

# Juvenile myelo-monocytic leukemia (JMML): No effect of granulocyte monocyte-colony stimulating factor (GM-CSF) on Wilms Tumor gene (*WT1*) by nested Polymerase Chain Reaction (nPCR) and flow cytometry

Sana Khan<sup>1</sup>, Marie Olszewski<sup>1</sup>, Wei Huang<sup>1</sup>, Morris Kletzel<sup>1,2\*</sup>

<sup>1</sup>Stem Cell Transplant Chimerism Laboratory, Depts. of Hematology-Oncology Transplant; Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, USA

<sup>2</sup>Northwestern University Feinberg School of Medicine, Chicago, USA

Email: [mkletzel16@northwestern.edu](mailto:mkletzel16@northwestern.edu)

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## ABSTRACT

This study was to determine whether GM-CSF induced *WT1* gene expression and to establish an association with markers of proliferation  $CD71^+CD34^+$  using nPCR and flow cytometry respectively, in samples obtained from 5 newly diagnosed JMML patients. Overtime (day 0 to day 14) there was an insignificant difference in *WT1* gene expression and  $CD71^+CD34^+$  in JMML samples when compared to peripheral blood of normal volunteers (n = 3). Our study suggests that there is a correlation between *WT1* gene expression and cellular proliferation and that GM-CSF *in vitro* does not create a significant difference in JMML samples.

## KEYWORDS

Juvenile Myelo-Monocytic Leukemia; Wilms Tumor; Nested PCR; JMML; *WT1*; GM-CSF; nPCR

## 1. INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) is a rare hematopoietic malignancy of early childhood with features exhibiting of both myeloproliferative neoplasm (MPN) and myelodysplastic syndrome (MDS) [1]. JMML affects very young children (median age at diagnosis is 2 years) with a significant male predominance (male:female = 2.5). This leukemia clinically presents with marked hepatosplenomegaly together with lymph-

denopathy, pallor and skin rash [2]. It is a representative of a clonal disease of pluripotent cell origin and *in vitro* granulocyte-macrophage colony-stimulating factor (GM-CSF) hypersensitivity is the hallmark of JMML [3-7].

*WT1* gene was first identified in patients with Wilms' tumor, codes for a zinc-finger transcription factor located on chromosome 11p13 [8]. Although expressed at low levels in bone marrow by normal progenitor cells, *WT1* has been reported to be expressed at high levels in almost all hematological malignancies, including JMML [9,10]. *WT1* plays an important role in regulating cellular growth and differentiation. Furthermore, *WT1* gene expression has been utilized as a diagnostic tool in assessing minimal residual disease (MRD) in varieties of leukemias [9,11-15] and disease progression in MDS [11-15].

In this study, we correlate the effect of GM-CSF stimulation and *WT1* gene expression over a period of time, obtaining samples from JMML patients, to establish a direct association of its expression with markers of proliferation ( $CD71^+CD34^+$ ) [16-20].

## 2. MATERIALS & METHODS

### 2.1. Patient Samples

Age at diagnosis ranged from 30 days to 9 years old. There were 3 males and 2 females. All five patients were diagnosed as JMML according to the World Health Organization (WHO) and met the laboratory criteria: Ph chromosome negative, no BCR/ABL rearrangement, peripheral blood monocyte count  $>1 \times 10^9/L$ , and bone marrow blasts  $<20\%$ . Additionally, all patients met at

\*Corresponding author.

least two laboratory findings: white blood count  $>10 \times 10^9/L$  and GM-CSF hypersensitivity of myeloid progenitors in vitro. Informed consent were obtained from the patients at Ann and Robert H. Lurie Children's Hospital of Chicago.

