The C allele of JAK2 rs4495487 is an additional candidate locus that contributes to myeloproliferative neoplasm predisposition in the Japanese population

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Abstract

Background: Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are myeloproliferative neoplasms (MPNs) characterized in most cases by a unique somatic mutation, JAK2 V617F. Recent studies revealed that JAK2 V617F occurs more frequently in a specific JAK2 haplotype, named JAK2 46/1 or GCCC haplotype, which is tagged by rs10974944 (C/G) and/or rs12343867 (T/C). This study examined the impact of single nucleotide polymorphisms (SNPs) of the JAK2 locus on MPNs in a Japanese population.

Methods: We sequenced 24 JAK2 SNPs in Japanese patients with PV. We then genotyped 138 MPN patients (33 PV, 96 ET, and 9 PMF) with known JAK2 mutational status and 107 controls for a novel SNP, in addition to two SNPs known to be part of the 46/1 haplotype (rs10974944 and rs12343867). Associations with risk of MPN were estimated by odds ratios and their 95% confidence intervals using logistic regression.

Results: A novel locus, rs4495487 (T/C), with a mutated T allele was significantly associated with PV. Similar to rs10974944 and rs12343867, rs4495487 in the JAK2 locus is significantly associated with JAK2-positive MPN. Based on the results of SNP analysis of the three JAK2 locus, we defined the “GCC genotype” as having at least one minor allele in each SNP (G allele in rs10974944, C allele in rs4495487, and C allele in rs12343867). The GCC genotype was associated with increased risk of both JAK2 V617F-positive and JAK2 V617F-negative MPN. In ET patients, leukocyte count and hemoglobin were significantly associated with JAK2 V617F, rather than the GCC genotype. In contrast, none of the JAK2 V617F-negative ET patients without the GCC genotype had thrombosis, and splenomegaly was frequently seen in this subset of ET patients. PV patients without the GCC genotype were significantly associated with high platelet count.

Conclusions: Our results indicate that the C allele of JAK2 rs4495487, in addition to the 46/1 haplotype, contributes significantly to the occurrence of JAK2 V617F-positive and JAK2 V617F-negative MPNs in the Japanese population. Because lack of the GCC genotype represents a distinct clinical-hematological subset of MPN, analyzing JAK2 SNPs and quantifying JAK2 V617F mutations will provide further insights into the molecular pathogenesis of MPN.

Keywords: JAK2 V617F, SNP, myeloproliferative neoplasms
Background
Myeloproliferative neoplasms (MPNs) represent a heterogeneous group of hematological malignancies characterized by clonal hematopoiesis and an increased number of mostly peripheral blood elements of myeloid origin [1]. The classic Philadelphia-chromosome-negative MPNs encompass three distinct diseases, namely polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) [2-5]. Identification of the V617F mutation of the \textit{JAK2} gene (\textit{JAK2} V617F) led to an important breakthrough in the understanding of MPN disease pathogenesis [2-5]. The \textit{JAK2} V617F mutation is present in the majority of PV patients, and about 50% of patients with ET and PMF are affected [2-5]. Because this somatic mutation is highly specific to MPNs, it has been designated as a major diagnosis criterion for PV, ET, and PMF according to the latest World Health Organization classification of MPNs [6].

Recent investigations revealed that somatic acquisition of genetic aberrations is one pathogenic mechanism, but inherited genetic factors also play an important role in the development of MPN. Several independent groups reported that a particular \textit{JAK2} haplotype, designated 46/1 or GGCC, is strongly associated with the development [7-9], or with MPN development, regardless of the \textit{JAK2} mutational status [10,11]. Olcaydu et al. [12] performed \textit{JAK2} haplotype analysis in familial MPNs, and they concluded that even if \textit{JAK2} 46/1 is related to the development of MPN independent of V617F status, it has to be regarded as only one of the genetic factors involved in the development of MPN. Moreover, Jones et al. [13] found correlations in \textit{JAK2} wild-type MPN between \textit{JAK2} 46/1 and both MPL exon 10 and \textit{JAK2} exon 12.

In the present study, we attempted to find novel single nucleotide polymorphisms (SNPs) of the \textit{JAK2} locus in a Japanese population. We then examined whether \textit{JAK2} SNPs are indeed associated with a predisposition to MPNs, especially in \textit{JAK2} V617F-positive MPNs.

