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Research Article

Wilms' Tumor 1 Gene Expression as a Predictive Marker for Clinical Outcome of Acute Myeloid Leukemia in Iraqi Population: A Prospective Study

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Abstract

Background: Wilms' tumor gene 1 (WT1), a tumor-associated antigen (TAA), is expressed in many types of cancer. Most acute leukemia patients have a quantitatively detectable and strong expression of it. ***Objectives***: To analyze WT1 expression levels as a predictor of clinical outcomes at the time of diagnosis of de novo leukemia and to monitor tumor progression during treatment. ***Methods***: A total of 71 patients with acute myeloid leukemia (AML) were separated into two groups: twenty-nine de novo AML patients upon presentation and 25 with AML at the time of initial induction. The second induction included 17 AML patients and ten healthy volunteers who served as controls in this study. The WT1 gene was tested using a real-time PCR with the Cyber Green assay. ***Results***: Patients with acute myeloid leukemia had considerably greater levels of WT1 gene expression than controls (27.3 *vs.* 5.5). In terms of clinical outcomes, WT1 gene overexpression was substantially related to non-responsive AML patients compared to complete response at diagnosis (27.3 *vs.* 22.15). However, there is no substantial difference between instances following induction. ***Conclusions***: The WT1 tumor antigen may serve as an early diagnostic for acute leukemia prognosis. Improved clinical outcomes have been linked to reduced WT1 levels. A high amount, on the other hand, was linked to a poor prognosis for people with AML, although more research is needed.

Keywords: Acute myeloid leukemia, Measurable molecular marker, Prediction, Wilms' tumor gene 1.

التعبير الجيني لورم ويلمز 1 كعلامة تنبؤية للنتائج السريرية لابيضاض الدم النخاعي الحاد لدى المواطنين العراقيين: دراسة مستقبلية

الخلاصة

الخلفية: يتم التعبير عن جين ورم ويلمز 1 (WT1)، وهو مستضد مرتبط بالورم (TAA)، في العديد من أنواع السرطان. معظم مرضى سرطان الدم الحاد لديهم تعبير قوي وقابل لأكتشافه كميا. **الأهداف**: تحليل مستويات تعبير WT1 كمؤشر على النتائج السريرية في وقت تشخيص سرطان الدم من جديد ومراقبة تطور الورم أثناء العلاج. **الطرق**: تم تقسيم ما مجموعه 71 مريضا يعانون من ابيضاض الدم النخاعي الحاد (AML) إلى مجموعتين: تسعة وعشرون مريضا من جديد AML عند الظهور و 25 مصابا ب AML في وقت التحريض الأولي. شمل التحريض الثاني 17 مريضا ب AML وعشرة متطوعين أصحاء عملوا كعناصر تحكم في هذه الدراسة. تم اختبار جين WT1 باستخدام تفاعل البوليميراز المتسلسل في الوقت الفعلي باستخدام مقايسة Cyber Green. **النتائج**: كان لدى المرضى الذين يعانون من AML مستويات أعلى بكثير من التعبير الجيني WT1 من الضوابط (27.3 مقابل 5.5). من حيث النتائج السريرية، كان التعبير المفرط عن جين WT1 مرتبطا ارتباطا كبيرا بمرضى AML غير المستجيبين مقارنة بالاستجابة الكاملة عند التشخيص (27.3 مقابل 22.15). ومع ذلك، لا يوجد فرق معنوي بين الحالات التي تلي الاستقراء. **الاستنتاجات**: قد يكون مستضد الورم WT1 بمثابة تشخيص مبكر لتشخيص AML. تم ربط النتائج السريرية المحسنة بانخفاض مستويات WT1. من ناحية أخرى ، تم ربط كمية كبيرة بسوء التشخيص للأشخاص المصابين بـAML ، مع الحاجة إلى مزيد من البحث.

