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ORIGINAL ARTICLE

A Proposed Kinetic Model for the Diagnostic and Prognostic Value of WT1 and p53 in Acute Myeloid Leukemia

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SUMMARY

Background: **Wilms tumor (WT1) and p53 proteins were identified in the pathogenesis of several malignancies, including hematological malignancies. As a result of their interaction and diverse context-specific functions, this study aimed to emphasize the diagnostic and prognostic impacts of WT1 and p53 expression in acute myeloid leukemia (AML).**

Methods: **Twelve bone marrow (BM) biopsies were obtained from AML patients who were diagnosed in accordance with the French-American-British diagnostic criteria. For comparative purposes, nine normal BM biopsies were included. The expression rate of WT1 and p53 were determined by an immunohistochemistry assay.**

Results: **A significantly higher ($p < 0.005$) and strongly correlated ($\rho = 0.855$, $p = 0.001$) expression rates of WT1 and p53 were observed in the BM of AML patients in comparison to control BM. Furthermore, relapsed AML patients had significantly higher expression of WT1, but not p53, when compared to newly diagnosed patients. In regard of patient's responsiveness to chemotherapy, no significant difference was reported between good and poor responders. However, the relative ratio of p53 to WT1 expression was evidently correlated to the responsiveness groups ($p < 0.05$), where the ratio was observed to be significantly higher among poor responders. Poor responders were characterized by a statistically significant and dominant p53 expression (p53/WT1 > 1.0) while both good responding patients and control subjects had a dominant WT1 expression (p53/WT1 < 1.0).**

Conclusions: **The enhanced expression levels of WT1 and p53 proteins in the BM of AML patients is supportive of their intermediate role in the pathogenesis of the disease. WT1 expression rate may encompass a negative prognostic value of the disease. Furthermore, the ratio of p53/WT expression may serve as a hallmark of the patient's responsiveness to chemotherapy, where a dominant WT1 expression may reveal good responsiveness to chemotherapy. Herein, we are proposing a kinetic model where the p53/WT1 ratio might be useful as a laboratory approach to evaluate the prognostic value of AML including the patient's responsiveness to chemotherapeutic regimen.**

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INTRODUCTION

Acute myeloid leukemia (AML) is a group of heterogeneous disorders that are characterized by uncontrolled proliferation and subsequent accumulation of malignant myeloid progenitors of affected cell lineages in the bone marrow and peripheral blood [1,2]. In accordance with the two-hit model of the pathogenesis of leukemia, studies have emphasized the presence of recurring mutations with pathologic and prognostic implications, particularly within the normal karyotype and/or intermediate-risk cytogenetic subsets [3]. These mutations include Fms-like tyrosine kinase 3 (FLT3) [4], nucleophosmin (NPM) [5], and Wilms tumor-1 (WT1) [6,7]. WT1 protein is a zinc finger DNA-binding protein that was first defined through the positional cloning and sequencing in patients with nephroblastoma (Wilms tumor) [8]. WT1 has been defined as a transcription regulator of several genes that are involved in cellular proliferation and differentiation [8,9]. However, the function of WT1, as a transcriptional activator or suppressor, is cellular and chromosomal context specific and requires the interaction of four WT1 isoforms with conserved structure and cellular levels [8,10].

The expression of WT1, in the context of several hematological malignancies and solid tumor, has been investigated. It has been shown that WT1 expression is markedly induced in most leukemia cells [11]. WT1 mRNA overexpression in leukemia is evident with oncogenic properties and the quantitative detection of WT1 transcripts could be helpful in monitoring and following up minimal residual disease [6,12]. It also acts as a useful predictor of leukemia-free survival rate following a treatment regimen [13].

WT1 has been shown to physically interact with p53, a tumor suppressor gene that has several biological functions such as the regulation of cell cycle arrest, apoptosis, and angiogenesis [14]. p53 mutations have been shown to provide a favorable environment for the propagation of other genetic mutations and subsequent development of tumors [15]. p53 has been reported to interact with the first two zinc-finger domains of WT1 in a manner that modulates their transcription regulatory functions of respective target genes [16]. Furthermore, the expression of WT1, along with the expression of wild-type p53, is not only associated with an increased stability of p53 but also inhibited pro-apoptotic properties that ultimately result in cellular tolerance to p53-mediated apoptosis [17].

This study is the first that emphasizes the clinical impacts of WT1 and p53 expression on the diagnosis and prognosis of AML. The expression rates of WT1 and p53 were evaluated. Furthermore, their correlations to the clinical status of patients as well as to the patient's responsiveness to chemotherapy were investigated.

