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High *WT1* Expression Associates with Unfavorable Prognosis in Acute Myeloid Leukemia

Shuqi Zhao[1], Hanzhang Pan[1], Qi Guo[2], Wanzhuo Xie[1], Jie Jin[1]* and Jinghan Wang[1]*

[1]Department of Hematology, The First Affiliated Hospital, Zhejiang University College of Medicine, China

[2]Department of Nephrology, The First Affiliated Hospital, Zhejiang University, China

Abstract

Background: Recently, *WT1*-specific Chimeric Antigen Receptor (CAR) - T cell has been identified and evaluated in clinical trials of Acute Myeloid Leukemia (AML). However, the clinical impact of Wilms' Tumor 1 (*WT1*) expression is still unclear. Therefore, we enrolled two cohorts of AML patients to evaluate the prognostic significance.

Methods: We measured *WT1* transcript by real-time quantitative PCR in 173 Cytogenetically Normal AML (CN-AML). To assess the prognostic value of *WT1* transcript, we classified patients into high and low groups based on the higher quartile of *WT1* expression. The independent prognostic value of *WT1* expression was investigated in the context of the well-established predictors including white blood cell counts, age, European Leukemia Net genotype (*NPM1* mutations and lacking *FLT3*-ITD or *CEBPA* double allele mutations), mutated genes of *IDH1*, *IDH2* and *DNMT3A*. In addition, the published GSE12417 data was used as an external validation.

Results: High *WT1* expressers have the higher levels of white blood cell counts, and higher percentage of *FLT3*-ITD and *NPM1* mutations. Moreover, high *WT1* expressions are positively correlated with the adverse overall survival in patients with CN-AML. This result was also validated in the independent cohort of 163 CN-AML patients from the public dataset GSE12417.

Conclusion: We found high *WT1* expression is associated with unfavorable overall survival in CN-AML patients.

Keywords: *WT1*; **Acute myeloid leukemia; Prognosis; mRNA**

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Correspondence:

Jinghan Wang, Department of Hematology, The First Affiliated Hospital, Zhejiang University College of Medicine, No. 79 Qingchun Road, Hangzhou 310003, Zhejiang, China, E-mail: 1513084@zju.edu.cn

Jie Jin, Department of Hematology, The First Affiliated Hospital, Zhejiang University College of Medicine, No.79 Qingchun Road, Hangzhou310003 Zhejiang, China, E-mail: NIL

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Introduction

Acute Myeloid Leukemia (AML) is a group of heterogeneous hematologic malignancy with the poor clinical outcome [1]. One of the underlying reasons is the biological and clinical heterogeneity. Hence, the lacks of reliable biomarker will hinder the improvement for prognostic prediction in clinical practice. Although there has been significant progression in the discovery of novel predictors, a useful biomarker is still required for AML prognostic stratifications. To date, specific chromosomal aberrations like t(8;21) and inv(16) abnormalities and genes mutations including *FLT3*-ITD, *NPM1* and *CEBPA* double allele mutations have been proved to be of prognostic significance [2]. Notably, the contribution of gene expression is still important in classification, prognosis and understanding of pathology, particularly in Cytogenetically Normal AML (CN-AML) [3].

The Wilms' Tumor 1 (*WT1*), which is located in 11p13 of the human chromosome, encodes a transcription factor. It is known that *WT1* plays a critical role in the normal development of the urogenital system. It has been reported that urinary exosomal *WT1* is a useful biomarker to improve risk stratification in patients with diabetic nephropathy [4]. Of interesting, this gene involved in cancer progression through regulation of vascular endothelial growth factor [5], implying the oncogene function. Additionally, *WT1* protein is abundantly expressed in numerous hematological malignancies. Several studies had demonstrated *WT1* expression associates with leukemia burden, predicts the disease relapse and promotes the progression and development of leukemia [6,7]. Therefore, *WT1* expression has been designed as a novel drug target and proved to be effective in clinical trials [8,9]. With respect to the development of targeted treatment, Chimeric Antigen Receptor T cells (CART) have been demonstrated as a successful treatment in B-lineage acute lymphoblastic leukemia by targeting CD19 [10]. Similarly, *WT1* has been used as a myeloid equivalent to CD19 [11]. Currently, a *WT1*-specific Chimeric Antigen Receptor (CAR) - T cell has been identified and evaluated in clinical trials [8,9]. Although *WT1* expression as a target has