Methods
Patients
In the current study conducted at the Tokyo Medical University Hospital, 138 constitutive Japanese MPN patients aged 30-87 years with known \textit{JAK2} V617F status were included: 33 patients with \textit{JAK2} V617F-positive PV, 57 patients with \textit{JAK2} V617F-positive ET, 39 patients with \textit{JAK2} V617F-negative ET, and 9 patients with PMF. The patients experienced no familial MPNs. We revised their classification at diagnosis according to the latest World Health Organization classification of MPNs. As controls, 107 healthy volunteers aged 24-86 years from the same demographic area in Japan were used. The \textit{JAK2} V617F mutation detection system used was reported elsewhere [14], and the \textit{JAK2} V617F mutational status was categorized according to the allele burden of mutated T allele. This study was approved by the institutional review board of Tokyo Medical University (no. 975). Written informed consent according to the Declaration of Helsinki was obtained from all patients prior to collection of the specimens.

PCR direct sequencing of the \textit{JAK2} locus
Genomic DNA was obtained from whole blood using an automated system (Qiagen). To identify novel SNPs in the \textit{JAK2} locus in this Japanese population, primer sets for the amplification of \textit{JAK2} were designed according to GenBank AL161450 (Figure 1A; Additional file 1A). PCR conditions were 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min for 36 cycles using High Fidelity PLUS PCR System dNTPack (Roche Diagnostics, Mannheim, Germany). The PCR products were purified by a High Pure PCR Product Purification Kit (Roche Molecular Biochemical Diagnostics, Indianapolis, IN, USA) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an Applied Biosystems 3130 Genetic Analyzer. The obtained sequences were compared with the \textit{JAK2} sequence.

Allele-specific PCR analysis
To determine whether minor alleles of \textit{JAK2} SNPs favor the \textit{cis} acquisition of \textit{JAK2} V617F, we performed allele-specific analysis of six SNPs in patients with PV. The sequence \textit{JAK2} nt51936-nt55084 (3158 bp) was amplified using forward primer int12-F and reverse primer V617F-R or exon14-R. The primer set for the amplification of \textit{JAK2} nt55038-nt55636 (598 bp) was forward primer V617F-F or exon14-F and reverse primer int14-R. Both primers V617F-R and V617F-F could amplify the T allele of \textit{JAK2} V617F (Figure 1B; Additional file 1A). The PCR products were purified and sequenced as described above.

Genotyping
Allele-specific PCR was performed with a common forward primer and two allele-specific reverse primers (Additional file 1B) using High Fidelity PLUS PCR System dNTPack (Roche Diagnostics) and SYBR Green I (Lonza, Rockland, ME, USA). The PCR conditions were 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min for 40 cycles using an iCycler IQ Real-time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). To avoid nonspecific PCR products, melting analysis was performed by denaturing at 95°C for 1 min and cooling to 55°C for 1 min followed by heating at the rate of 0.5°C/10 s from 55 to 95°C.
Statistical analysis
GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis. Associations with risk of MPNs were estimated by odds ratios (ORs) and their 95% confidence intervals (95% CIs) using logistic regression. A Mann-Whitney U-test was used to determine the statistical significance of differences between the control and test groups. P-values less than 0.05 were considered to indicate statistically significant differences. We also performed multivariate analysis using College analysis software (version 4.5, Fukuyama Heisei University, Fukuyama, Japan) to exclude possible false correlation between genotype and clinical manifestations.

Results
Minor allele frequency of SNPs from the JAK2 locus in Japanese PV patients
We first screened for 24 SNPs of the JAK2 locus around exons 12 to 14 in 28 Japanese patients with JAK2 V617F-positive PV from whom we obtained sufficient DNA for this analysis (Figure 1A). Among them, minor allele frequencies in six SNPs (rs10974944, rs12686652, rs12335546, rs4495487, rs1028730, and rs12343867) were significantly higher in PV patients than in healthy volunteers (Table 1). There were no significant differences in age or sex between the PV population and healthy controls (data not shown). Minor allele frequency was estimated by total cases that had at least one minor allele (heterozygous) or two minor alleles (homozygous). The JAK2 SNP rs4495487, which has not been reported previously in Caucasian populations, showed the highest OR (13.8, 95% CI: 3.79-50.21) among the six SNPs.

