Wilms Tumor 1 Expression at Diagnosis Correlates With Genetic Abnormalities and Polymorphism But Is Not Independently Prognostic in Acute Myelogenous Leukemia: A Hokkaido Leukemia Net Study

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Wilms tumor 1 expression at diagnosis correlates with genetic abnormalities and polymorphism but is not independently prognostic in acute myelogenous leukemia: a Hokkaido Leukemia Net study

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ABSTRACT

The association between Wilms tumor 1 (WT1) expression, genetic abnormalities and homozygous single polymorphism (SNP) in WT1 gene was evaluated in 252 acute myelogenous leukemia (AML) patients. WT1 expression correlated with prognostic genetic abnormalities. Homozygous WT1 SNP rs16754 was associated with lower expression of WT1. WT1 expression had no prognostic impact in any cytogenetic group or SNP status.

Background: The prognostic impact of WT1 expression at diagnosis of acute myelogenous leukemia (AML) has been controversial. The aim of this study was to determine the correlations of WT1 expression at diagnosis of AML with established prognostic alterations.

Patients and Methods: We analyzed diagnostic bone marrow samples from 252 patients. WT1 expression, SNP in WT1 gene (rs16754), and Flt3-ITD mutation were analyzed for all patients. NPM1 mutation and CEBPA double mutation were analyzed for cytogenetically normal (CN)-AML. KIT mutation was analyzed for core-binding factor (CBF)-AML. Results: Within the cytogenetically favorable prognosis group, WT1 expression in AML with inv(16) or t(15;17) was significantly higher than that in AML with t(8;21). In cases with CN-AML, Flt3-ITD and NPM1 mutations were both correlated with higher expression of WT1, whereas CEBPA double mutation was related to lower WT1 expression. The existence of both Flt3-ITD and NPM1 mutations showed synergistically higher expression of WT1 in CN-AML. SNP in WT1 gene (rs16754) was significantly associated with lower expression of WT1. WT1 levels were not prognostic factors in the total cohort, in any cytogenetic group nor SNP status. Conclusion: Since WT1 expression correlated to known prognostic factors, the prognostic impact of WT1 levels might be misunderstood depending on the distribution of collaborative mutations in each cohort. We conclude that the prognostic significance of WT1 at diagnosis of AML is weak compared to other established prognostic factors.

Keywords: WT1, prognostic stratification, AML, Hokkaido Leukemia Net (HLN), North Japan Hematology Study Group (NJHSG)
Introduction

Wilms tumor 1 (WT1) gene located in chromosome 11p13 encodes a DNA-binding protein with 4 zinc finger domains.\(^1,2\) The WT1 protein is a unique transcription factor that can activate or repress target promoters depending on the cellular cofactors it binds to.\(^3\) More than 70% of acute myelogenous leukemia (AML) patients have overexpression of WT1 at diagnosis.\(^4-7\) Whether WT1 transcript levels at diagnosis predict outcomes of AML patients is controversial.\(^8-15\) On the other hand, cytogenetic categories and certain molecular mutations were proven to be risk factors in AML.\(^16\) Patients with core-binding factor (CBF) AML or AML with t(15;17) have better overall survival. Fms-like tyrosine kinase receptor-3 internal tandem duplication (Flt3-ITD), nucleophosmin 1 (NPM1), and CCAAT/enhancer-binding protein alpha (CEBPA) double mutations are established prognostic risk factors in cytogenetically normal (CN)-AML. Risk stratification by these cytogenetic categories and molecular abnormalities has already been integrated in clinical practice\(^16\). The relationship between WT1 expression and each cytogenetic category has not been fully determined. In this study, we analyzed data for 252 AML patients and determined the prognostic significance of WT1 levels at diagnosis in each cytogenetic category.