MATERIALS AND METHODS

Patients and sample collection

A total of twenty-one (n = 21) bone marrow (BM) trephine biopsies were obtained of which twelve were from hospitalized de novo AML patients who were previously diagnosed with the disease, in accordance with the French-American-British (FAB) diagnostic criteria. BM biopsies were originally taken from patients to confirm AML diagnosis.

AML patients were under an induction chemotherapeutic regimen with intravenous administration of antineoplastic agents, including Cytarabine (ara-C), Mitoxantrone, and Etoposide. The patient's responsiveness to chemotherapy was evaluated by the supervising physician based on the peripheral blood laboratory findings that primarily included the achievement of blasts percentage of less than 5% of all nucleated cells (ANC) as well as circulatory granulocytes count of less than 1,000 cells/μL and platelets counts of less than 100,000 cells/μL.

For comparative purposes, nine normal non-pathological bone marrow biopsies were kindly provided by the histopathology department at King Abdullah University Hospital, Jordan. These biopsies were taken from patients with unexplained anemia with no BM abnormalities, lymphoma with no BM involvement, or solid non-hematological malignancies with no BM metastasis. Upon histopathological examination, BM biopsies were confirmed to be normal with no hematopathological abnormalities.

Patients and/or their guardians were informed about the study objectives and that any obtained data would be kept confidential and be used for scientific purposes. Following their agreement to participate, they were asked to fill a consent form in that regard. This study was pre-approved by the institutional review board (IRB) at Jordan University of Science and Technology (JUST).

WT1 and p53 expression

BM sections were deparaffinized, rehydrated, and then subjected to heat-induced antigen retrieving. Sections were washed with phosphate buffer saline (PBS) for five minutes prior to their incubation with 3% hydrogen peroxide (H_2O_2) for five minutes to block endogenous peroxidase activity. Then, sections were washed again with PBS after which they were incubated for one hour at room temperature with p53 or WT1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Prior to their usage, the antibodies were diluted with PBS in 1/100 dilutions as instructed by the manufacturer. Subsequently, sections were washed in PBS and incubated with biotinylated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 minutes at room temperature after which they were washed with PBS. The sections were incubated with streptavidin horse radish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 minutes at room temperature and then washed with PBS. Next, 3,3'-Diaminobenzidine (DAB)

Table 1. Comparison and correlation analysis of WT1 and p53 expression rate.

	Percentage (%) of expression rate		p-value
	AML	Control	
WT1 (Mean ± SEM)	8.30 ± 1.94%	0.71 ± 0.2%	(p < 0.005)
p53 (Mean ± SEM)	10.07 ± 3.38%	0.19 ± 0.08%	(p < 0.05)
Spearman's Coefficient (ρ)	0.855		0.001

Comparison and correlation of WT1 and p53 expression rate in the bone marrow of acute myeloid leukemia (AML) patients and the control group. Results are presented as the mean expression ± standard error mean (SEM).

substrate was applied for two minutes after which sections were washed with tap water to stop the reaction. Finally, sections were counterstained with hematoxylin and examined under 400 x magnification power using a light compound microscope.

Based on histopathological evaluation, selected areas of interest of hematopoietic tissue in all samples were photographed using a digital camera (Kodak 10-megapixels resolution, Japan). Adobe Photoshop CS5 software was used to semi-quantify WT1 and p53 expression in the total hematopoietic cell population. As described previously, the percentage of expression was determined by the average number of pixels with positive staining where the omission from the primary antibody in the counterstained area served as a negative control [18,19].

Statistical analysis

Data were analyzed using the statistical package for the social sciences (SPSS). Independent Student's *t*-test and univariate general linear model were used for comparative purposes and the results were considered significant when p-value was less than 0.05. A correlation analysis was conducted using Spearman's *rho* correlation. Power analysis and effect size analysis were performed to investigate the validity and reliability of obtained results. Graphs were prepared using GraphPad Prism 6 software.

