



Prognostic Implications of Expression of the Wilms Tumor 1 (*WT1*) Gene in Acute Leukemia (Experience from South Egypt)

Abeer Ibrahim¹, Hosny Badrawy^{2*} and Heba Sayed³

¹Department of Medical Oncology and Hematological Malignancies, South Egypt Cancer Institute, Assiut University, Egypt.

²Department of Clinical Pathology, South Egypt Cancer Institute, Assiut University, Egypt.

³Department of Pediatrics medical oncology and hematological malignancies South Egypt Cancer Institute, Assiut University, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author AI wrote the manuscript and author HB designed the study, performed the practical analysis, review the manuscript and published it. Author HS review the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background: Wilms' tumor (*WT1*) gene expression has been reported in the majority of acute leukemia patients at diagnosis and has been evaluated as a prognostic and minimal residual disease (MRD) marker but its role is still controversial.

Methods: Real-time quantitative polymerase chain reaction was used on bone marrow samples from 100 newly diagnosed adults and pediatrics acute leukemia patients (50 AML and 50 ALL patients). *WT1* expression were examined at diagnosis and at the end of induction.

Results: *WT1* was expressed in (14%) ALL and in (36%) AML patients. We found no statistically significant impact of *WT1* expression at diagnosis on response $p=0.054$, 0.057 , DFS ($P=0.591$, 0.858), or OS ($p=0.339$, $p=0.331$) in ALL and AML patients respectively. Persistence of *WT1* expressions at the end of induction didn't show any effect on relapse rate in AML however, it

*Corresponding author: Email: badrawyh@yahoo.com;

showed significant results in ALL $p=0.045$.

Conclusion: Our results suggest that WT1 expression in patients with acute leukemia doesn't have any implication on response or survival however, significant association was found in predicting ALL relapse but the small sample size should be considered.

Keywords: Wilms tumor gene 1; acute leukemia; real-time quantitative polymerase chain reaction.

1. INTRODUCTION

The prognosis of acute leukemia used to be dependent on its morphological immunophenotypical classification or on their chromosomal aberration [1]. In AML, it is typically divided into three different risk groups, based on the types of chromosomal aberrations. However, inconsistencies were found among this group of patients in their responses to chemotherapy and prognosis that sometimes makes it difficult to make the right decision for therapeutic treatment and/or assessment of the possible treatment outcome of the patients [1]. Adding examination of molecular aberrations is thought to be helpful in addressing these differences. However, they still controversial [2]. Thus, identification of novel markers for risk stratification and therapeutic targeting is still needed [2]. One potential marker is the mutational status of Wilms tumor 1 (*WT1*), which is a gene located at chromosomal band 11p 13 and encodes a transcription factor with an N-terminal transcriptional regulatory domain (exons 1 to 6) and a C-terminal 4-Cys2His2 zinc finger domain (exons 7 to 10) [3]. *WT1* functions as a potent transcriptional regulator for genes involved in cellular growth and metabolism [4]. Although its role in normal hematopoiesis has not been clarified, disruption of *WT1* function is currently considered to promote stem-cell proliferation and to hamper cell differentiation [3,5]. Acquired mutations of this *gene* have been reported in approximately 10% of cytogenetically normal AML (CNAML) patients [6,7] and have been associated with poor prognosis in both adult and pediatric CNAML patients [8-10]. However, another large study was done recently contradicted these results and found no prognostic impact of *WT1* mutations in AML patients [11].

On the other hand, few studies have evaluated the prognostic relevance of *WT1* expression in ALL and have suggested an association between high expression and an inferior outcome [12,13] while other studies showed non-significant affection on response or survival [14-16].

This study was conducted to evaluate the effect of expression of WT1 gene on the outcome of our patients with acute leukemia

2. METHODS

From May 2011 to May, 2014, we prospectively assessed the WT1 transcript expression of leukemic cells. Bone marrow samples were collected from 100 patients (50 ALL and 50 AML), from both Adults and Pediatrics Medical oncology and Hematological Malignancies Departments, South Egypt Cancer Institute. Assiut University. The age of the patients ranged from 2-60 years, all of them were de-novo acute leukemia.