Materials and methods

Patients

Hokkaido Leukemia Net (HLN) consists of 15 hospitals covering the entire Hokkaido prefecture in Japan. Bone marrow (BM) samples of AML patients were collected from all over Hokkaido and studied for relevant molecular markers at the central laboratory of Hokkaido University Hospital. Patients who were diagnosed with AML during the period from October 2007 to May 2017 were enrolled in this study. WT1 expression and Flt3-ITD mutation were analyzed using diagnostic bone marrow samples of all patients. This study was conducted in compliance with the ethical principles based on the Declaration of Helsinki. Approval of the institutional review board of each hospital participating in this study was obtained beforehand. Written consent was obtained from each patient after sufficient explanation from an investigator or sub-investigator before taking a sample.

Molecular analysis

Flt3-ITD, NPM1, CEBPA and KIT mutations were analyzed using a genome DNA template. The relevant region of Flt3 was amplified by PCR and gel electrophoresis was run to verify the presence of the ITD band. After cytogenetic analysis, NPM1 mutation and CEBPA double mutation were analyzed for CN-AML. NPM1 exon 12 and entire CEBPA
were directly sequenced. KIT mutation was analyzed for CBF-AML. KIT exons 8, 9, 11, 17 were directly sequenced. TP53 mutation was not determined in this study.

**WT1 expression analysis**

RNA was extracted from diagnostic bone marrow using a QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA). Complimentary DNA (cDNA) was synthesized from 1 µg of extracted RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Primer and probe sequences used for RQ-PCR were as follows: WT1, WT1-For GGGTACGAGAGCGATAACCACA, WT1-Rev TCTCAGATGCGACCCTACAA, WT1-MGB CAGAATACACACGCACGGT. As a copy number control, GAPDH was amplified using GAPDH-For GAAGGTGAAGGTCGGAGT, GAPDH-Rev GAAGATGGTGATGGGATTTC, and GAPDH MGB AAGCTTCCCGTTCTCA. RQ-PCRs were carried out on the real-time PCR system Step One Plus (Applied Biosystems, Foster City, CA). For quantification of WT1 transcripts in patient samples, samples were always amplified simultaneously with a standard dilution of K562 cDNA. K562 is a human erythroleukemic cell line that consistently expresses WT1.17 The WT1 transcript levels in samples were determined by reference to the corresponding transcript levels of K562 cells. The level of WT1 expression in each sample was normalized against GAPDH, and the relative value was calculated by calibrating to that of K562 RNA. The levels of WT1 were shown as relative WT1 expression per 10^4 K562 RNA (/10^4 K562 RNA). WT1 expression higher than K562 (≥1,000 / 10^4 K562 RNA) was defined as high expression, and WT1 expression lower than K562 (<1,000 / 10^4 K562 RNA) was defined as low expression according to receiver-operating characteristics analysis. K562 was purchased from Japanese Collection of Research Bioresources (JCRB) and cultured in RPMI medium with 10% fetal calf serum. RNA of K562 cells was reverse-transcribed into cDNA. This standard cDNA was prepared in large amounts and stored at -20 °C.

**WT1 SNP analysis**

WT1 SNP (rs16754) was analyzed using genome DNA. Primers for PCR at WT1 gene exon 7 was as follows: forward TACTCCAGTCTCAGTTTCC, reverse GGCAACATGGTCAGAGCT. PCR product was digested by HpyCH4IV (New England Biolabs, Ipswich, Massachusetts). Because the SNP broke HpyCH4IV recognition site “ACGT” to “GCGT”, SNP status can be identified by gel electrophoresis of digested PCR product (FIGURE S1A-C).

**Statistical analysis**
Differences between two groups of data of continuous variables were analyzed by the Mann-Whitney U test. Differences between two groups of categorical data were analyzed by Fisher’s exact test. A Cox proportional hazard model was used to investigate survival time. Overall survival (OS) was defined as the time from diagnosis to death or last follow-up. P-values < 0.05 were considered as statistically significant. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