RESULTS

Study subjects were acute myeloid leukemia patients who were previously diagnosed with the disease, in accordance with the FAB diagnostic criteria. The mean age of patients was 32.3 ± 23.6 years old with a female to male ratio of 1:3. Eight patients (66.7%) were newly diagnosed with the disease of whom four (33.3%) were relapsed patients. Furthermore, four patients had poor responsiveness to chemotherapy of whom two were relapsed patients and two were newly diagnosed patients. As shown in Table 1, AML patients had shown significantly enhanced expression rates of both WT1 and p53, as compared to their expression among the control group. The mean WT1 expression rate among AML patients was 8.30 ± 1.94% compared to 0.71 ± 0.2% the control group ($p < 0.005$). Similarly, the mean p53 expression rate among AML patients was 10.07 ± 3.38% compared to 0.19 ± 0.08% expression rate among the control group ($p < 0.05$). Correlation analysis has revealed a monotonic and strong linear relationship between WT1 and p53 expression rates among study subjects, as represented by Spearman's rho and Pearson's coefficients of 0.855 ($p < 0.001$) and 0.795 ($p < 0.001$), respectively.

Based on their clinical status, AML subjects were sub-categorized into either newly diagnosed or relapsed AML patients. Spearman's ranked correlation analysis has revealed that WT1 and p53 expression rates were both having equally strong and statistically significant monotonic relationships with the clinical status of study subjects [ρ (rho) = 0.837, p = 0.001]. As illustrated in Figure 1, relapsed AML patients had significantly higher WT1 expression rates with a mean expression of 15.43 ± 1.67% as compared to 4.23 ± 1.22% among newly diagnosed patients ($p = 0.001$) and 0.71 ± 0.20 ($p = 0.001$) among the control group. Furthermore, WT1 expression rate among newly diagnosed subjects was significantly higher than the expression rate among control subjects ($p = 0.026$). Regarding p53, the expression rate of 22.18 ± 6.23% among relapsed patients was significantly higher than the expression rate of 3.15 ± 1.10% ($p = 0.001$) and 0.19 ± 0.08 ($p = 0.001$) among newly diagnosed subjects and control subjects, respectively. However, there was no significant difference in the expression rate between newly diagnosed AML patients and the control group ($p = 0.58$).

Considering their responsiveness to chemotherapeutic regimen, AML subjects were sub-categorized into either good or poor responders. There was no significant difference in the mean expression rates of both WT1 and p53 between the two groups. As shown in Figure 2, good responder AML patients had mean WT1 and p53 expression rates of 7.6 ± 2.63% and 4.60 ± 4.6% in comparison to 9.48 ± 3.10% ($p = 0.96$) and 19.62 ± 7.61% ($p = 0.36$) among patients with poor responsiveness, respectively ($p > 0.05$).

We noticed that p53 expression among patients with poor responsiveness to chemotherapy was dominant over WT1 expression. This is compared to a dominant WT1 expression among AML patients with good re-

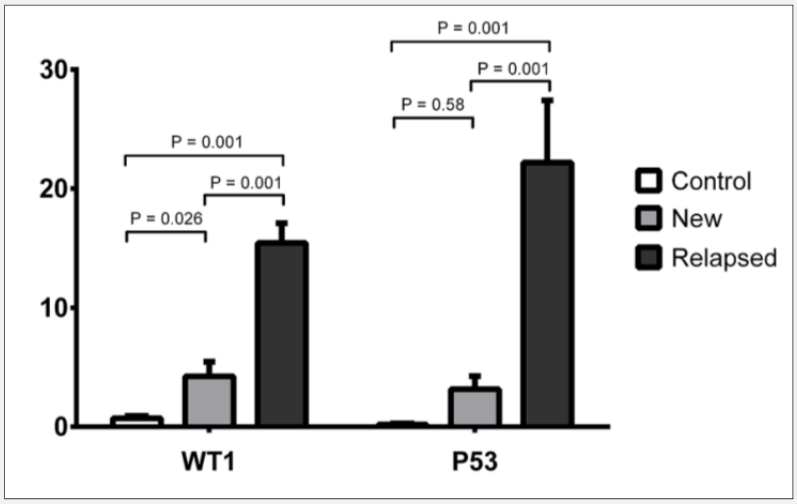

Figure 1. The expression rate of WT1 and p53 among newly diagnosed (New) and relapsed AML patients.

Expression rate is presented in percentage (%) as mean ± standard error mean (SEM).