The study was performed after obtaining approval from the local Institutional Review Board Committee and in accordance with the Declaration of Helsinki, the Good Clinical Practices, and local ethical and legal requirements. All patients provided written informed consents.

ALL patients were classified according to their immunophenotyping into B-lineage and T-lineage. The risk stratification was based mainly on each patient's initial presenting features and immunophenotypical data. Patients with non-T cell immunophenotype, age between 1 year and <10 years in pediatric patients, and leukocyte counts $<50 \times 10^9$ in B lineage or $< 100 \times 10^9$ in T lineage were assigned to the standard-risk group. Patients with t (9;22) or the presence of BCR-ABL fusion, were assigned as very high-risk group. The remaining patients were assigned to the high-risk group.

In AML patients the diagnosis was based on the presence of blast cells at $\geq 20\%$ in bone marrow (BM) smears and French-American-British subtype was used for classification [17] and the diagnosis was confirmed by immunophenotyping.

Pretreatment BM samples were also analyzed by fluorescence in situ hybridization for the presence t (15;17) (q22;q12) to exclude M3.

2.1 Treatment Regimens

All patients were treated according to the protocols of the pediatrics and adults protocols of South Egypt Cancer Institute.

In ALL group adults patients received modified BFM regimen [18] while, patients with ALL were treated according to the institute treatment protocol modified from the study XIII-B of St. Jude Children Research Hospital [19]. Patients with very high risk who have t (9-22) received their standard protocol with addition to imatinib followed by bone marrow transplant after first remission.

Regarding AML patients, all patients received intensive induction therapy (cytarabine, and mitoxantron [20] , while pediatric received ADE protocol [21], if they achieved remission consolidation therapy was given in the form of (cytarabine, 3 g/ m²/12 h for 3 days repeated for 3-6 cycles).

CR was defined as the absence of clinical manifestations of acute leukemia accompanied with neutrophil count higher than 1.5x10⁹/L, platelet count higher than 150x10⁹/L and hemoglobin levels higher than 100 g/L and morphological examination of bone marrow shows less than 5% of blast cells.

Patients with blast cells in BM greater than 5% at the end of the induction phase were considered induction failures [22].

Disease-free survival (DFS) was defined as survival without relapse or death from the date of first CR or censoring patients alive in continuous complete remission at last follow-up date. Overall survival was defined as the time from diagnosis until date of death or censoring patients' alive at last follow-up date.

2.2 RNA Extraction and cDNA Synthesis

Aspirated bone marrows were collected at South Egypt cancer Institute, Assiut Egypt. in EDTA tubes . Mononuclear cells (MNC) from bone marrow (BM) were isolated by density gradient centrifugation (LymphPrep), then the samples underwent total RNA extraction using the RNeasy mini kit, following the manufacturer's instructions (Qiagen, GmbH, Hilden, Germany). cDNA was synthesized by reverse transcription using Transcriptor First Strand cDNA synthesis

kit (Roche Diagnostics GmbH, Mannheim Germany) and then stored at -80°C until use.

2.3 Real Time Quantitative PCR of WT1

RQ-PCR was performed using Light Cycler TaqManMaster [ready-to-use hot start reaction mix for PCR on the Light Cycler Carousel-based system with Hydrolysis TaqMan Probes (Roche Diagnostics GmbH)]. Using the primers and probe for WT1 (Roche, Genebank Accession: NM_000378) and Standard, primers and probes of ABL (Roche, Genebank Accession:M33197) and the Light Cycler TaqMan Master protocol was used according to manufacture's instructions.