Results

WT1 Expression and Cytogenetic Abnormalities

A total of 252 AML patients were enrolled in this study. The patients were divided into 6 groups depending on cytogenetic abnormalities, including t(8;21), inv(16), t(15;17), cytogenetically normal, poor cytogenetics, and others. Poor cytogenetics was defined as a complex karyotype (≥3 clonal chromosomal abnormalities), monosomal karyotype, or karyotypes containing -5, 5q-, -7, 7q-, 11q23 non t(9;11), inv(3), t(3;3), t(6;9), and t(9;22) according to the National Comprehensive Cancer Network (NCCN) guidelines. Patient characteristics are shown in TABLE 1. Age distribution was not different among the groups. Expression of WT1 at diagnosis of AML varied from <1 to 25118.9 (median, 1156.15). According to our criteria, 136 cases had high WT1 expression (≥1,000 / 10^4 K562 RNA) and 116 cases had low WT1 expression (<1,000 / 10^4 K562 RNA) at diagnosis of AML. Flt3-ITD positivity was higher in the t(15;17) and CN-AML groups and relatively low in CBF-AML and poor cytogenetics groups. KIT mutation rate was higher in the t(8;21) group (26.4%) than in the inv(16) group (4.3%) (p=0.038).

We then divided patients into 3 risk groups based on validated cytogenetics and molecular abnormalities in the NCCN guidelines except for TP53 mutation. This classification showed a clear prognostic difference in our cohort (FIGURE 1A). On the other hand, there was no significant difference in overall survival between patients in the WT1 high group and low group, though the low WT1 group tended to show a slightly poorer prognosis (p=0.393, FIGURE 1B). We then analyzed WT1 expression in each cytogenetic risk group (FIGURE 2). WT1 levels were varied in the cytogenetic categories, with particularly high levels in the inv(16) group (median, 1778.3) and the t(15;17) group (median, 3388.4) compared to the level in the t(8;21) group (median, 468.95). Patients with KIT mutation in the t(8;21) group tended to show lower WT1 expression and poor prognosis, but the tendency was not statistically significant (FIGURE S2A, B).

WT1 Expression in Cytogenetically Normal AML
For the cases with CN-AML, positivity of Flt3-ITD and NPM1 mutations was correlated with higher expression of WT1, whereas CEBPA double mutation was correlated with lower WT1 expression \((p<0.001, p<0.001, \text{ and } p=0.023, \text{ respectively, FIGURE 3})\). Combined analysis of Flt3-ITD and NPM1 mutations showed synergistically high WT1 expression in patients with both mutations (FIGURE S3A). As previously shown and classified in guidelines\(^{16}\), Flt3-ITD-positive CN-AML patients showed a poor prognosis, whereas Flt3-ITD-negative and NPM1-positive patients showed a better prognosis (FIGURE S3B). Flt3-ITD positivity was also high in the t(15;17) group. However, positivity of Flt3-ITD was weakly correlated to high WT1 in the t(15;17) group and the poor prognostic impact of Flt3-ITD was not significant in this group (FIGURE S4A, B).

**Prognostic Significance of WT1 expression in Each Cytogenetic Risk Group**

Since WT1 expression correlated with both a poor prognostic factor (Flt3-ITD in CN-AML) and better prognostic factors (inv(16), t(15;17), NPM1 and CEBPA double mutation in CN-AML), the prognostic impact of WT1 could be neutralized depending on the distribution of cytogenetic abnormalities in the cohort. We then examined the prognostic impact of WT1 expression for overall survival in each cytogenetic category, but the expression levels of WT1 did not show a significant prognostic impact (FIGURE 4).

**WT1 SNP status**

WT1 SNP could be analyzed in 225 (89.3%) cases. Thirty-two (14.2%) cases were homozygous Wt/Wt, 93 (41.3%) cases were heterozygous Wt/SNP, and 100 (44.4%) cases were homozygous SNP (FIGURE 5A). WT1 expression of the patients with homozygous SNP was significantly lower compared to those with homozygous Wt/Wt (median 825.15 vs. 1833.15, \(p=0.043\)) (FIGURE 5B). However, no prognostic impact was observed by each SNP status (FIGURE 5C).