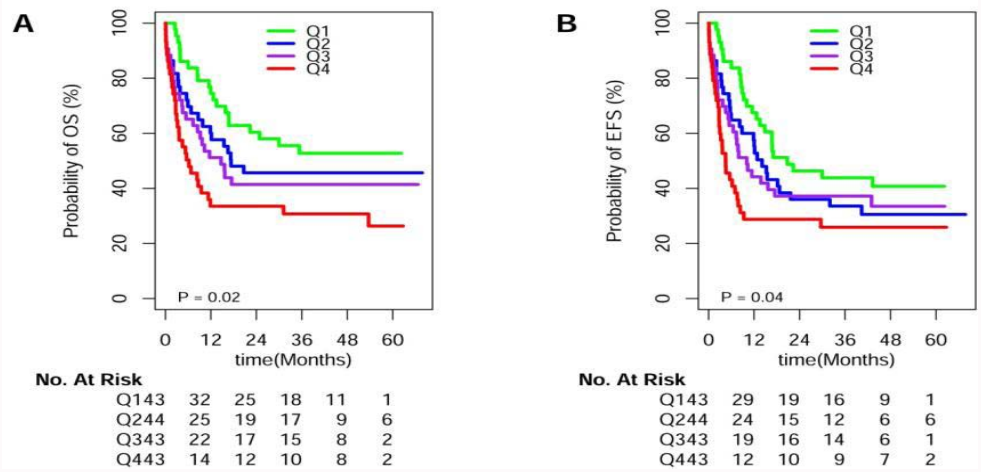

Figure S1: Survival analyses of patients with CN-AML. Kaplan-Meier estimates of OS (A) and EFS (B) by the four quartile subgroups for CN-AML patients, respectively.

been reported in previous studies, there is a paucity of knowledge regarding its prognostic significance in CN-AML patients treated in our hospital. Therefore, we analyzed the prognostic value of *WT1* expression.

Materials and Methods

Patients

In our cohort, we enrolled 173 patients with CN-AML in the first affiliated hospital of Zhejiang university college of medicine during 2013 and 2016. Bone Marrow (BM) samples were obtained at the time of diagnosis. Patients received anthracycline and cytarabine induction chemotherapy, and consolidations chemotherapy based on the treating physician's choice in an individualized manner. Details of the treatment protocols were reported in previous studies [12,13]. We excluded patients who had severe hepatic disease, severe renal insufficiency, previous malignancy, transformed from myelodysplastic syndromes, or who were older than 65 years. All of the subjects were well-informed about the study and provided written informed consent to participate in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. The study was approved by the Institutional Review Boards of the First Affiliated Hospital of Zhejiang University.

Cytogenetic and gene mutation analysis

Cytogenetic and molecular studies were performed centrally at ZIH molecular laboratories. Mononuclear cells were isolated from the BM samples by Ficoll-Hypaque density-gradient centrifugation and DNA and RNA were extracted as described previously [14]. Mutation analyses of *NPM1*, *FLT3*-ITD, *CEBPA, IDH1/IDH2* and *DNMT3A* were carried out as described previously [14].

Quantitative real-time PCR

Bone Marrow (BM) mononuclear cells were purified by Ficoll density gradient centrifugation and total RNA was isolated using Trizol reagents (Invitrogen, Shanghai, China) according to the manufacture's protocol. The first-strand complementary DNA synthesis was performed using the MMLV systems (Life Technologies). The assay was carried out on an ABI 7500 Real Time PCR machine (BIO-RAD, US). The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 30 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 10 sec. Relative quantification was calculated using $2^{-\Delta\Delta CT}$ method and ABI was used for normalization. The following primers were used for quantitative PCR: *WT1* 5′-GATAACCACACAACGCCCATC-3′ (sense) and 5′-CACACGTCGCACATCCTGAAT-3′ (antisense); ABI (control), 5′- TGGAGATAACACTCTAAGCATAACTAAAGGT-3′ (sense) and 5′-GATGTAGTTGCTTGGGACCCA-3′ (antisense).