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INTRODUCTION

Leukemia is the abnormal production of leukocytes, which can occur as a primary or secondary process. Based on the rate of proliferation [1], it is characterized by an aberrant buildup of undifferentiated blasts that can proliferate uncontrollably in the bone marrow [2]. We can classify them as acute or chronic, myeloid or lymphoid, depending on the originating cell [1]. Previously, substantial study was focused on describing tumor-associated antigens (TAA) in human tumors and developing new methodologies for investigating these antigens. Wilms' tumor gene 1 (WT1) is a universal tumor-associated antigen (TAA) that appears in a wide range of cancers. It is also highly expressed in most acute leukemia patients and can be quantified, making it a possible MRD

signal for AML in the absence of specific molecular markers [3,4]. Minimal residual disease (MRD) monitoring is the most significant tool for detecting early signs of relapse, which may help prevent post-transplant relapse [5]. The diagnosis of minimal residual disease (MRD) in acute myeloid leukemia (AML) is critical for risk assessment and therapy planning. More than half of AML patients do not have a distinct or detectable molecular marker for monitoring MRD. WT1 has been studied as a potential MRD marker because it is overexpressed in more than 80% of AML patients, albeit its role in this situation is still debated [6]. The WT1 gene, found on chromosome 11p13, encodes a zinc finger transcriptional factor [7-9]. This zinc finger transcriptional factor is present in mesothelial cells, gonads, and developing kidneys. In addition, WT1 is detected in the spleen and CD34+ progenitor cells, but not in mature leukocytes [10]. Recent discoveries of specific genetic anomalies in AML patients enable more sensitive detection of MRD through quantitative RT-PCR (qRT-PCR) or next-generation sequencing (NGS) [11]. Thus, the purpose of this study is to evaluate WT1 gene expression in follow-up AML patients at diagnosis and after induction using quantitative real-time PCR. We intended to determine the WT1 cutoff value for predicting clinical outcomes in AML patients. This will help anticipate the patient's reaction before administering treatment. If the patient does not respond, the treatment regimen is changed, which causes a delay in treatment, and a marrow transplant may be recommended.

METHODS

Study design and setting

This is a prospective cohort study in which, for the WT1 gene expression analysis, peripheral blood samples were taken from 10 healthy controls and 71 patients with acute myeloid leukemia. Twenty-nine patients received their AML diagnosis recently, and 25 already had it at the time of their first induction. At the second induction, there were 17 AML patients enrolled in this study. The estimated value (percentage) of blast cells in bone marrow and peripheral blood was 13-97 (67%) and 14-98 (77.7%), respectively Table 1.

Table 1: Characteristics of AML patients before and after treatment and controls

Variable	Samples	n (%)	Blast average (%)
Groups	Newly diagnosed	29(35.8)	59.08 Ranged (13-97)
	1st induction	25(30.86)	
	2nd induction	17(20.99)	
	Control	10(12.34)	
	Total	81(100)	

Inclusion criteria

Patients with acute myeloid leukemia have recently been diagnosed at the time of their first induction and those at the second induction. The patients' ages range was 16-72 years.

Exclusion criteria

Patients are early diagnosed with pre-leukemia, in addition to those who died after the first or second induction.

Outcome measurements

During follow-up, we documented complete remission (CR) and not-responding patients (NR) from tumor registry files with the help of consultant hematologists. Clinical information about the patient included blast percent in the peripheral blood and bone marrow, WBC count, platelet count, and hemoglobin. Each patient's response differed depending on whether they were treated according to the chemotherapy regimens specified by the Baghdad Teaching Hospital, Hematology Unit. Each patient underwent two cycles of induction and subsequent consolidation. The therapeutic response was classified as complete remission (CR), which occurred under extraordinary conditions for more than six months. Following induction chemotherapy and the lack of leukemia in other places, the bone marrow aspirate was more than 20% cellular and fewer than 5% blast cells. After at least two chemotherapy treatments, a patient is labeled a non-responder (NR) if there are more than 5% blast cells in the bone marrow or evidence of leukemia in other places [12]. We measured CR and NR after each cycle of induction.

