and CD86 in adult AML. Assem et al. (2013) demonstrated the absence of CD80 on AML blasts and CD86 was significantly lowered in adult AML cases compared to normal controls ($P < 0.001$) and ($p = 0.014$) respectively. Their absence or decrease could be the cause of the suppressive effect of Tregs, In this study although FOXP3 expression was comparative in both pediatrics and adults AML cohorts (43.7% vs. 43.6%), the negative impact of WT1 overexpression on CR induction ($P = 0.020$) andDFS ($P = 0.035$) and OS ($P = 0.019$) in univariate analysis was clear only in adults. i.e Tregs mere presence does not hinder WT1 overexpression effect on CR, DFS and OS unless it is properly functioning in adults. This might possibly be the cause of non-agreement between WT1 and FOXP3 expression observed in adult AML. i.e. An effective suppressive function of FOXP3 (regulatory T cells) in adults AML is impaired allowing WT1 to exert its detrimental effects on CR induction, DFS and OS in adult AML.

Splenomegaly, in the present study, accounts for 33/63 (54.1%) in adults and 17/34 (51.5%) in pediatrics. A significant association was demonstrated in adults only where the median survival estimate for DFS was 16 months for those with splenomegaly and 9 months for those without splenomegaly ($p = 0.019$). The median survival estimate for OS for adult AML with splenomegaly was 15 months; while those with no splenomegaly had a median survival estimate was 3 months only the difference was non-significant. Causes of splenomegaly in AML could be either extramedullary hematopoiesis or immune cells proliferation (Reticulo Endothelial system RES) or encroachment of spleen by malignant myeloblasts. The first cause could be excluded since splenomegaly was not significantly associated with the Total leukocytes count. Since splenomegaly was significantly associated with improved DFS then the most likely explanation would be the second cause. But not Tregs since splenomegaly was not significantly associated with FOXP3 (Table 4). However, the third cause cannot be excluded since a significant association between BM blasts% and splenomegaly ($p = 0.039$) was documented in the present study. In our pediatric group patients with splenomegaly had a median survival estimate for OS of 12 months while those with no splenomegaly median survival estimate was 4 months the difference was non-significant. i.e. neither DFS nor OS was significantly affected by splenomegaly in pediatric group.

Hepatomegaly, in the present study, accounts for 34/63 (55.7%) in adults and 20/34 (60.6%) in pediatrics. Like splenomegaly, hepatomegaly had no significant effect on OS. Unlike splenomegaly, hepatomegaly did not significantly influence DFS. Most adult AML patients (68.4%) with hepatomegaly had overexpression of FOXP3 while most cases of AML (77.8%) without hepatomegaly showed down expression of FOXP3. This association with hepatomegaly was statistically significant ($p = 0.005$) only in the adult group. This association was insignificant in the pediatric cohort. Thus, one can assume that liver enlargement might be possibly due to its invasion by Tregs in adults, a finding which can be confirmed by postmortem autopsy. In further support of this notion was the finding in our study that most of the hepatomegaly in adult AML had over-expression of two or three markers (WT1, FOXP3, and FLT3-ITD) while most of AML patients without of hepatomegaly had either none or only one marker overexpressed and the difference between the two groups were statistically significant ($p = 0.009$). Matthaios et al., (2011) demonstrated a local Treg response i.e an overexpression of FOXP3 in the liver biopsy sections which was independent of the initial inducer of liver inflammation whether HBV, HCV, non-alcoholic fatty liver disease, autoimmune diseases, and methotrexate - related toxicity. They showed that FOXP3 expression in liver correlates with the degree but not the cause of inflammation. Our cases were recruited at initial diagnosis prior to receiving any chemotherapy although previous liver affection cannot be ruled out. Further research towards the elucidation of the causal relationship between overexpression of FOXP3 and hepatomegaly in adult AML is warranted. Up to our best knowledge, this is the first report to show an association between FOXP3 and Hepatomegaly in adult AML.

Regarding the FAB classification and up to our best knowledge this is the first report to demonstrate the association between higher FOXP3 expressions in FAB subtype M0+M1 (adult 69%, pediatric 66% and whole group 68%) VS lower expression in M2 (adults 76%, pediatric 80% and whole group 77%) also M4+M5 (adults 62.5%, pediatric 100% and whole group 66.7%). The difference was statistically significant $p = 0.039$ in adults,

p =0.008 in the whole group with NO p-value detected in the pediatric group due to the small number of various FAB subtypes. Some studies indicate that increased Tregs are a poor prognostic indicator (Pandiyan et al., 2007; Szczepanski et al., 2009). In the present study FOXP3, expression did not significantly affect CR induction in the whole group. Interestingly, however in our whole group most 69% M2 and 62.5% M4+M5 achieved CR while most M0+M1 61.8% did not achieve CR (p =0.023). Since most M2and M4+M5 who achieved CR had mostly down expression of FOXP3 (77.3% in M2 and 66.7% in M4+M5) and since most M0+M1who had higher expression of FOXP3 68.2% did not achieve CR. one can conclude that reduced Tregs (FOXP3) can possibly contribute to complete remission induction in FAB- subtypes M2 and M4+M5. Whether the decrease expression of Tregs (FOXP3) could possibly be the cause of achievement CR is worth mentioning.

WT1 expression in the present study, however, did not significantly vary among the various FAB- subtypes. Lapillonne et al. (2006) demonstrated a significant (p <0.0001) lower expression of WT1 in their pediatric M5 FAB- subtype. We did not have any M5 - FAB subtype in our pediatric cases. In another Egyptian cohort, WT1 down expression was demonstrated in M5 FAB- subtype in 13 out of their 18 M5 cases (Ibrahim et al., 2015). In Lapillonne cohort, all of their M2 pediatric AML had significantly overexpression of WT1 at diagnosis (p = 0.04). This was not the case in our study.

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Conflict of interest

No conflict of interest to disclosure

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