To determine whether minor alleles of JAK2 SNPs favor the cis acquisition of JAK2 V617F, we next sequenced six SNPs using allele-specific primers (Additional file 1A). In accordance with a previous report [8], in the genotype with minor allele in all six SNPs, the T allele was more frequently observed in JAK2 V617F than the G allele in normal controls; the OR was 7.74 (95% CI: 2.32-25.75) (Additional file 2).

JAK2 SNP distribution in MPN patients and controls
We genotyped 138 MPN patients with known JAK2 mutational status and 107 controls for JAK2 SNP rs449587 in addition to two SNPs that are known to be part of the 46/1 haplotype (rs10974944 and...
Table 1 Minor allele frequency of SNPs from the JAK2 locus in PV

| No. | SNP         | Minor alleles in PV (n = 28) | Minor alleles in control (n = 28) | P valuea (Chi square test) | Odds ratio (95% CI) |
|-----|-------------|-----------------------------|----------------------------------|---------------------------|---------------------|
|     |             | No | Yes (homo/hetero) | %  | No | Yes (homo/hetero) | %  |                     |                     |
| 1   | rs10974944  | 7  | 21 (15/6)       | 75 | 17 | 11 (3/8)        | 39.2 | 0.0069 | 4.64 (1.49-14.55)  |
| 12  | rs12686652  | 6  | 22 (21/1)       | 78.6 | 14 | 14 (5/9)        | 50    | 0.0257 | 3.67 (1.14-11.79)  |
| 13  | rs12335546  | 6  | 22 (21/1)       | 78.6 | 19 | 9 (1/8)         | 32.1   | 0.0005 | 7.74 (2.33-25.75)  |
| 19  | rs4495487   | 7  | 21 (21/0)       | 75 | 23 | 5 (3/2)         | 17.8    | <0.0001 | 13.8 (3.79-50.21)  |
| 22  | rs1028730   | 6  | 22 (21/0)       | 75 | 17 | 11 (4/9)        | 39.3   | 0.0069 | 4.64 (1.48-14.55)  |
| 24  | rs12343867  | 7  | 21 (21/0)       | 75 | 17 | 11 (4/9)        | 39.3   | 0.0069 | 4.64 (1.48-14.55)  |

aP values were calculated by the cases having minor alleles (homozygous and heterozygous) and cases without minor alleles.

Table 2 Genotype-specific association of SNPs from the JAK2 locus in MPN

| Case population | Control population | SNP         | P  | Genotypea | Odds ratio (95% CI) |
|-----------------|--------------------|-------------|----|-----------|---------------------|
|                 |                    | Major       | Homo | Major vs Hetero | Major vs Homo |
| JAK2 V617F-positive PV | Healthy volunteers | rs10974944 | 0.0126 | CC CG GG | 2.75 (1.06-7.14)* | 4.24 (1.51-11.92)* |
| (n = 33) | Japanese (n = 107) | rs4495487 | <0.0001* | TT TC CC | 4.26 (1.56-11.61)* | 11.31 (3.60-35.57)* |
|                |                   | rs12343867 | 0.0032* | TT TC CC | 2.26 (0.88-5.77) | 5.68 (1.98-16.35)* |
| JAK2 V617F-positive ET | Healthy volunteers | rs10974944 | 0.0371 | CC CG GG | 2.42 (1.20-4.92) | 1.10 (0.40-3.02) |
| (n = 57) | Japanese (n = 107) | rs4495487 | 0.0009* | TT TC CC | 3.97 (1.86-8.49) | 4.00 (1.44-11.14)* |
|                |                   | rs12343867 | 0.0013* | TT TC CC | 3.52 (1.68-7.38) | 3.55 (1.33-9.92)* |
| JAK2 V617F-negative ET | Healthy volunteers | rs10974944 | 0.068 | CC CG GG | 2.58 (1.14-5.84) | 1.49 (0.50-4.46) |
| (n = 39) | Japanese (n = 107) | rs4495487 | 0.0097* | TT TC CC | 3.28 (1.45-7.43) | 3.05 (0.95-9.58) |
|                |                   | rs12343867 | 0.112 | TT TC CC | 2.26 (1.01-5.04) | 2.08 (0.67-6.40) |
| JAK2 V617F-positive MPN | Healthy volunteers | rs10974944 | 0.0138* | CC CG GG | 2.42 (1.29-4.55) | 2.24 (1.03-4.88)* |
| (PV, ET, and PMF, n = 95) | Japanese (n = 107) | rs4495487 | <0.0001* | TT TC CC | 4.08 (2.13-7.84) | 6.88 (2.88-16.14)* |
|                |                   | rs12343867 | 0.0002* | TT TC CC | 2.89 (1.53-5.45) | 5.54 (2.35-13.06)* |
| JAK2 V617F-negative MPN | Healthy volunteers | rs10974944 | 0.1961 | CC CG GG | 2.01 (0.92-4.37) | 1.14 (0.39-3.37) |
| (PV, ET, and PMF, n = 43) | Japanese (n = 107) | rs4495487 | 0.024* | TT TC CC | 2.79 (1.28-6.08) | 2.48 (0.78-7.80) |
|                |                   | rs12343867 | 0.3060 | TT TC CC | 1.78 (0.82-3.84) | 1.63 (0.58-4.94) |
| JAK2 V617F-positive MPN | JAK2 V617F-negative MPN | rs10974944 | 0.5493 | CC CG GG | 1.21 (0.55-2.66) | 1.82 (0.70-5.35) |
| (PV, ET, and PMF, n = 95) | (PV, ET and PMF, n = 43) | rs4495487 | 0.1762 | TT TC CC | 1.46 (0.66-3.31) | 2.78 (0.93-8.29) |
|                |                   | rs12343867 | 0.1572 | TT TC CC | 1.62 (0.72-3.61) | 2.71 (0.93-7.90) |