Statistical analysis

The main objective is to investigate the prognostic impact of *WT1* expression on Overall Survival (OS) of AML patients. OS was measured as the date from disease diagnosis to death from any cause, or censoring for patients alive at their last follow-up. To characterize patients with high expression, we subdivided our cohort into four quartiles (Q1: <25%; Q2: 25%~50%; Q3: 50%~75% and Q4: >75%) based on *WT1* expression values to determine the group with the best overall survival (Figure S1A). As a result, patients in Q1, Q2 and Q3 respectively had favorable survival compared with those in the fourth quartile Q4, and no difference was found among Q1, Q2 and Q3. The similar result was seen in event-free survival (Figure S1B). Thus, 43 (25%) cases in Q4 were refined into the high expressed group while those in Q1-Q3 as low group (Figure 1A, 1B). Patient characteristics were summarized using descriptive statistics, which included frequency counts, median and range. The nonparametric test and Chi-square test was used to estimate the characteristics of patients with distinct *WT1* expression. In order to evaluate the prognostic value of *WT1* expression, Kaplan-Meier method was used in the univariate analysis and Cox proportional hazard regression model in the multivariate analysis. GSE12417 raw data were downloaded from the GEO database (www.ncbi.nlm.nih.gov/geo) [15]. *WT1* mRNA expression was calculated by transcript levels of the probe 206067_s_at. All statistical analyses were conducted with R statistic package, version 3.3.1 (www.r-project.org). P<0.05 demonstrated statistical difference.

Results

Characteristics of patients with high *WT1* expression

The median of *WT1* expression in this study is 0.12 with the range from 0.001 to 0.88. Among 173 patients, 43 (25%) cases were classified as high and 130 (75%) low *WT1* expression. Clinical features of patients with high *WT1* expression are summarized in Table 1.

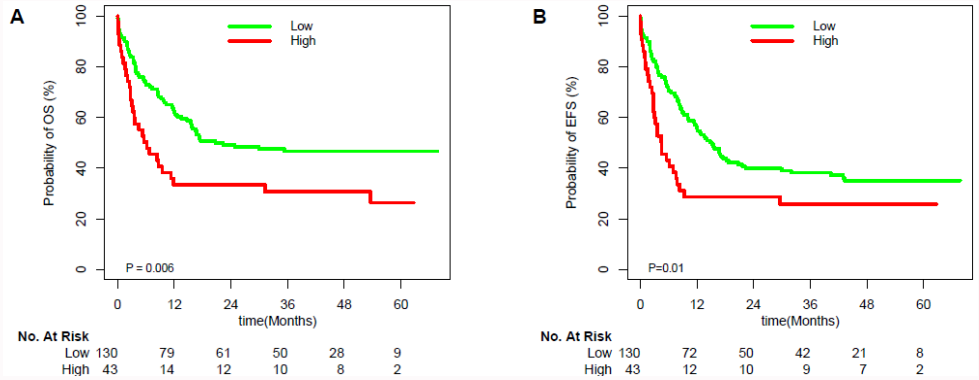

Figure 1: Survival analyses of patients with CN-AML. Kaplan-Meier estimates of OS (A) and EFS (B) by high and low *WT1* expression groups for CN-AML patients, respectively.