Sample preservation

At the genetic laboratory of the National Center for Early Detection of Tumors in the medical city of Baghdad/Iraq, the blood samples were preserved in TRIzol. Two milliliters of peripheral blood were taken, and after being treated with TRIzol, all samples were stored at -80 ºC. The samples were investigated by the Molecular Oncology Unit at Guy's Hospital, King's College, London, UK, extracting RNA followed by utilizing reverse transcription and real-time PCR for molecular analysis.

RNA extraction and RT-PCR assay

Following the manufacturer's instructions, the TRIzol® LS Reagent (Life Technologies-Ambion USA) was used to extract the total RNA from all groups of blood samples. The high-capacity cDNA reverse transcription kit (Life Technologies, Ambion, USA) was used to reverse transcribe total RNA, as the manufacturer's recommendations. Following that, cDNA was kept at -80 ºC until it was required. Specific primers created with Primer 3 were used to evaluate the gene expression (Table 2) by SYBR Green (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The standard curve was created using sequential dilutions of the cDNA. For target and endogenous control genes [ABL], standard curves were produced. The Applied Biosystems 7900 Real-

Time PCR machine was used to perform the quantitative real-time PCR experiments in triplicate.

Table 2: Primers sequences

Prim	Sequence
WT1	5'-AGGCTTTGCTGCTGAGGAC-3'
WT1	5'-CAGGTCATGCATTCAAGCTG-3'
ABL	5'-TGGAGATAACACTCTAAGCATAACTAAAGGT-3'
ABL	5'-GATGTAGTTGCTTGGGACCCA-3'

Quantitative RT-PCR data analysis

The quantity of the target WT1 gene was standardized to the endogenous reference ABL gene, and it is dependent on a calibrator non-treated normal control given by 2-ΔΔCt ABI PRISM (7700) Sequence Detection System (1997) (User Bulletin No. 2, 1997). [13]. The comparative expression level was measured using the comparative CT method (threshold cycle) and compared with a calibrator to assess the level of expression for various blood samples. When the PCR efficiency of the target and reference genes is equal, the comparative CT way eliminates the need for standard curves for relative quantification. The fold change in gene expression was determined using the 2-ΔΔCt method, where ΔΔCt= ΔCt target - Ct non-treated for calibration, and was then standardized using ΔCt = Ct target gene - Ct endogenous [13].

Relative Efficiency of CA9 and ABL genes

For the ΔΔCT computation to be accurate, the efficiency of both target and reference amplification should be roughly equivalent to the ABI PRISM (7700) Sequence Detection System [13]. Using the essential data from the standard curves for WT1 and ABL, the efficiency of both target and reference amplification was computed using the formula E= 10^ (-1/slop).

Ethical consideration

The study was approved by the local institutional research ethics committee according to reference No. MEC-28.

Statistical analysis

Data was analyzed using SPSS program version 20. Categorical variables were presented as frequencies and percentages. The *t*-test was used for comparison between two groups that were normally distributed. While the Shapiro-Wilk test was significant for *WT1* expression, indicating that it is not normally distributed, the Mann-Whitney U test was used for comparisons between the two groups. Meanwhile, the Kruskal-Wallis test was used for comparisons among all study groups. Statistical significance was regarded at *p* less than 0.05.

RESULTS

The study comprised twenty-nine de novo acute myeloid leukemia patients with a blast cell percentage of 59.08% in their peripheral blood at presentation, as well as 25 AML patients during initial induction. In this prospective trial, the second induction included 17 AML patients and 10 healthy volunteers as control subjects. The AML patients were separated into two groups: 11 girls (68.8%) and 18 males (78.1%). The participants ranged in age from 17 to 61 years old, with a mean of 33.7±15.9. The control group includes five girls (31.2%) and five males (21.9%). The percentage varied from 24 to 48 years old, with a mean of 34.4±8.5. There were no significant differences between age and gender in AML patients compared to control groups (Table 3).

Table 3: The Characteristics of case-control according to age and gender

		Before Treatment (n=29)	Controls (n=10)	Total	*p*
Gender	female	11(68.8)	5(31.2)	16(100)	0.7
	Male	18(78.1)	5(21.9)	23(100)	
Age		33.7±15.9	34.4±8.5		0.9

Values were expressed as frequency, percentage and mean±SD.