2.4 Principles

Hydrolysis probes, also called the TaqMan assay, use a single probe containing two labels: a fluorescent reporter dye and a fluorescent quencher. While the probe is intact, the quencher is close to the reporter dye and suppresses reporter fluorescence by fluorescence resonance energy transfer. When the probe is hybridized to the target sequence, the 5' nuclease activity of the polymerase can cleave the hydrolysis probe, separating the reporter and quencher. With an increasing amount of target sequence during PCR, more probes are cleaved and the fluorescence signal of the unquenched reporter dye increases.

2.5 Standards and Normalisation of WT1 Expression

A standard curve for the housekeeping gene ABL was set up, normalized WT1 expression (WT1_N) was determined as a ratio between WT1 and ABL levels assessed by RQ-real time PCR in each individual sample. Sensitivity of the assay reached 10⁻⁴ (= one cell among 10⁴ normal pooled mononucleated cells) in all experiments, therefore all calculated WT1_N values 10⁻⁵ and lower were considered as zero ((Roche, Genebank Accession:M33197).

2.6 Statistics

The cut-off date for follow-up observations was May 31, 2014. All statistical analyses were performed using SPSS 16. The comparison of WT1 levels and clinical parameters was analyzed by a non-parametric test (Mann-Whitney), to evaluate the significant difference between

clinical parameters. The probability of survival was estimated by the Kaplan–Meier method [23]. A *p*-value of <0.05 was considered to be statistically significant.

3. RESULTS

3.1 Patients' Characteristics

Bone marrow samples were collected from 100 consecutive adults and pediatric patients (65 males and 35 females). The age of patients ranged from 2-60 years, all of them are de-novo acute leukemia. We divided the patients into 2 groups according to the type of leukemia. ALL group: included 50 patients and AML group: included 50 patients. The expression of WT1 was analyzed in all patients according to their sex, age, total white blood cell, percentage of blast in bone marrow, and immunophenotyping

classification in ALL patients, and FAB classification in AML patients (Tables 1 and 2).

Regarding ALL group; included 34 males and 16 females. 27 patients treated in pediatric department aged 2-16 years and 23 patients treated in adults department aged 17-40 years old. 82% of the patients were B lineage while, T cell lineage accounted for 18%. According to their risk we stratified the patients into 3 groups, "standard risk group" included 30 patients, 'high risk group' group included 11 patients "very high risk group" included 9 patients (Table 1).

AML group, it included 31 males and 19 females, 11 patients treated in pediatric department aged 2-16 years old while, 39 patients aged 17-60 treated in adult department no one showed high risk feature of t (9-22), FLT3 was done in 13 patients only 3 of them showed positive mutation (Table 2).

Table 1. ALL patients characteristics

	N(%)	WT1 (-)	WT1 (+)	P
Sex				
Male	37(74)	32	5	0.321
Female	13(26)	11	2	
Age				
0-10	24(48)	21	3	0.053
10-20	11(22)	8	3	
20-30	10(20)	9	1	
30-40	5(10)	5	0	
40-50	0			
50-60	0			
Immunophenotype				
B lineage	41(82)	36	5	0.064
T lineage	9(18)	7	2	
WBCx10⁹/L				
<50	34(28)	30	4	0.051
>50-<100	10(20)	8	2	
>100	6(12)	5	1	
Risk classification				
SR	30(60)	27	3	0.754
HR	11(22)	9	2	
VHR	9(15)	7	2	
Bone marrow blast				
30-49	7(14)	5	2	0.416
50-67	5(10)	4	1	
>70	38(76)	34	4	
Remission after induction				
CR	42(84)	38	4	0.054
Not CR	7(14)	4	3	
Death during induction	1(2)	1	0	