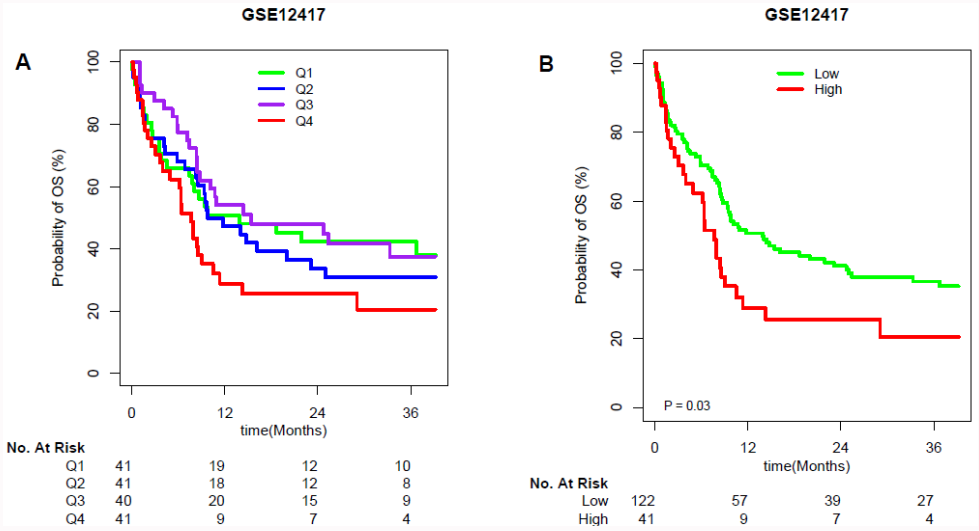

Figure 2: Kaplan–Meier survival analysis by the four quartile subgroups (A) and high and low *WT1* expression (B) For CN-AML patients, respectively. These patients were used as a validation cohort treated according to the German AMLCG-1999 protocol.

High expressers were associated with a significantly higher levels of White Blood Cell (WBC) counts, and higher percentage of *FLT3*-ITD (51.2% *vs.* 19.2%, P<0.001) and *NPM1* (39.5% *vs.* 23.1%, P=0.047) mutations, compared with patients with low expression. There was no statistically significant correlation between *WT1* expression and other variables including age, sex, percentage of blasts, Hemoglobin (HB), platelet counts, FAB subtypes, NEL favorable risk group (*NPM1* mutations and lacking *FLT3*-ITD or *CEBPA* double allele mutations) and mutations of genes like *IDH1, IDH2, DNMT3A* and *CEBPA* double allele (Table 1).

Association of *WT1* expression with clinical outcome

With a median follow-up for CN-AML patients of 496 days, patients with high expression (n=41) had more adverse Overall Survival (OS) and Event Free Survival (EFS) compared to low expressers (Figure S1 and Figure 1). In order to exclude the potential confounders, we conducted the univariate analysis and multivariate Cox proportional hazard regression analysis. As a result, there were significant associations between survivals and these factors including the well-established predictors like age, WBC and ENL favorable risk group. Even if we taken the potential predictors as confounders, *WT1* expression was still as an independent prognostic factor in multivariate analysis after adjusting for age, WBC, ENL favorable risk groups, and genes of *IDH1*, *IDH2,* and *DNMT3A* mutations (for OS HR (95% CI), 1.708 (1.057, 2.761); P=0.029 (Table 2); for EFS HR (95% CI), 1.627 (1.018, 2.6); P=0.042, (Table S1)). With respect to the induction remission rate, high expressers had lower complete remission rate compared with low expressers in the univariate analysis (67.6% *vs.* 80%, P=0.16, Table 1).

Validation of *WT1* expression in an independent cohort of CN-AML

In this study, 163 patients treated in the German AMLCG 1999 trial [15] were used to validate the prognostic value of *WT1* expression. Transcript values of the probe 206067_s_at were taken as the *WT1* expression values. Consistently, we classified these patients into four subgroups based on the quartiles (Q1: <25%; Q2: 25%~50%; Q3: 50%~75% and Q4: >75%) of *WT1* expression values (Figure 2A). Notably, patients in Q1, Q2 and Q3 had the similarly favorable survival, while those in the fourth quartile obtained the unfavorable OS. Thus, we refined 163 patients into the high (41 (25%)) and low (122 (75%)) expressed groups (Figure 2B). Kaplan-Meier analysis for OS demonstrated that high *WT1* expression was still a significant unfavorable prognostic predictor compared with low expression (P=0.01).

