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Risk-Reducing Genetic Variant of *Wilms Tumor 1* Gene rs16754 in Korean Patients With *BCR-ABL1*-Negative Myeloproliferative Neoplasm

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The genetic variant rs16754 of *Wilms tumor* gene 1 (*WT1*) has recently been described as an independent prognostic factor in AML patients. It is of great interest to test whether *WT1* single nucleotide polymorphism can be used as a molecular marker in other types of cancer, to improve risk and treatment stratification. We performed sequencing analysis of exons 7 and 9 of *WT1*, which are known mutational hotspots, in a total of 73 patients with *BCR-ABL1*-negative myeloproliferative neoplasm (MPN) and 93 healthy controls. No previously reported *WT1* mutations were identified in the present study. In Korean patients with *BCR-ABL1*-negative MPN, *WT1* genetic variant rs16754 had no significant impact on clinical outcomes. We observed a significant difference in the allelic frequencies of *WT1* rs16754 in Koreans between *BCR-ABL1*-negative MPN cases and healthy controls. Individuals carrying variant G alleles of *WT1* rs16754 showed a relatively low prevalence of *BCR-ABL1*-negative MPN, compared with those carrying wild A alleles of *WT1* rs16754 (Hazard ratio 0.10-0.65, $P<0.05$). Therefore, possession of the variant G allele of *WT1* rs16754 may reduce the risk of developing *BCR-ABL1*-negative MPN.

Key Words: Myeloproliferative neoplasm, *WT1*, rs16754

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Recently, molecular markers have been described that likely contribute to disease pathogenesis and determine the clinical outcome in *BCR-ABL1*-negative myeloproliferative neoplasms (MPN). Most common are genetic mutations in exon 12 of *JAK2*, myeloproliferative leukemia virus (*MPL*), casitas B-lineage lymphoma proto-oncogene (*CBL*), Src homology 2 B3 (*SH2B3*), *Wilms tumor 1* (*WT1*), serine/arginine-rich splicing factor 2 (*SRSF2*), and epigenetic mutations in TET oncogene family member (*TET2*), isocitrate dehydrogenase (*IDH*)1/2, additional sex combs-like 1 (*ASXL1*), enhancer of zeste homolog 2 (*EZH2*), and DNA methyltransferase (*DNMT3A*).

The *WT1* gene, on chromosome 11p13, is a potent transcriptional regulator of genes involved in cell survival, differentiation, and proliferation [1]. The precise role of *WT1* in hematopoiesis and its contribution to leukemogenesis are not fully understood. Recently, interest in *WT1* has grown, with the discovery of mutations (most in a "hotspot" in exon 7) in patients with AML [2, 3]. However, despite the large number of patients analyzed, controversies remain about the prognostic impact of these mutations in AML. *WT1* mutations are also seen in MDS; increased *WT1* expression is associated with higher blast counts and portends an early progression to acute leukemia [4]. Recently, several publications have emphasized the possible impact of the genetic variant rs16754, located in exon 7 of *WT1*, on the outcome for both pediatric and adult AML patients [5]. Becker *et al*. [6] demonstrated that AML patients carrying homozygotes for a genetic

variant of rs16754 (GG) had a more favorable outcome in a study of a subset of patients with *FLT3*-ITD. However, in a Korean cohort, different genotypes of rs16754 did not have a significant impact on clinical outcome in AML [7]. Therefore, it is of interest to test whether the *WT1* rs16754 genetic variant shows potential as a molecular marker in *BCR-ABL1*-negative MPN, to improve risk and treatment stratification.

We performed sequencing analyses of *WT1* mutational hotspots in exons 7 and 9 in 75 patients with *BCR-ABL1*-negative MPN and 93 healthy controls from a Korean population; we also included genetic variant rs16754, located in exon 7 of *WT1*.