aP based on chi-square test. Asterisks indicate statistically significant values.
bGenotype was divided into three groups. Major: no existence of minor allele; Hetero: existence of one minor allele; Homo: existence of two minor alleles.
V617F-negative ET patients without the GCC genotype had thrombosis ($p = 0.0446$), and splenomegaly was more frequently seen in this subset of ET patients ($p = 0.0448$), indicating that JAK2 V617F-negative ET without genetic variation shows a distinct clinical feature. White blood cell counts were significantly elevated in patients with JAK2 V617F-positive ET, regardless of GCC genotype status ($p = 0.0399$) (Figure 2A). Hemoglobin levels were significantly elevated in patients with both JAK2 V617F and the GCC genotype compared to those with the GCC genotype but lacking JAK2 V617F ($p = 0.002$) (Figure 2B). There was no significant difference in platelet counts among the four groups. These results indicate that the proliferative nature of MPNs, such as increased white blood cell and hemoglobin counts, may be linked to JAK2 V617F, regardless of JAK2 genetic variations.

Because all the PV patients in the current study were JAK2 V617F positive, we compared the clinical and hematological features between PV patients with or without the GCC genotype (Table 6). Although there were no significant differences in age, sex, clinical manifestations, or survival between the two groups, platelet count was significantly elevated in PV patients without the GCC genotype ($p = 0.015$) (Figure 2). These findings suggest that germline genetic variation also affects PV patients.

**Discussion**

This is the first study to provide evidence of an association between somatic JAK2 V617F mutation and JAK2 SNPs in a Japanese population of MPN patients. We found a candidate SNP, rs4495487, that may contribute to MPN phenotype in this population. A contribution of this SNP has not been reported in Caucasian populations; however, because it is located between rs1097944 and rs12343867, rs4495487 might be included in the 46/1 haplotype. As in previous reports, we found a significant association between JAK2 SNPs and MPN phenotype in JAK2 V617F-positive MPNs [7-9] and in JAK2 V617F-negative MPNs [10,11].

Although the occurrence of JAK2 V617F greatly contributes to the diagnosis of MPNs, it remains unclear why this single genetic change represents at least three clinical phenotypes (i.e., PV, ET, and PMF). It also remains uncertain whether JAK2 V617F is the primary genetic change responsible for MPNs. Thus, the major obstacle to clarifying the molecular pathogenesis of MPNs is the substantial complexity of the genetic changes, including germline genetic variation of the JAK2 locus.

In the present study, we demonstrated an association between germline genetic variation in the JAK2 locus and MPN phenotype in a Japanese population. Although the clinical manifestation largely depends on JAK2 V617F mutation rather than SNPs in the JAK2 locus of ET patients, we noted that JAK2 V617F-negative ET without the GCC genotype showed a distinct clinical feature, suggesting an underlying genetic change that has not yet been identified. Tefferi et al. [11] demonstrated that nullizygosity for the JAK2 46/1 haplotype is associated with inferior survival. Taken together, these findings suggest that the lack of certain germline genetic variation in JAK2 may contribute to the distinct clinical features observed in JAK2 V617F-negative ET patients. 