## 2.2. Sample Preparations

Peripheral blood samples were collected in EDTA tube and prepared by density gradient preparation to isolate mononuclear cells using Ficoll-Paque Plus (Pharmacia Amersham Bioscience AB, Uppsala, Sweden). A cell concentration of  $2 \times 10^6/mL$ /plate mononuclear cells was used as a seeding density on a 6-well culture plate. Samples were conducted in RPMI-1640 containing 100 U/mL penicillin, 100 ug/mL streptomycin, 10% FBS and 10 ng/mL GM-CSF added at day 0. Cells were cultured with and without the addition of GM-CSF to assess *WT1* gene expression. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> for a period of 14 days. At days 0 and 14 culture cells were harvested for total RNA extraction using a QIAamp RNA Blood Mini kit (QIAGEN Inc., Valencia, CA, USA) to determine *WT1* gene expression. Cells were additionally stained for CD 34<sup>+</sup> early stem cell markers and CD 71<sup>+</sup> a transferrin receptor, all indicators of cellular proliferation [16-20] were executed by flow cytometry using a FACSort flow cytometer (FacSort Becton-Dickinson, San Jose, CA, USA) and was analyzed with CELLQuest software (Becton Dickinson).

## 2.3. Nested PCR (nPCR)

The composition of the reaction buffer was 2 µL 10× PCR buffer II, 4 µL of 25 mM MgCl<sub>2</sub>, 8 µL dNTP, 1 µL Oligo d (T), 1 µL MuLV reverse transcriptase, and 1 µL RNAase inhibitor (Applied Biosystem, Foster City, CA, USA). Primer pairs were devised to locate precise nucleotide fragments for mRNA of the *WT1* gene illustrated in **Table 1** [21].

RT-PCR reaction was performed in two rounds in a 20-µL reaction volume containing 1 ug/µL total RNA from each sample. In preparation of the first round, RT was incubated at 42°C in a waterbath for 60 min, followed by denaturation at 96°C for 10 min. The first step included amplification of 10 µL of cDNA which was then subjugated to 30 cycles of denaturation for 5 min, amplification for 5 seconds at 95°C, annulment at 55°C for 5 seconds, and extension at 72°C for 10 seconds. Step 2 was then conducted by taking 1 µL of the amplified product from the first step and which was further re-amplified with inner primers using the LightCycler System with SYBR-Green I RNA master mix reagent, acquired from Roche Diagnostics, Biochemica (Indianapolis, IN, USA) [21]. After the termination of PCR the

**Table 1.** *WT1* Primer Sequences [21].

Primer Sense	Primer Sequence
Outer Sense	5'-GGCATCTGAGACCAGTGAGAA-3'
Outer Anti-Sense	5'-GAGAGTCAGACTTGAAAGCAGT-3'
Inner Sense	5'-GCTGTCCCACTTACAGATGCA-3'
Inner Anti-Sense	5' TCAAAGCGCCAGCTGGAGTTT-3'

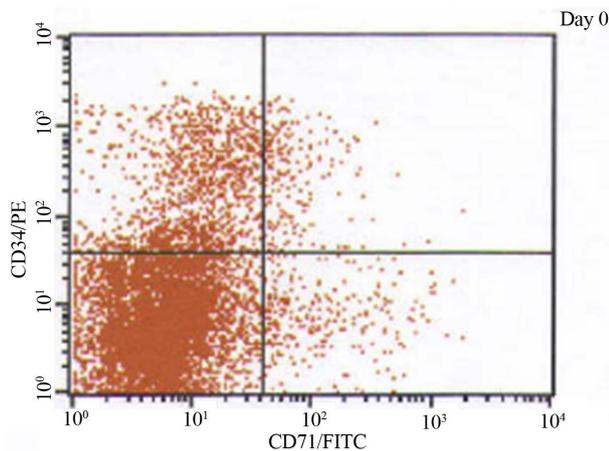
LightCycler software through computation from the external curve figured the titration of target molecules. The housekeeping gene GAPDH was admitted as an internal control to establish the integrity of the RNA in question. A tenfold serial dilution of the K562 leukemia cell line yielded a standard curve wherein sample results were interpolated. Values ranging from  $1 \times 10$  ng/µL and higher were considered positive for *WT1* mRNA expression (**Table 2**) [21]. The level of *WT1* gene expression in K562 was arbitrarily defined as 1.0 [14-15,21-26]. Samples were analyzed in triplicate. Any PCR results illustrating a discrepancy greater than one cycle threshold were expelled and reprised.