A total of 75 patients (32 with essential thrombocytosis [ET], 25 with polycythemia vera [PV], 10 with primary myelofibrosis [PMF], and eight with unclassifiable MPN) were enrolled. The diagnoses of PV, ET, and PMF were made according to the WHO criteria [8, 9]. All patients were diagnosed between June 2007 and March 2012 at Pusan National University Hospital, Busan, Korea. The *JAK2* V617F mutation was identified in 20 (80%) of 25 patients with PV, 18 (56.2%) of 32 patients with ET, five (50%) of 10 patients with PMF, and five (62.5%) of eight patients with unclassifiable MPN. The patients comprised 40 males and 35 females (median age=57.3 yr, range=19-83 yr). All patients provided informed consent. This research was reviewed and approved by full committee review of the Institutional Review Board at Pusan National University Yangsan Hospital (No. 05-2014-058).

A total of 75 DNA samples were extracted from the bone marrow of MPN patients between 2007 and 2012. All samples were obtained at initial diagnosis of MPN. Genomic DNA was extracted from cryopreserved mononuclear cells using an AccuPrep Genomic DNA extraction kit (Bioneer, Daejeon, Korea). The *BCR-ABL1* gene rearrangement was assessed by reverse transcription-polymerase chain reaction using an in-house method. Mutational analysis of coding regions previously described as mutational hotspots for *WT1* (exons 7 and 9) was performed by using PCR amplification and bidirectional direct sequencing. Primers were designed by using the Primer 3 software (http://frodo.wi.mit.edu/primer3/). The 20-μL reaction mixture for amplification contained 1 μL of DNA template, 1 μL of each primer, 17 μL of water-solubilized AccuPower HotStart PCR Premix (Bioneer). The amplification conditions were: one initial cycle of 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 45 sec at 55°C, and 30 sec at 72°C, with one final cycle of 10 min at 72°C. PCR products were purified by using standard methods and directly sequenced in both directions on an ABI 3100 analyzer using BigDye chemistry (Applied Biosystems, Foster City, CA, USA). The sequence data files were analyzed by using SEQUENCHER software (Gene Codes Corporation, Ann Arbor, MI, USA). Electropherograms were also read manually to identify mutations below the detection threshold of the software.

No mutations were detected in *WT1* exons 7 or 9, but c.1107A>G was detected in 88.0% of patients; this known genetic variant (rs16754) is listed in the National Center for Biotechnology Information Single Nucleotide Polymorphism (SNP) Database (dbSNP; http://ncbi.nlm.nih.gov/projects/SNP/). *WT1* rs16754 is located in the mutational hotspot of *WT1* exon 7. We aimed to genotype the *WT1* rs16754 locus and analyze the clinical impact of *WT1* rs16754 genotypes on clinical outcomes in Korean adult patients with MPN. A total of 93 DNA samples were acquired from healthy controls. The MPN patients and healthy controls were divided into three groups based on genotype at rs16754 in *WT1* exon 7: wild type (AA), heterozygotes (AG), and homozygotes (GG).

The frequencies of the three genotypes for *WT1* rs16754 were 12.0% (nine patients) AA, 49.3% (37 patients) AG, and 38.7% (29 patients) GG in the 75 patients with MPN (Table 1). In the 93 healthy controls, the AA, AG, and GG genotypes were observed in 1.1% (one individual), 40.9% (38 individuals), and 58.1% (54 individuals), respectively. The genotype frequencies showed no significant difference. The A allele was present at a frequency of 36.7% in MPN patients, compared with 21.5% in controls ($P=0.002$). A significant difference in the allelic frequencies of *WT1* rs16754 was noted in Koreans between *BCR-ABL1*-negative MPN cases and healthy controls.

Genotype-specific risks were estimated as odds ratios (OR) for the heterozygote and homozygote types, with the wild type as the baseline category, using the χ^2 test and logistic regression for the three different genetic models (co-dominant, dominant, and recessive). The results showed a risk-reducing association with MPN for *WT1* rs16754 with an OR of 0.11 (95% confidence in-

Table 1. Allele frequencies for *WT1* rs16754 (n=168)

	MPN (n=75)	Control (n=93)
Genotype		
GG	29 (38.7%)	54 (65.1%)
GA	37 (49.3%)	38 (40.9%)
AA	9 (12.0%)	1 (1.1%)
Allele		
G	95 (63.3%)	146 (78.5%)
A	55 (36.7%)	40 (21.5%)

Abbreviation: MPN, myeloproliferative neoplasm.