Figure 1 depicts the Fab categorization for AML patients. Most of them were AML-M3 (28.6%).

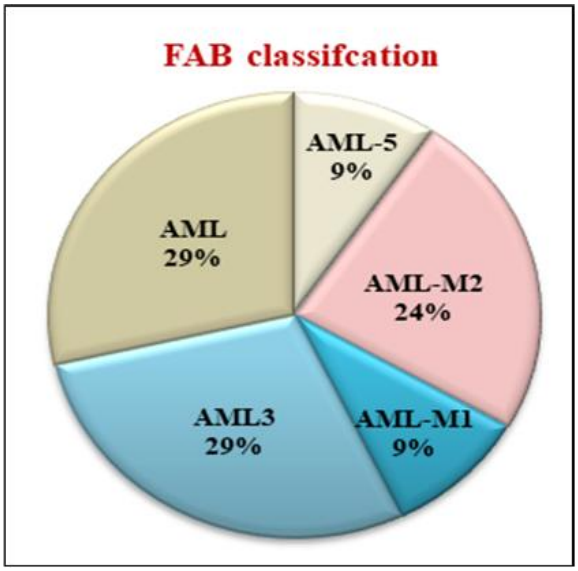

Figure 1: Distribution of AML patients according to FAB classification

The WT1 gene expression levels in 81 peripheral blood samples were determined using qRT-PCR. The WT1 product's Ct values (cycle threshold) are inversely proportional to the amount of target nucleic acid in the sample (e.g., the lower the Ct level, the more target nucleic acid is present). Ct values less than 29 indicate significant positive reactions, implying an abundance of the target nucleic acid in the sample. Positive reactions with Cts ranging from 30 to 37 indicate moderate quantities of the target nucleic acid. Cts of 38-40 indicate weak reactions with small quantities of target nucleic acid. The WT1 transcript has a lower Ct value in AML patients than in controls (Figure 2). The level of WT1 expression was calculated in three comparison groups.

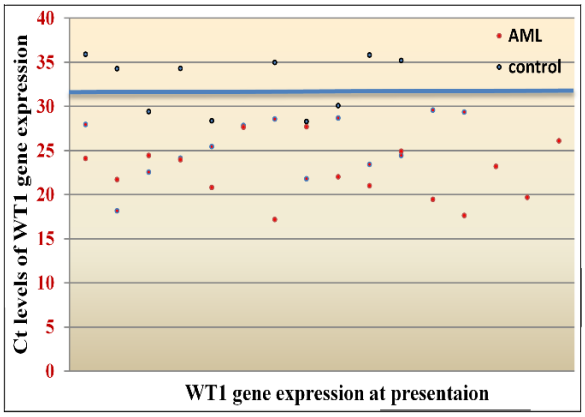

Figure 2: The Ct value of WT1 expression

Prior to treatment, there were two inductions with the control group: the first and second. The mean Ct value of the WT1 transcript in non-responding patients at presentation was 22.6±3.2. The complete answer rate was 25.6±3.5, significantly higher than the control rate of 32.7±3.2 (p= 0.0001). Statistical analysis showed no significant differences (p= 0.6) or (p= 0.7) between AML patients at the first and second induction compared to control (Table 4).

Table 4: Differences of Mean Ct value of *WT1* Gene Expression among CR and NR AML Patients with control

		Course		
		Before treatment	1st induction	2nd induction
AML	Non-response (NR)	22.6±3.2	30.7±7.1	31.2±8.7
	Complete response (CR)	25.6±3.5	30.6±3.3	33.6±5.1
Control		32.7±3.2	32.7±3.2	32.7±3.2
p-value		0.0001	0.6	0.7

Values were expressed as mean±SD.

The mean Ct value of the WT1 transcript differs significantly between NR (22.6±3.2) and CR (25.6±3.5) AML patients before therapy (p= 0.03) versus after induction treatment (Table 5).