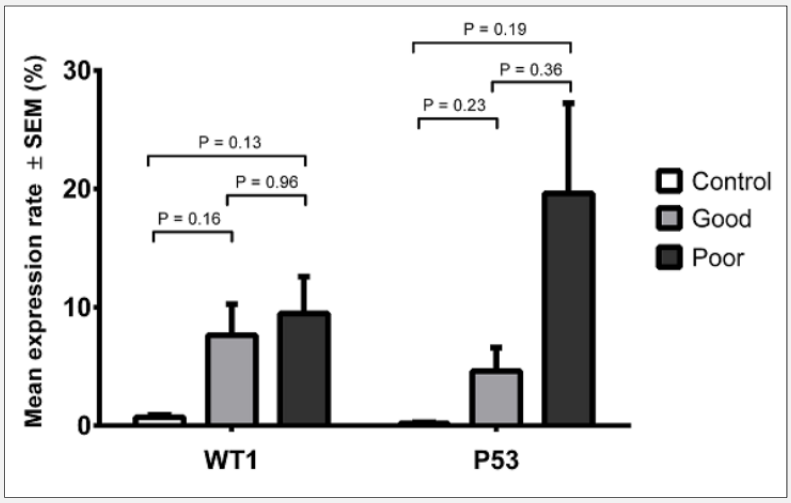

Figure 2. The expression rate of WT1 and p53 among AML patients with good and poor responsiveness to chemotherapy.

Percentage (%) of expression rate is presented as mean ± standard error mean (SEM).

sponsiveness as well as among control subjects. Based on this observation, the relative ratio of p53 to WT1 expression (p53/WT1) was calculated for study subjects. As shown in Figure 3, Univariate Generalized Linear Model analysis has revealed that patients with poor responsiveness to chemotherapy had a mean p53/WT1 ratio of 1.98 ± 0.27%, which is significantly higher than the mean ratio among patients with good responsiveness, who showed a mean relative ratio of 0.67 ± 0.13%, as well as the mean ratio among the control

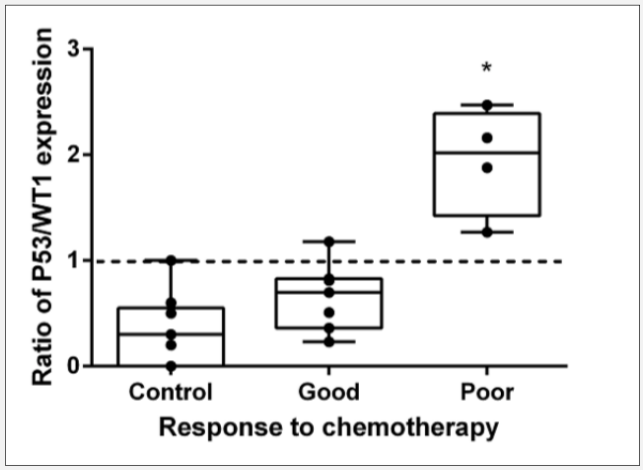

Figure 3. The relative ratio of p53/WT1 expression rate among AML patients with good and poor responsiveness to chemotherapy.

Boxes represent the 25th to the 75th percentile and the middle line is median while whiskers represent the minimum and maximum values per group. Dots are the individual value for each subject. Horizontal dotted line represents a reference value where p53/WT1 equals one. * - p-value < 0.05.

group, who showed a mean relative ratio of 0.32 ± 0.13% ($p < 0.001$). There was no significant difference between good responder and control subjects ($p > 0.05$). In support of this, correlation analysis has revealed that the relative p53/WT1 ratio was significantly correlated to the patient's responsiveness status [ρ (rho) = 0.744, $p = 0.001$].

To evaluate the statistical reliability and the clinical significance of our obtained data, statistical effect size as well as the observed power analysis were conducted. The effect size analysis showed a strong effect size with a partial Eta squared value of 0.759, which reveals a strong clinical significance of our analysis. Furthermore, the power analysis revealed a power value that equals to 1.00 (> 0.80), indicating a statistically accurate and reasonable analysis of our obtained results.

DISCUSSION

Considering its controversial role as a transcriptional activator or repressor, the diversity of WT1 expression has been shown to be cellular and context specific [10]. WT1 expression has been shown to be prominent in the majority of hematological malignancies, including AML [20-22]. Several studies have proposed that WT1 gene expression is a useful marker for the detection and monitoring of minimal residual disease (MRD) in acute leukemia [23,24], while others have concluded that it has no impact on the diagnosis of AML and, accordingly, has no prognostic significance in patients with complete remission [25]. Ellisen et al. reported variable stage-specific WT1 expression levels in hematopoietic cells with diverse effects [26]. Contrary to molecular WT1 gene expression, the impact of WT1 protein level in AML is poorly investigated. This current study aimed to resolve the dilemma of WT1 protein expression in AML as well as to evaluate its diagnostic and prognostic significance in accordance with p53 expression.