SR: standard risk, HR High risk, VHR: very high risk

Table 2. AML Patients characteristics

	N (%)	WT1 (-)	WT1 (+)	P
Sex				
Male	31(62)	21	10	0.931
Female	19(38)	11	8	
Age				
0-10	3(6)	2	1	0.192
10-20	14(28)	10	4	
20-30	18(36)	12	6	
30-40	8(16)	3	5	
40-50	5(10)	3	2	
50-60	2(4)	2	0	
FAB				
M0	0 (0)	0	0	0.469
M1	2(4)	2	0	
M2	18(36)	11	7	
M4	12(24)	6	6	
M5	18(36)	13	5	
M6	0(0)	0	0	
M7	0(0)	0	0	
WBCx10⁹/L				
<10	20(40)	13	7	0.129
10-50	24(48)	15	9	
50-100	6(12)	4	2	
>100				
Bone marrow blasts				
20-50	12(24)	8	4	0.612
50-70	22(44)	14	8	
>70	16(32)	10	6	
FLT3				
+ve	3 (6)	2	1	0.091
-ve	10(20)	8	2	
Not done	38(76)	22	15	
Remission after induction				
CR	36(72)	22	14	0.067
Not CR	10(20)	7	3	
death during induction	4(8)	3	1	

3.2 WT1 expression at diagnosis

WT1 expression at diagnosis was found in 14 % of ALL patients and in 36% of AML patients.

Our results showed no significant difference between WT1 expression among ALL patients regarding different prognostic variables (sex: p=0.321, age: p= 0.053 total white blood cell count: p= 0.051, immunophenotyping: p=0.064, percentage of BM blast: p=0.416 and different risk group: p=0.754).

Also in AML group we couldn't find any significant difference between WT1 expression and (sex: p= 0.931, age: p= 0.192, FAB subtypes: p=0.469, percentage of blast in BM: p=0.61, and FLT3 mutation status in examined patients: p=0.091.

Regarding remission after induction, no significant differences was found among patients with WT1 expression at diagnosis and the response to 1st induction in ALL group p= 0.054 and AML group p=0.067 p=0.067 (Table 1&2).

3.3 Status of WT1 Gene Expression (WT1) at the End of Induction

After induction chemotherapy in ALL, of 7 patients who had positive baseline WT1 expression 4 of them achieved CR while 3 patients did not. 3(75%) out of 4 patients who achieved CR, the expression remained positive while, the 3 patients who didn't achieved CR, the expression persisted in all of them 100% p= 0.049.

In AML After induction chemotherapy, of 18 patients who had positive baseline WT1 expression 15 of them achieved CR while 3 patients did not entered in CR and 1 patients died during treatment, WT1 expression persist in 12 (80%) out 14 patients who achieved CR Also it persist in 2(66%) out 3 of patients who didn't respond to first induction therapy $p= 0.049$.

After median follow up of 20 months, in ALL group, one patient out of 3 patients (33%) who had CR with persistence of WT1 expression relapsed after 6 months of finishing maintenance, while 5 out of 39 patients (12%) who had CR with negative WT1 expression at the end of induction relapsed after median 20 months of follow up $p=0.042$ Table 3, while in AML patients with same period of follow up relapse occurred in 4 patients out of 12 (33%) who had CR with persistence WT1 expression, while it occurred in

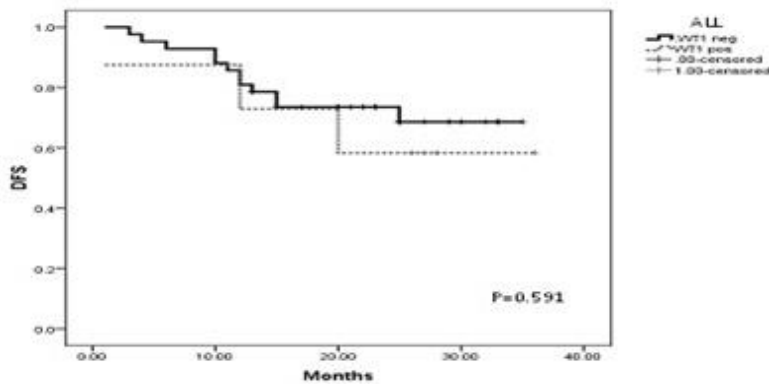
7 patients out of 21 (32%) who had CR with negative expression of WT1 $p=0.988$ (Table. 3).