Table 5: Association between mean Ct value of WT1 gene expression and AML patients' clinical outcome

		Course			*p*-value
		Before treatment	1st induction	2nd induction	
AML	Non-response (NR)	22.6±3.2	30.7±7.1	31.2±8.7	0.001
	Complete response (CR)	25.6±3.5	30.6±3.3	33.6±5.1	0.0001
p-value		0.03	0.9	0.5	

Values were expressed as mean±SD.

Of the 29 de novo AML patients, 16 (55.17%) were NR and 13 (44.83%) were CR. This suggests that the mean rank of WT1 mRNA expression was considerably greater in NR (27.3) and CR (22.15) than in control (5.5) (p= 0.0001). After treatment, 15 (60%) of the 25 AML patients at the first induction were NR and 10 (40%) were CR, with a significantly higher mean rank (21.13 and 21.6, respectively) than the control (9.7) (p= 0.01). At the second induction, no significant changes were seen between AML patients and controls (Table 6). The average fold change for healthy controls is approximately 0.296856. The average fold in non-response AML patients changed by around 7064.9, while in complete-response AML patients, it changed by about 2656.9.

Table 6: Mean rank Wilms' tumor 1-fold change of AML patients and controls during treatment follow up

		Course		
		Before treatment	1st induction	2nd induction
AML	Non-response (NR)	27.3	21.13	14.86
	Complete response (CR)	22.15	21.6	16.2
Control		5.5	9.7	11.2
p-value		0.0001	0.01	0.4

DISCUSSION

The expression of the Wilms' tumor 1 gene has been recognized as a potential diagnostic and prognostic marker in AML. Regardless of treatment response, a significant proportion of AML patients exhibit its high expression levels. This makes it a useful tool for disease monitoring and assessing treatment outcomes. The expression level of WT1 in acute AML can be complex and varies among different patients. Normally expressed during embryonic development, WT1, a transcription factor that regulates gene expression, is either absent or expressed at very low levels in most adult tissues. However, in AML, WT1 is often found to be highly expressed [11]. The WT1 gene is involved in various cellular and biological mechanisms, such as cell apoptosis, differentiation, and proliferation. Theoretically, malignant conditions should exhibit higher WT1 gene expression, leading to reports of WT1 overexpression in a variety of tumors, including pediatric tumors, mesothelioma, ovarian cancer, and hematopoietic cancers like AML [10]. This study found no significant differences in age between AML patients and controls. The Fab classification revealed that AML-M3 (28.6%) was the most common subtype. Some studies have suggested a close relationship between the WT1 mutation and younger age, as well as the FAB M6 subtype, but this relationship is reversed for the (M0) subtype [14]. The cycle threshold (Ct) data of patients in this study revealed a significant variation in WT1 level between de novo AML patients and those treated after induction. The mean Ct value of WT1 reveals significant differences between NR and CR AML patients before treatment compared to after induction. Additionally, the fold change of WT 1 gene expression was significantly higher in AML patients compared to control cases. The most important outcome for treatment is complete remission (CR), which is defined as normocellular bone marrow with <5% blasts, a neutrophil count of 1×10^9/L, and a normal platelet count of 100×10^9/L [9,10]. According to some authors, using peripheral blood samples may give better results because the normal level of detectable WT1 expression is about 1 log lower in peripheral blood than in leukemia-free samples [15]. This is due to the presence of WT1-expressing red blood cells in the bone marrow. The current study reported that out of 29 new cases of AML, 16 (55.17%) were non-responders (NR) and