**Discussion**

Expression of WT1 is detected in the majority of AML patients.\(^{5,13,19}\) It was hypothesized that high WT1 expression originated from a high leukemic cell population, but a direct correlation between blast percentage or CD34 positivity and WT1 expression was not confirmed.\(^{20}\) In our study, WT1 expression at diagnosis of AML varied by more than a 4 log range. High WT1 expression could not have originated from only the large number of leukemic blasts in the specimen because the blast percentage at diagnosis varied within the range of 20% to 100%. Thus, researchers speculated that levels of WT1 expression would reflect the biological difference of AML in each patient. Many studies were carried out to try
to determine the clinical impact of WT1 levels for survival or disease progression. In patients with myelodysplastic syndrome (MDS), it was shown that higher expression of WT1 was consistently correlated with earlier disease progression to AML and poor prognosis. However, the prognostic significance of WT1 in patients with AML at diagnosis is still controversial. There were several inconsistent reports concerning the prognostic impact of WT1 in AML patients (TABLE 2). Some reports showed a greater prognostic impact of higher WT1 expression, whereas other reports showed a greater prognostic impact of lower WT1 expression. The prognostic impact of WT1 was discussed by using the entire AML cohort in most of these reports. However, as we showed and some previous studies also showed, WT1 expression levels could be correlated to certain cytogenetic abnormalities. Previous studies showed that high WT1 expression correlated to AML with inv(16), NPM1 mutation, and 11q23 abnormality and that low WT1 expression correlated to AML with t(8;21). Another group showed that low WT1 expression at diagnosis predicted poor outcomes in AML with t(8;21). That group also showed that a KIT mutation was correlated with low WT1 expression. KIT mutation in patients with CBF leukemia is known as a poor prognostic factor, especially in t(8;21). In our series, patients with KIT mutation in t(8;21) tended to have lower WT1 expression and a poorer outcome, but the difference was not significant (FIGURE S2). The higher allogeneic hematopoietic stem cell transplantation (allo-HSCT) ratio in KIT-mutated t(8;21) than in KIT-non-mutated t(8;21) (44.4% vs 28.0%) could improve the poor outcome of the KIT-mutated group in our cohort. In our study, patients with inv(16) and t(15;17) showed higher WT1 expression than that in patients with t(8;21). Flt3-ITD or NPM1 mutation in CN-AML was independently associated with high WT1 expression, whereas CEBPA double mutation was correlated with lower WT1 expression. It is important to know the correlations of WT1 levels with background cytogenetic abnormalities because the sensitivity of WT1 as a minimal residual disease (MRD) marker might be different in AML with high WT1 expression and AML with low WT1 expression.

A biological explanation for the prognostic impact of WT1 transcript level at diagnosis of AML remains unclear. Over the years, many apparently contradictory functions have been described for the WT1 gene, which has been considered to be an oncogene or a tumor suppressor gene. Evidence is in fact emerging that the WT1 protein can exhibit both properties under different cellular conditions. These complex functions of WT1 were cited to explain contradictory results. Because of the inconsistent results for the prognostic impact, WT1 had not been integrated into AML risk stratification. Our cohort is the second-largest cohort in this field of research and includes unbiased patients in the region. The main limitation of this study was the retrospective study design, in which patients with a
poor risk group classification could be treated intensively, resulting in improved outcome. However, NCCN risk stratification was still effective even in our heterogeneously treated cohort in which 32.9% of the patients underwent allo-HSCT. The relatively large sample size enabled us to determine WT1 levels in each cytogenetic category. After dividing the patients into cytogenetic abnormality groups, WT1 levels still did not have any prognostic impact in each group. We also analyzed the cohort excluding patients who underwent allo-HSCT. However, prognostic significance of WT1 expression at diagnosis was not verified (data not shown).

A single nucleotide polymorphism (SNP) in the WT1 gene (rs16754) has initially been reported as a positive prognostic factor in AML patients. However, following publication showed inconsistent prognostic impact of the SNP (TABLE S1). This SNP was disproportionately distributed across ethnic groups, with higher prevalence in Asian and Hispanic patients. Our cohort had high prevalence of the SNP reflecting Japanese ethnicity. The SNP correlated to lower WT1 levels in AML, however the SNP status was not correlated to survival in our cohort.