3.4 Impact of WT1 Gene Expression on Survival

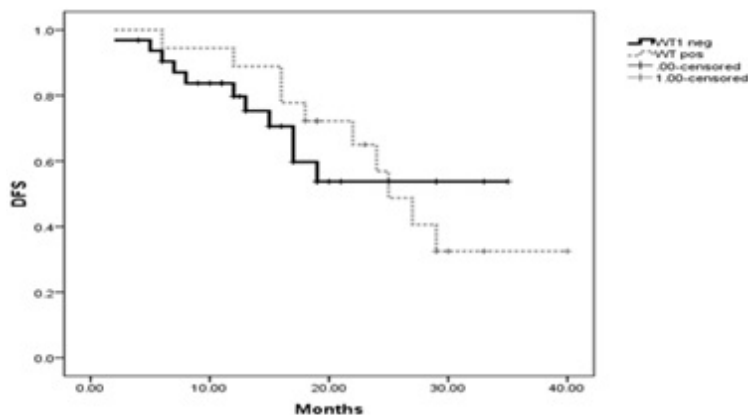
The median duration of follow-up was 20 months. We couldn't find any significant impact of positive WT1 expression at diagnosis on DFS in ALL group $p=0.591$ Figs. (1a and b) or AML patients $p=0.858$ Figs. (2a and 2b) and in OS $p= 0.339$ and 0.331 in ALL and AML groups respectively (Fig. 2).

4. DISCUSSION

In this work, WT1 expression was evaluated in ALL and AML patients at diagnosis and after induction chemotherapy to identify its impact on prognosis and relapse.



(A)



(B)

Fig. 1. DFS in different WT1 expression at diagnosis A) in ALL B) in AML

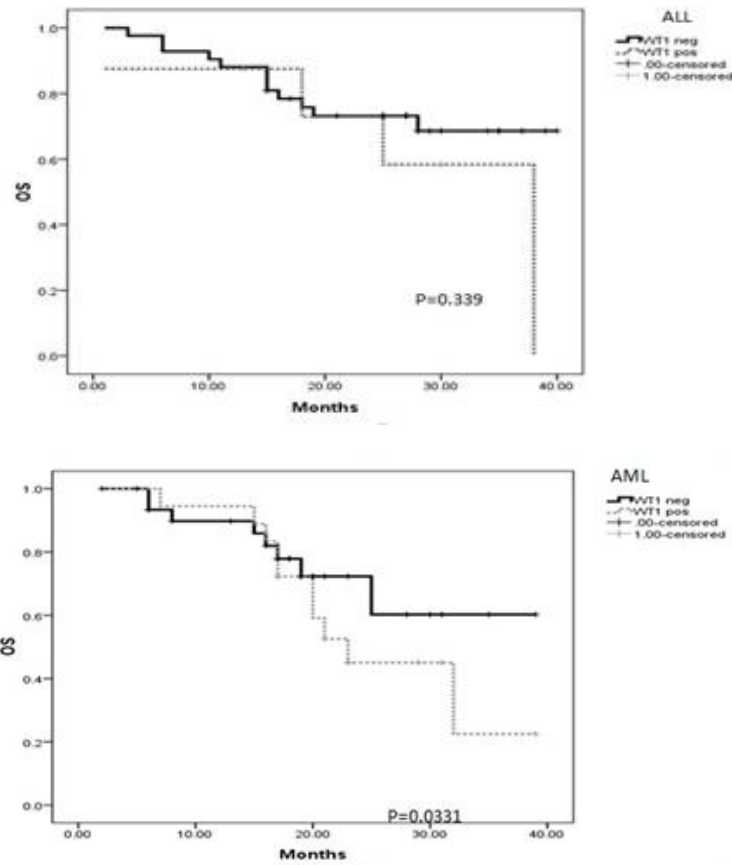


Fig. 2. OS in different WT1 expression at diagnosis a) in ALL, b) in AML

Table 3. WT1 expression at the end of induction remission and its affection on response and relapse