The markedly increased levels of WT1 in the BM of AML patients, as compared to the lower levels among control subjects, are suggestive of the intermediate role of WT1 in the pathogenesis of AML. In this regard, several studies have reported WT1 overexpression in the context of the majority of AML subtypes and is mutant in a proportion of investigated cases [27,28].

Similarly, the higher p53 expression rate in the BM of AML patients, as compared to its expression rate in the BM of control group, is additional evidence of the implications of p53 on the pathogenesis of the disease. In a bioinformatics study, where semi-quantitative immunohistochemistry scoring (SQ scoring) was conducted, significantly increased levels of p53 have been reported in leukemic BM as a result of the higher fraction of p53-expressing leukemic cells among the total cell population in the BM [29]. Earlier studies have postulated that p53 overexpression may contribute to the survival of malignant cells and, subsequently, the prognosis of malignant diseases [30]. On the contrary, others have demonstrated that the loss of p53 expression is incurporated in the pathogenicity of many hematological malignancies including AML [31,32]. It is worthy to mention

here that the impairment of the p53 pathway is primarily a result of either quantitative impairment or functional inactivation of the p53 protein [29].

There was a significantly strong correlation between WT1 level and the clinical status of AML patients; WT1 expression rates among relapsed patients were significantly higher in comparison to newly diagnosed patients. This may represent an additional diagnostic and prognostic value of WT1, especially when considering the wide diversity in p53 expression. Paschka et al. proposed that WT1 mutations are associated with extremely poor prognostic impacts in intensively treated and young, cytogenetically normal AML patients [33]. Gary et al. demonstrated that enhanced levels of WT1 in the peripheral blood at the time of diagnosis of AML as well as the presence of detectable WT1 levels post-consolidation are associated with poorer leukemia free survival (LFS), independent of age or cytogenetic risk-group [13]. Accordingly, we demonstrate that WT1 and p53 represent valuable diagnostic and prognostic markers in AML where WT1 may augment the significance of p53 expression, particularly when considering the observed diversity in p53 expression rates among AML patients.

On the other hand, there was an evident lack of correlation of both WT1 and p53 with the responsiveness to chemotherapeutic regimens. In that regard, there were insignificant differences in WT1 and p53 expression rates between AML patients with good responsiveness and those with poor responsiveness as well as with control subjects. Accordingly, it can be concluded that WT1 and p53 do not contribute in evaluating patient's responsiveness to chemotherapy. However, based on their defined physical interaction and subsequent stabilization effects, we have noticed that the relative ratio of p53 to WT1 is strongly correlated to the patient's responsiveness status. Additionally, we observed that all patients with poor responsiveness were characterized by dominant p53 expression that overwhelmed WT1 expression compared to patients with good responsiveness and control subjects who were characterized by dominant WT1 expression.

The significantly elevated levels of WT1 and p53 among AML patients, as compared to control subjects, may suggest their intermediate interaction in the pathogenicity of AML. Statistically, we reported a significantly monotonic and linear correlation between WT1 and p53 expressions among study subjects, which further support their joint implication in the pathogenicity of the disease. In 1993, Maheswaran et al. demonstrated a complex formation of WT1 and p53 proteins in baby rat kidney (BRK) cell line transfected with adenovirus E1A [16]. Later in 1995, they demonstrated that this physical interaction stabilizes p53 expression leading to an induced resistance to p53 mediated apoptosis in affected cells, even in the absence of p53 mutations [17].

CONCLUSION

In agreement with earlier studies, our study demonstrates that WT1 and p53 expression in the context of AML is a hallmark and is suggestive of their intermediate incorporation in the pathogenesis, the clinical presentation, as well as the prognosis of the disease. The enhanced expression rates of WT1 and p53 are prognostic markers of the disease with negative impacts. Furthermore, we are proposing a laboratory kinetic model of WT1 and p53 expression that may predict patient's responsiveness to chemotherapy, where a dominant p53 expression may suggest a poor responsiveness to chemotherapy. The clinical significance of our proposed model is that it may provide laboratory monitoring of patient's responsiveness to specific chemotherapeutic protocol. In addition, it may be a useful prognostic marker to follow up AML patients for possible relapse upon achieving complete remission.

We understand that the small sample size is a limitation of our study. Therefore, effect size and power analysis were conducted. Results have revealed a strong clinical significance as well as a statistical accuracy of our obtained data analysis. However, further investigation on a larger sample size is required for an auxiliary authentication of our proposed kinetic model of WT1 and p53 interaction in the pathogenesis and progression of AML.

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Declaration of Interest:
All authors declare that there is no potential conflict of interest.

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