Conclusion

In this study, we found that WT1 levels at diagnosis of AML were significantly associated with certain cytogenetics, collaborative leukemic mutations or existence of SNP rs16754. In any cytogenetic category, WT1 expression did not show clear prognostic significance. We conclude that the prognostic significance of WT1 at diagnosis of AML is weak compared to other established prognostic factors. Correlations between WT1 expression and cytogenetic abnormalities need to be confirmed in other series to determine whether our findings can be reproduced.

Clinical Practice Points

- WT1 expression in AML correlated with prognostic genetic abnormalities.
- Homozygous WT1 SNP rs16754 was associated with lower expression of WT1.
- WT1 expression did not have a prognostic impact in any genetic category or SNP status.

Acknowledgements

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Authors’ contributions

Designed the study: M.O., T.K.

Analyzed the data and wrote the manuscript: D.H., M.O.

Performed experiments: J.H., S.F., T.K.


Revised and approved the manuscript: T.T.

All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no competing financial interests.
References


**TABLE 1. Patient characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>t(8;21)</th>
<th>inv(16)</th>
<th>t(15;17)</th>
<th>CN-AML</th>
<th>Poor cytogenetics</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>252</td>
<td>34</td>
<td>23</td>
<td>27</td>
<td>93</td>
<td>34</td>
<td>41</td>
</tr>
<tr>
<td><strong>Age (median)</strong></td>
<td>17-89 (57)</td>
<td>17-75 (50.5)</td>
<td>25-74 (47)</td>
<td>17-86 (62)</td>
<td>20-80 (57)</td>
<td>17-89 (60.5)</td>
<td>24-76 (61)</td>
</tr>
<tr>
<td><strong>Sex M/F</strong></td>
<td>141/111</td>
<td>19/15</td>
<td>19/4</td>
<td>15/12</td>
<td>41/52</td>
<td>22/12</td>
<td>25/16</td>
</tr>
<tr>
<td><strong>Kit mutation</strong></td>
<td>NA</td>
<td>9 (26.4%)</td>
<td>1 (4.3%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>CEBPA double mutation</strong></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>19 (20.4%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>NPM1 mutation</strong></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>30 (32.3%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Flt3-ITD</strong></td>
<td>53 (21.0%)</td>
<td>3 (8.8%)</td>
<td>1 (4.3%)</td>
<td>8 (29.6%)</td>
<td>31 (33.3%)</td>
<td>2 (5.9%)</td>
<td>8 (19.5%)</td>
</tr>
<tr>
<td><strong>WT1 (High/Low)</strong></td>
<td>136/116</td>
<td>9/25</td>
<td>17/6</td>
<td>24/3</td>
<td>45/48</td>
<td>18/16</td>
<td>22/19</td>
</tr>
<tr>
<td><strong>WT1 median</strong></td>
<td>1156.15</td>
<td>468.95</td>
<td>1778.3</td>
<td>3388.4</td>
<td>891.3</td>
<td>1068.5</td>
<td>1148.2</td>
</tr>
<tr>
<td><strong>Allo-HSCT</strong></td>
<td>83 (32.9%)</td>
<td>11 (32.4%)</td>
<td>5 (21.7%)</td>
<td>2 (7.4%)</td>
<td>39 (41.9%)</td>
<td>12 (35.3%)</td>
<td>14 (34.1%)</td>
</tr>
</tbody>
</table>

**Abbreviation**, CN: Cytogenetically normal; AML: acute myelogenous leukemia; NA: not analyzed; Allo-HSCT: allogeneic hematopoietic transplantation
### TABLE 2. Inconsistent prognostic significance of WT1 at diagnosis of AML