Table S1: *WT1* expression associated with EFS in CN-AML patients.

Variables	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	P value	HR (95% CI)
WT1 High *vs.* low	0.013	1.69 (1.115, 2.561)	0.042	1.627 (1.018, 2.6)
Age	<0.001	1.04 (1.025, 1.055)	<0.001	1.046 (1.031, 1.063)
WBC[1]	0.043	1.49 (1.013, 2.193)	0.141	1.403 (0.894, 2.202)
Blast%	0.469	1.003 (0.995, 1.011)	0.712	0.998 (0.989, 1.008)
ENL favorable subtype	0.051	0.638 (0.406, 1.002)	0.108	0.675 (0.418, 1.09)
IDH1	0.879	1.042 (0.612, 1.774)	0.944	1.02 (0.578, 1.803)
IDH2	0.702	1.106 (0.659, 1.857)	0.181	0.689 (0.399, 1.19)
DNMT3A	0.296	1.309 (0.79, 2.17)	0.632	1.136 (0.675, 1.912)

WT1 Expression: high *vs.* low

[1]WBC: White Blood Cells; HR: Hazard Ratio; CI: Confidence Intervals; ENL: European Leukemia Net genotype (*NPM1* mutations and lacking *FLT3*-ITD or *CEBPA* double allele mutations)

Table 1: Clinical characteristics of CN-AML.

Variable	Low expression	High expression	P value
Number	130	43	
WT1 expression, (median [IQR])	0.08 [0.03,0.15]	0.42 [0.36,0.52]	<0.001
Age, median (range), years	53.00 [42.75,63.00]	56.50 [38.25,66.25]	0.779
Sex, male, n (%)	72 (56.2)	24 (57.1)	1
BM blast, median (IQR), %[1]	59.00 [38.88,80.00]	74.00 [49.62,83.25]	0.089
WBC, median (IQR), × 10^9/L[2]	8.05 [2.25,38.20]	32.50 [4.95,106.25]	0.004
HB, median (IQR), g/L[3]	85.00 [70.00,103.00]	89.00 [72.25,104.50]	0.316
PLT, median (IQR), × 10^9/L[4]	57.00 [28.75,97.25]	38.00 [20.75,72.00]	0.1
ENL favorable, n (%)	36 (27.9)	7 (16.3)	0.186
FAB classification, n (%)[5]			0.123
M0	11 (8.5)	1 (2.3)	
M1	7 (5.4)	5 (11.6)	
M2	55 (42.3)	23 (53.5)	
M4	1 (0.8)	1 (2.3)	
M5	50 (38.5)	10 (23.3)	
AML	6 (4.6)	3 (7.0)	
Gene mutations, n(%)			
FLT3-ITD	25 (19.2)	22 (51.2)	<0.001
NPM1	30 (23.1)	17 (39.5)	0.047
CEBPA[DM6]	6 (4.7)	2 (4.7)	0.915
DNMT3A	20 (15.5)	4 (9.5)	0.476
IDH1	18 (14.5)	6 (14.6)	1
IDH2	19 (15.1)	5 (12.2)	0.841
Complete remission	96 (80.0)	23 (67.6)	0.16

[1]BM: Bone Marrow; [2]WBC: White Blood Cell; [3]HB: Hemoglobin; [4]PLT: Platelet Counts; [5]FAB: French-American-British classification systems; [6]DM: Double-allele; IQR: Interquartile Range; ENL: European Leukemia Net genotype (NPM1 mutations and lacking *FLT3*-ITD or *CEBPA* double allele mutations)