13 (44.83%) were in complete remission (CR). The high mean rank of WT1 mRNA expression was significantly higher in NR (27.3) and CR (22.15) compared to control (5.5). Most of the researchers who investigated the expression of the WT1 gene in leukemia noticed that it is overexpressed in a high percentage of patients, which is close to 80% or more [5,6,8,11,15-20]. The association between a higher rate of relapse in AML and the inability to lower high levels of WT1 transcript after therapy led to the adoption of WT1 as a biomarker of minimal residual disease (MRD) in AML [17]. In some cases, WT1 expression levels may remain high after treatment, even in patients who achieve complete response. The treatment may not completely eradicate residual leukemic cells or subclones. These remaining cells may continue to express WT1, leading to its persistence at high levels of post-treatment. There are a few reasons for this observation: AML is considered a heterogeneous disease; it differs significantly from patient to patient in terms of its molecular features and response to treatment. Numerous factors, including many genetic alterations and molecular subtypes of AML, can affect WT1 expression. Therefore, other genetic or molecular features of the disease may influence the treatment response, not just the high expression of WT1. Yoon *et al*. revealed that WT1 expression has been associated with a higher relapse rate and inferior disease-free survival in AML patients [21]. The frequency of WT1 gene mutations does not differ significantly between patients with early mortality, relapse, and persistent complete remission [9,10]. In solid cancer patients, WT1-positive expression showed a significant association with worse OS, DFS/RFS/PFS, and a borderline association with poor DSS. The analysis of 14 datasets revealed a significant association between WT1 positive expression and worse OS [20,22]. Making a correct diagnosis of WT can pose a significant challenge for pathologists. The presence of several subtypes of WT (morphological heterogeneity) [7] and leukemia, a collection of heterogeneous hematopoietic stem cell malignancies [10], are significant contributing factors. Also, the main mutations connected to WT1 are heterozygous events in exons 7 and 9. These usually cause nonsense-mediated mRNA decline, or if any protein is made, they play a possible role in leukemogenesis through a pro-proliferation effect [19,22]. For the AML prognostic stratifications, we still require a significant biomarker [10,23]. In addition to these reasons, there are several downstream effectors of WT1 genes. For example, the heparin-binding growth factor midkine (MK) gene is a prognostic biomarker for many cancers. The insulin-like growth factor I receptor [IGF-I-R] is another known downstream effector [9]. Additionally, accumulating evidence suggests that changes in iron metabolism are a crucial feature of leukemia [24]. Another factor that can complicate the diagnosis of WT for pathologists is the variation in isoform patterns, specifically the four major WT1 isoforms, across different samples [20]. Isoform profiles, which were independent of total WT1 expression, shared common features such as the overexpression of isoform D and EX5 (+) variants [25]. Overexpression of Wilms' Tumor 1 has been found in about 70–90% of people with acute myeloid leukemia, and 6–15% of those people had WT1 mutations. Many regard WT1 as a marker for tracking minimal residual disease in acute myeloid leukemia. Many researchers were interested in developing WT1-targeting therapy [26]. It is well known that acute leukemia patients with WT1 mutations express high levels of wild-type WT1 protein. Therefore, it is tempting to hypothesize that the expression of the wild-type allele solely influences leukemogenesis [19]. Commonly accepted is the role of WT1 in the pathogenesis of AML, but its molecular details remain essentially unknown. One probable explanation is that WT1 blocks the expression of interferon regulatory factor 8 [IRF8], an important tumor suppressor in myeloid leukemogenesis. An unfamiliar observation led to the approval of a negative WT1 expression in 11 out of 25 cases that achieved a complete remission. In one case, the decreased WT1 expression increased before a relapse occurred; at 10 weeks, they identified marginal positivity (0.149) and strong positivity (53.9). Six weeks before the relapse, these findings led to the conclusion that WT1 positivity varies over time [15]. Also, some patients who were thought to have attained CR on BM testing remained WT1-positive after induction. The expression of WT1 on CD34-positive blast cells may indicate that WT1 monitoring is more sensitive than morphological examination of the BM. Candoni *et al*. supported this idea when they discovered that 24% of their AML patients in remission remained WT1-positive [16].

Study limitations

The following restrictions apply to our study: The number of patients included in the study was not large enough for future randomized studies, and we were unable to conduct a test set analysis due to the small size of our study. Additionally, these inconsistencies may be explained by the different sample sizes and the limited ability to follow up with AML patients for a longer period.

Conclusion

Our findings strongly demonstrate that the WT1 tumor antigen might act as an early potential marker for acute myeloid leukemia prognosis. The overexpression of WT1 was linked to poor clinical outcomes, whereas a low level was associated with a good prognosis in AML patients. However, this level did not align with their response to induction chemotherapy, indicating the need for further testing.

Conflict of interests

No conflict of interest was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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