<table>
<thead>
<tr>
<th>Author</th>
<th>AML</th>
<th>Number of cases</th>
<th>Age (median)</th>
<th>Prognostic impact of higher WT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barragan E et al.</td>
<td>non-M3</td>
<td>77</td>
<td>NA</td>
<td>poor</td>
</tr>
<tr>
<td>Rodrigues PC et al.</td>
<td>pediatric non-M3</td>
<td>41</td>
<td>1-19 (9)</td>
<td>favor</td>
</tr>
<tr>
<td>Galimberti S et al.</td>
<td>AML</td>
<td>24</td>
<td>23-87 (58)</td>
<td>poor</td>
</tr>
<tr>
<td>Miglino M et al.</td>
<td>non-M3</td>
<td>100</td>
<td>17-84</td>
<td>favor</td>
</tr>
<tr>
<td>Damm F et al.</td>
<td>non-M3</td>
<td>181</td>
<td>17-60</td>
<td>poor</td>
</tr>
<tr>
<td>Nomdedeu JF et al.</td>
<td>non-M3</td>
<td>584</td>
<td>16-70 (53)</td>
<td>poor</td>
</tr>
<tr>
<td>Ujj Z et al.</td>
<td>AML</td>
<td>60</td>
<td>19-80 (57.5)</td>
<td>no impact</td>
</tr>
<tr>
<td>Qin YZ et al.</td>
<td>AML with t(8;21)</td>
<td>88</td>
<td>14-60 (36)</td>
<td>favor</td>
</tr>
<tr>
<td>Marjanovic I et al.</td>
<td>CN-AML</td>
<td>104</td>
<td>19-78 (54)</td>
<td>poor</td>
</tr>
<tr>
<td>This study</td>
<td>AML</td>
<td>252</td>
<td>17-89 (57)</td>
<td>no impact</td>
</tr>
</tbody>
</table>
FIGURE 1. OS by NCCN risk stratification and WT1 expression

A

Overall survival

Year

favorable (N=103)
intermediate (N=84)
poor (N=65)

p<0.001

B

Overall survival

Year

High (N=136)
Low (N=116)

p=0.393
FIGURE 2. WT1 expression in each cytogenetic category

- $p<0.001$
- $p=0.001$

<table>
<thead>
<tr>
<th>Cytogenetic Category</th>
<th>WT1 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(8;21) N=34</td>
<td></td>
</tr>
<tr>
<td>inv(16) N=23</td>
<td></td>
</tr>
<tr>
<td>t(15;17) N=27</td>
<td></td>
</tr>
<tr>
<td>CN-AML N=93</td>
<td></td>
</tr>
<tr>
<td>Poor cytogenetics N=34</td>
<td></td>
</tr>
<tr>
<td>Others N=41</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 3. WT1 expression in CN-AML

$\ p<0.001 \quad p<0.001 \quad p=0.023$

WT1 expression

- Fit3-ITD (+) N=31
- Fit3-ITD (-) N=62
- NPM1 mut (+) N=30
- NPM1 mut (-) N=63
- CEBPA double mut (+) N=19
- CEBPA double mut (-) N=74
FIGURE 4. OS by WT1 expression in each cytogenetic category

- **t(8;21)**
  - High (N=9)
  - Low (N=25)
  - \( p=0.556 \)

- **inv(16)**
  - High (N=17)
  - Low (N=6)
  - \( p=0.949 \)

- **t(15;17)**
  - Low (N=3)
  - High (N=24)
  - \( p=0.352 \)

- **CN-AML**
  - High (N=45)
  - Low (N=48)
  - \( p=0.924 \)

- **Poor cytogenetics**
  - Low (N=18)
  - High (N=16)
  - \( p=0.493 \)

- **Others**
  - Low (N=19)
  - High (N=22)
  - \( p=0.409 \)
FIGURE 5. WT1 expression and OS by WT1 SNP

A

- Wt/Wt (14.2%)
- Wt/SNP (41.3%)
- SNP/SNP (44.4%)