Table 2. Association between *WT1* rs16754 and MPN (n=168)

Model	Genotype	Control (%)	MPN (%)	OR (95% CI)	*P*
Co-dominant	AA	1 (1.1)	9 (12.0)	1.00	0.001
	AG	38 (40.9)	37 (49.3)	0.11 (0.01-0.90)	
	GG	54 (58.1)	29 (38.7)	0.06 (0.01-0.49)	
Dominant	AA	1 (1.1)	9 (12.0)	1.00	0.002
	AG+GG	92 (98.9)	66 (88.0)	0.08 (0.01-0.64)	
Recessive	AA+AG	39 (41.9)	46 (61.3)	1.00	0.012
	GG	54 (58.1)	29 (38.7)	0.46 (0.24-0.85)	

Abbreviations: MPN, myeloproliferative neoplasm; OR, odds ratio; CI, confidence interval.

terval [CI]: 0.01-0.90) and 0.06 (95% CI: 0.01-0.49) for heterozygotes and homozygotes, respectively (Table 2).

We used Cox proportional hazards analyses to evaluate the effect of genotype. The Cox model showed that the GG and AG genotypes of *WT1* rs16754 were significantly associated with a lower risk of MPN (hazard ratio [HR]: 0.23, 95% CI: 0.13-0.52) than AA genotypes (Table 3).

Survival curves were defined by using the Kaplan-Meier method and compared by using the log-rank test. Overall survival (OS) was calculated as the time (months) between the date of diagnosis and the date of death (for patients who were deceased) or last follow-up (for censored patients). With a median follow-up duration of 22.3 months (range, 0-69 months) among surviving patients, there were no significant differences in OS between patients with the GG type and those with the AG and AA types. Patients with the AA type, however, tended to show a longer OS as the estimated OS at 5 yr were 79.4% for GG genotype, 85.7% for AG genotype, and 100% for AA genotype, although the difference was not statistically significant ($P=0.459$).

None of the previously reported *WT1* mutations were identified in the present study; however, this study has limitations to estimate the real incidence of *WT1* mutations in *BCR-ABL* negative MPN patients. This study investigated only exons 7 and 9, not the entire coding sequence, in a small number of MPN patients.

Although no mutations were identified in *WT1*, 66 patients (88.0%) and 92 healthy controls (98.9%) carried at least one variant G allele(s) of *WT1* rs16754 in exon 7. The International HapMap 3 Consortium genotyped 1.6 million common SNPs and observed wide variations in allele frequencies among different populations. In Asian populations, G was the major allele for rs16754. The frequency of the G allele ranged from 58.9% to 64.1% in a Japanese (JPT) population, 72.6% to 76.8% in a Han Chinese (CHB) population, and 14.6% to 16.7% in a European (CEU) population (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=16754) [10]. The allele frequencies observed in this study are similar to those for the other Asian populations and differ significantly from those for the CEU population. G was a major allele in the Korean population, but a minor allele in the CEU population. The differences were observed in both MPN patients and healthy controls.

Table 3. Genotype-specific hazard ratio in *WT1* rs16754 in patients with MPN

Genotype	Hazard ratio	95% CI	*P*
GG	0.22	0.10-0.47	<0.001
AG	0.31	0.15-0.65	0.002
GG+AG	0.26	0.13-0.52	<0.001

Abbreviations: MPN, myeloproliferative neoplasm; CI, confidence interval.

We found evidence of a risk-reducing association with *BCR-ABL1*-negative MPN for *WT1* rs16754, consistent with the G allele functioning as an MPN-protective allele. No significant difference in OS was detected between genotypes. The genetic function of *WT1* rs16754 has not been fully elucidated. The prognostic impacts of *WT1* rs16754 on clinical outcomes for AML have been investigated in several studies [5, 11, 12], but the results were conflicting. These contradictory results indicate that the influence of *WT1* rs16754 should be investigated further.

In conclusion, we observed a significant difference in the allelic and genotypic frequencies of *WT1* rs16754 in an Asian population, relative to the frequencies reported for a western population. The individuals carrying variant G alleles of *WT1* rs16754 showed a relatively low prevalence of MPN, compared with those carrying A alleles of *WT1* rs16754 (HR: 0.10-0.65, $P<0.05$); the G allele for *WT1* rs16754 might reduce the risk of developing MPN.

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