## 2.4. Statistical Analysis

Statistical analysis was performed using SPSS software (IBM Corporation, Armonk, New York, USA) to obtain a mean, median, ± SEM and range along with 95% confidence interval (CI) by column statistics. To compare and contrast sample groups and cultured groups an F-test and paired t-test were analyzed respectively for the determination of *WT1* expression. A p value of  $\leq 0.05$  was considered statistically significant.

## 3. RESULTS

JMML (n = 5) and negative control (n = 3) PB samples were tested on days 0 and 14 for *WT1* gene expression (**Figure 1**). Differentiation of cultures, un-stimulated vs. stimulated by GM-CSF was measured on day 14. There was a difference found on day 14 in JMML samples for *WT1* transcription in assessment to the initial day. The detection of *WT1* transcription in JMML cultured groups on day 14 represented an insignificant p-value ( $p < 0.738$ , 95% CI -63.9 to 49.3) signifying that despite of the GM-CSF stimulation *WT1* transcripts remained persistently positive. In contrast, normal PB measured low to undetectable levels of *WT1* transcripts from day 0 to day 14 in both culture groups ( $p < 0.0182$ , 95% CI -0.259 to -0.0669). Furthermore, this level of expression was significantly lower in comparison to the positive control, K562 cell line. A significant difference was noted when comparing *WT1* transcriptions of patient samples to the negative control (**Table 3**) throughout the study on day 0 ( $P < 0.0001$ ), day 14 un-stimulated ( $p < 0.0001$ ) and sti-



**Figure 1.** Comparison of *WT1* gene expression in JMML patients vs. negative control (CTL) samples with a significant ( $p < 0.0001$ ) difference to be found at days 0 and 14: un-stimulated vs. stimulated by GM-CSF. The bars indicate standard errors.

**Table 2.** Value ranges for *WT1* gene expression analysis.

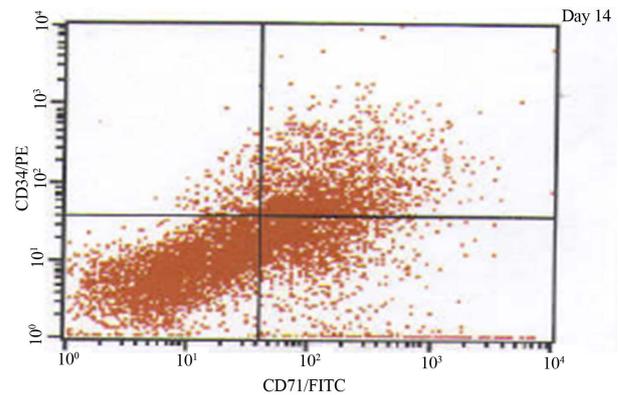
Range	<i>WT1</i> Gene Expression
$\geq 1 \times 10^0$ ng/ $\mu$ L	Positive
$9.9 \times 10^{-1}$ ng/ $\mu$ L - $6.5 \times 10^{-1}$ ng/ $\mu$ L	Weakly Positive
$< 6.5 \times 10^{-1}$ ng/ $\mu$ L	Negative

**Table 3.** Measurement of *WT1* transcription by nPCR.

	JMML (ng/ $\mu$ l)	Negative Control (ng/ $\mu$ l)	P value
<b>Day 0:</b>			
Mean $\pm$ SEM	16.4 $\pm$ 5.76	0.0347 $\pm$ 0.00116	
Median	11.3	0.034	<0.0001
Range	(1.70 - 35.0)	(0.0332 - 0.037)	
<b>Day 14</b>			
<b>Un-stimulated:</b>			
Mean $\pm$ SEM	25.4 $\pm$ 12.2	0.0304 $\pm$ 0.0139	
Median	19.0	0.026	<0.0001
Range	(1.60 - 65.7)	(0.0087 - 0.0564)	
<b>Day 14</b>			
<b>Stimulated:</b>			
Mean $\pm$ SEM	32.7 $\pm$ 13.0	0.193 $\pm$ 0.0219	
Median	34.0	0.210	<0.0001
Range	(2.53 - 71.7)	(0.150 - 0.220)	

ulated ( $p < 0.0001$ ).