B

- p=0.043

C

- Overall survival vs Year

- Wt/Wt
- Wt/SNP
- SNP/SNP

- p=0.728
FIGURE S1. WT1 SNP analysis

A

SNP rs16754 A>G

Forward primer

TAATCCCCAGTGGCTCACTCTTCTACGACACCT

CCTCCCTTCTCCTCTCTCTGCCTGAGATGCACGG

CCGACTCTGTACGATCGCCATCTGAGACCAGTGAGAAACGCCCCTTTCATGTGCT

TACCCCAGGCTGCAATAAGAGATATTATAAGCTGTCCACTTACAGATGCACACAGGA

AGCAGCTGGTAGTGGCGCCGCCGCTGTCCAGCTGCCGCAACATGGTAAAGCT

ACGT: HpyCH4IV recognition site

Reverse primer

B

300 bp

200 bp

Wt/Wt

Wt/SNP

SNP/SNP

C

Homozygous Wt/Wt

Heterozygous Wt/SNP

Homozygous SNP/SNP

Primers for WT1 SNP analysis was shown (Fig S1A). SNP rs16754 break HpyCH4IV recognition site. After digestion of PCR product by HpyCH4IV, Wt product was digested into 3 bands (189, 64, 31 bp), whereas SNP product was digested into 2 bands (253, 31 bp). So 189 bp band represented Wt, and 253 band represented SNP rs16754 (Fig S1B). Representative sample of each SNP status were sequence verified (Fig S1C).
FIGURE S2. WT1 expression in AML with t (8;21)

A

\[ p = 0.073 \]

B

Overall survival

\[ p = 0.409 \]

Kit mut (+)  
Kit mut (-)
FIGURE S3. WT1 expression by FLT3-ITD and NPM1 mutation status

A

\[ p < 0.001 \]
\[ p = 0.049 \]

WT1 expression

<table>
<thead>
<tr>
<th>Flt3-ITD</th>
<th>NPM1 mut</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>49</td>
</tr>
</tbody>
</table>

B

Overall survival

- Flt3 (-), NPM1 (+)
- Flt3 (-), NPM1 (-)
- Flt3 (+), NPM1 (-)
- Flt3 (+), NPM1 (+)

\[ p = 0.562 \]

Year

Flt3 (+); Flt3-ITD positive
NPM1 (+); NPM1 mutation positive
FIGURE S4. WT1 expression in AML with t (15;17)

A

$p=0.089$

B

Overall survival

Year

Fli3-ITD (−)
Fli3-ITD (+)

$p=0.264$
<table>
<thead>
<tr>
<th>Author</th>
<th>AML</th>
<th>Country</th>
<th>Number of cases</th>
<th>Rate of homozygous SNP</th>
<th>Prognostic impact of SNP positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Damm F et al.</td>
<td>CN-AML</td>
<td>Germany</td>
<td>249</td>
<td>1.6%</td>
<td>favor</td>
</tr>
<tr>
<td>Hollink IH et al.</td>
<td>non-M3</td>
<td>Europe</td>
<td>232</td>
<td>2.2%</td>
<td>no impact</td>
</tr>
<tr>
<td>Ho PA et al.</td>
<td>non-M3</td>
<td>USA</td>
<td>790</td>
<td>4.8%</td>
<td>favor</td>
</tr>
<tr>
<td>Renneville A et al.</td>
<td>non-M3</td>
<td>France</td>
<td>511</td>
<td>3.6%</td>
<td>no impact</td>
</tr>
<tr>
<td>Becker H et al.</td>
<td>CN-AML</td>
<td>USA</td>
<td>433</td>
<td>2.8%</td>
<td>favor</td>
</tr>
<tr>
<td>Choi Y et al.</td>
<td>CN-AML</td>
<td>Korea</td>
<td>73</td>
<td>53.4%</td>
<td>no impact</td>
</tr>
<tr>
<td>Luna I et al.</td>
<td>non-M3</td>
<td>Spain</td>
<td>175</td>
<td>none</td>
<td>no impact</td>
</tr>
<tr>
<td>Luo S et al.</td>
<td>non-M3</td>
<td>China</td>
<td>122</td>
<td>65.6%</td>
<td>favor</td>
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<tr>
<td>Zhang D-Y et al.</td>
<td>non-M3</td>
<td>China</td>
<td>205</td>
<td>52.7%</td>
<td>poor</td>
</tr>
<tr>
<td>Petti J et al.</td>
<td>AML</td>
<td>Italy</td>
<td>87</td>
<td>NA</td>
<td>favor</td>
</tr>
<tr>
<td>This study</td>
<td>AML</td>
<td>Japan</td>
<td>225</td>
<td>44.4%</td>
<td>no impact</td>
</tr>
</tbody>
</table>

Abbreviation, CN: cytogenetically normal, NA: not analyzed