WT1 status at the end of induction	N (%)	WT1 (-)	WT1 (+)	P
<u>Response</u>				
ALL group				
CR	42(84)	39	3	0.049
Not CR	7(17)	4	3	
Death during induction	1(2)	1	0	
AML group				
CR	36(72)	24	12	0.097
Not CR	10(20)	8	2	
Death during induction	4(8)	3	1	
<u>Relapse</u>				
ALL group	7(16)	6	1	0.045
AML group	11(30)	7	4	0.965

Our study showed WT1 expression is less frequently expressed at diagnosis in BM samples in patients with ALL which is contradict many studies which reported more than 80% expression of WT1 [12,13,24], but our results agree to some extend with other studies [25-27]

which reported lower WT1 expression in newly diagnosed ALL patients in 40% of patients or less, they attributed this to ethnic difference or different sample sizes [28]. Regarding the immunophenotypic subtypes of ALL and WT1 expression in our patients, no difference between

T-ALL and B-ALL was found regarding WT1 expression both in adult and pediatric patients. Several studies showed controversial results with higher WT1 overexpression detected in B-ALL in some of them [29] and in T-ALL in others. [30] However, these studies investigated WT1 mostly in adult ALL patients or in heterogenous groups of children and adults, in PB samples, using potentially less sensitive PCR techniques for WT1 detection [31,32].

Our data shows the WT1 expression at diagnosis has no significant correlation to clinical complete remission rate or disease relapse rate in ALL, which is similar to the results of Gaiger et al. [31]. On the other hand, our data indicate that the WT1 level at the end of remission induction has significant relationship with disease relapse in ALL patients $p= 0.045$ and this correlated with Omaran et al. [25]. But contradict other study by Chen et al. [33] who didn't find any correlation between WT1 expression at the end of induction and relapse. However the small number of our study should be considered.

In the present work, patients were followed up for a median of 27 months, and the influence of WT1 expression levels at diagnosis on the DFS was determined. There was no statistically significant impact of WT1 gene's expression on DFS in ALL patients. This comes consistent with the studies of Magyarosy et al. [14] Imashuku et al. [15] and Boublikova et al. [16] who reported that higher levels of WT1 gene expression at diagnosis were not associated with shorter DFS.

Regarding AML, the WT1 transcript was overexpressed in 34% of AML patients at diagnosis, which is much lower than other studies reported WT1 is overexpressed in approximately 70–90% of AML patients [34]. No significant associations were encountered between WT1 overexpression at diagnosis and other prognostic factors including age, total leukocyte count, and blast percentage. This is in accordance with the previous studies which could not find an association between the gene overexpression and prognostic factors. [35,36] Also FAB classification showed no statistical difference in WT1 expression which is in accordance with results reported by Grag et al. [37] and Noronha et al. [38] although this contradict the results of Weisser et al. [39] and others, [40,41] who found significant lower level in M5 subtype being more differentiated compared to more undifferentiated subtypes.

No observed significant difference in CR in patients expressing the gene compared to those without overexpression. This was similar to findings of Schmid et al. [42] Barragan et al. [43] and Cilloni et al. [35]. They reported no difference in WT1 transcript at diagnosis in patients resistance compared to responders to chemotherapy. Our study didn't show any significant difference between WT1 expression and DFS and OS in AML patients which is in line with several studies which could not find a significant association between overexpression of the gene and DFS and OS, [44,45], in spite of other data reported worse outcome with WT1 overexpression [30,38,46].

5. CONCLUSION

Our results suggest that WT1 expression in patients with acute leukemia doesn't have any implication on response or survival however, significant association was found in predicting ALL relapse but the small sample size should be considered.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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