In vitro patient samples were examined for the expression of *WT1* and CD71<sup>+</sup>CD34<sup>+</sup> simultaneously throughout the study. Phenotypic analysis was completed to establish the level of cellular proliferation using markers CD71<sup>+</sup>CD34<sup>+</sup> (Figure 2). Although cellular proliferation increased as time progressed from day 0 (mean 9.00  $\pm$  SEM 3.81, median 6.20, range 3.30% - 24.0%) to day 14, under the influence of GM-CSF un-stimulated (mean 51.5  $\pm$  SEM 6.59, median 46.9, range 39.2% - 77.0%) vs. stimulated (mean 56.4  $\pm$  SEM 2.20, median 55.7, range



**Figure 2.** A phenotypic analysis of one JMML patient using cellular proliferative markers CD71<sup>+</sup>CD34<sup>+</sup> measured on day 0 and day 14, stimulated by GM-CSF.

49.7% - 63.1%) there was no significant difference ( $p < 0.4689$ , 95% CI -22.1 to 12.2). CD71<sup>+</sup>CD34<sup>+</sup> levels were further compared with *WT1* transcripts to understand the relationship between cellular proliferation and gene expression in JMML. The insignificance found between cultured groups on day 14 concurs with the results of *WT1* expression.

#### 4. DISCUSSION

*WT1* transcription is associated with presence, persistence or reappearance of leukemic hematopoiesis [12]. Tamaki *et al.* [22] as well as Cilloni *et al.* [12] reported a strong correlation between leukemic progression and *WT1* gene expression. It is over expressed in all leukemic cell lines regardless of lineage and is also the most reliable risk index of disease progression available for adult MDS and JMML patients [23-26].

In order to establish *WT1* transcription as a proliferative marker, phenotypic analysis was performed using CD71<sup>+</sup>CD34<sup>+</sup> [16-20]. *WT1* expression was measured with a standard K562 cell line, arbitrarily defined as 1.0 as a positive expression [14-15,21-26], which was demonstrated in all 5 JMML patients. Measuring CD71<sup>+</sup>CD34<sup>+</sup> coherently with *WT1* using K562 clearly shows the consistency of proliferation throughout the study. *WT1* is an unalterable gene that is highly expressed irrespective of the presence of GM-CSF, allowing us to assume that it is a proliferative marker not only in the genotypic level as well as in the phenotypic level. This observation concurs with the nature of JMML, being that is a myeloproliferative neoplasm (MPN) [1,3,4]. The fact that there is no statistical significance between the cultured groups defines the characteristic of random proliferation in JMML [3,4]. The inability of *WT1* expression as a quantitative assay using nPCR does not show the degree of proliferation but proliferation by itself.

Although the *WT1* gene expression was positively

amplified regardless of GM-CSF stimulation, due to insignificant p-values our observations are invalid. Minimal response to GM-CSF can be attributed to the fact that our study included a small number of patients. Thus, we cannot conclude that *WT1* gene expression has a relationship with GM-CSF.

## 5. CONCLUSION

Our findings suggest that over time there is a trend of *WT1* gene expression in JMML cells, and that GM-CSF does not induce higher transcription in liquid culture. Phenotypic analysis concurs with these results. Thus our study supports the hypothesis that *WT1* gene expression is a marker of proliferation and may not have any role in the pathogenesis of JMML.

## DECLARATION OF INTEREST

The authors report no conflicts of interest.

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## ABBREVIATIONS

JMML  
WT1  
GM-CSF  
nPCR