Discussion

Wilms' Tumor 1 (*WT1*) gene encodes a zinc-finger transcriptional factor that has a strong impact on the growth and differentiation of normal and malignant cells. In fact, *WT1* has been proved to have an oncogenic role in various types of tumors including pancreatic ductal [16], ovarian cancers [17], acute leukemia [18]. Increased expression of this gene is observed in leukemia. To date, the prognostic effect of *WT1* expression of AML has been controversial. Some studies demonstrated that *WT1* acts as a tumor suppressor gene while others as an oncogene. For example, one study reported that it has poor prognostic implication in AML [19]. It is also reported that *WT1* gene had an ant-oncogenic function and possibly associated with an enhanced response to chemotherapy in AML with intermediate karyotype [20]. Notably, a meta-analysis including 1,497 AML patients demonstrated high *WT1* expression conferred a trend toward shorter OS in total AML, and had negative effect on overall survival in the adult AML and non-M3 AML patients [21]. These inconsistent results may be attributed to the different selected cases with AML. Notably, there is still a lack of a systematical analysis about the

Table 2: *WT1* expression associated with overall survival in CN-AML patients.

Variables	Univariate analysis		Multivariate analysis	
	P value	HR (95% CI)	P value	HR (95% CI)
WT1 expression	0.006	1.833 (1.19, 2.823)	0.029	1.708 (1.057, 2.761)
Age	<0.001	1.04 (1.025, 1.056)	<0.001	1.045 (1.029, 1.062)
WBC[1]	0.028	1.578 (1.05, 2.372)	0.242	1.324 (0.827, 2.12)
Blast %	0.312	1.005 (0.996, 1.014)	0.836	0.999 (0.989, 1.009)
ENL favorable subtype	0.025	0.523 (0.297, 0.922)	0.044	0.584 (0.346, 0.985)
IDH1	0.602	1.158 (0.666, 2.013)	0.763	1.095 (0.606, 1.981)
IDH2	0.953	1.017 (0.576, 1.796)	0.115	0.619 (0.341, 1.124)
DNMT3A	0.141	1.484 (0.877, 2.509)	0.415	1.253 (0.728, 2.156)

WT1 expression: high *vs.* low

[1]WBC: White Blood Cells; HR: Hazard Ratio; CI: Confidence Intervals; ENL: European Leukemia Net genotype (*NPM1* mutations and lacking *FLT3*-ITD or *CEBPA* double allele mutations)

prognostic value of *WT1* expression in CN-AML. In this study, the *WT1* mRNA level measurement at the disease diagnosis is associated with poor survival, implying *WT1* can be used as a predictor to guide each patient care.

Recently, *WT1* expression has been used to monitor leukemia burden after bone marrow transplantation [11]. This study is in line with our founding that high *WT1* expression is positively correlated with WBC levels. Interestingly, the epigenetic modulators like *DNMT3A* and *IDH1/IDH2* mutations occur as early founder events in preleukemic progenitor cells before leukemogenic events [22]. Hematopoietic stem cell and progenitor cells accumulated these mutations still retain the ability to differentiate into the full spectrum of mature myeloid and lymphoid cells. In addition, acquisition of additional cooperating mutations like *FLT3*-ITD and *NPM1* converts a pre-Leukemic Stem Cell (pre-LSC) into a fully transformed LSC. Here, we also found *WT1* expression positively correlated with genes of *FLT3*-ITD, *NPM1* mutations, instead of *IDH1/IDH2* and *DNMT3A* mutations. These results implied that *WT1* aberrant expression may play an oncogene role in leukemia progression. Thus, *WT1* expression has the clinical and biological significance in CN-AML.

There are still some limitations in this study. Firstly, we only examine genes of *FLT3*-ITD, *NPM1, CEBPA, IDH1* and *IDH2* mutations, thus we could not exclude other mutated genes, particularly *WT1* mutation which will confound the prognostic value in AML patients [23]. Secondly, further large-scale and prospective research is still required to clarify the exact roles of *WT1* expression in clinical outcome. Finally, we did not perform the functional study of the silencing *WT1* expression on proliferation in leukemia cell lines *in vitro* and *in vivo* models are also required to investigate the oncogenesis of *WT1*. Therefore, caution in application of our findings is still warranted.

In conclusion, we found high *WT1* expression is associated with poor overall survival in CN-AML patients.

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