### Table 3 Summary of the JAK2 genotype in MPN patients

| Case Population  | GCC genotype  | non-GCC genotype | $P$ (Chi-square test) | Odds ratio | 95% CI       |
|------------------|---------------|------------------|-----------------------|------------|--------------|
|                  | JAK2V617F-positive PV (n = 33) | 46/1 haplotype rs4495987 (+) | 0.0023 | 3.63 | 1.59-8.29 |
|                  | JAK2V617F-positive ET (n = 57) | 46/1 haplotype rs4495987 (+) | 0.0043 | 2.72 | 1.40-5.32 |
|                  | JAK2V617F-negative ET (n = 39) | 46/1 haplotype rs4495987 (+) | 0.0076 | 2.91 | 1.36-6.19 |
|                  | JAK2V617F-positive MPN (n = 95) | 46/1 haplotype rs4495987 (+) | 0.0001 | 3.07 | 1.73-5.46 |
|                  | JAK2V617F-negative MPN (n = 43) | 46/1 haplotype rs4495987 (+) | 0.0289 | 2.26 | 1.01-4.70 |
| Control: non-MPN (n = 107) | 46/1 haplotype rs4495987 (+) | 0.0023 | 3.63 | 1.59-8.29 |

### Table 4 JAK2 V617F allele burden and GCC genotype

| Category | Genotype | JAK2 V617F allele burden | $P$ value |
|----------|----------|--------------------------|-----------|
| PV       | GCC (-)  | 0 | 0 | 7 | 3 | 0.0198 |
|          | GCC (+)  | 0 | 0 | 18 | 5 | 0.4198 |
| ET       | GCC (-)  | 17 | 8 | 14 | 1 | 0.4198 |
|          | GCC (+)  | 22 | 14 | 15 | 5 | 0.4198 |
| PMF      | GCC (-)  | 4 | 1 | 1 | 0 | 0.0198 |
|          | GCC (+)  | 0 | 0 | 2 | 1 | 0.0198 |
| MPN      | GCC (-)  | 21 | 8 | 22 | 4 | 0.6815 |
|          | GCC (+)  | 22 | 15 | 36 | 10 | 0.6815 |
variation may play an important role in the pathogenesis of MPNs. In a study by Trifa et al. [15], the 46/1 haplotype was associated with mutant allele burden > 50% in JAK2 V617F-positive MPN patients. However, we could not find any relationship between allele burden and germline genetic variations. Although we found an association between splenomegaly and JAK2 V617F-negative ET without the GCC genotype, a previous report by Vannucchi et al. [16] demonstrated JAK2 V617F mutation was related to larger spleen size in ET. In addition, we found no significant differences in platelet count among the ET groups, unlike previous reports [16,17]. These discrepancies could be related to differences in the size or ethnics of the analyzed patient cohorts. Therefore, larger studies of Japanese patients should be conducted to clarify the association between JAK2 V617F allele burden and JAK2 haplotype.

According to a recent report by Colaizzo et al. [18], in patients with splanchic venous thrombosis, the JAK2 V617F mutation is frequently found in women and, when interacting with the 46/1 haplotype, it may represent a gender-related susceptibility allele for splanchic venous thrombosis. In the current study, we found no significant differences in platelet count among the ET groups, unlike previous reports [16,17]. These discrepancies could be related to differences in the size or ethnics of the analyzed patient cohorts. Therefore, larger studies of Japanese patients should be conducted to clarify the association between JAK2 V617F allele burden and JAK2 haplotype.

In conclusion, we demonstrated that the C allele of the JAK2 rs4495487 is an additional candidate locus that contributes to MPN predisposition in the Japanese

### Table 5 Demographic and clinical characteristics of ET patients

|                      | JAK2 V617F negative | JAK2 V617F positive |
|----------------------|----------------------|---------------------|
|                      | GCC genotype        | GCC genotype        | P value   |
|                      | No (n = 19)          | Yes (n = 23)        | No (n = 22) | Yes (n = 35) |
| Age, mean (SD)       | 51.2 (18.3)          | 57.9 (17.5)         | 64.8 (14.9) | 66.1 (16.2)  | 0.6466    |
| Sex                  |                      |                     |           |             |
| Female               | 10 (52.6%)           | 14 (60.9%)          | 8 (36.4%)  | 22 (62.9%)  | 0.2965    |
| Male                 | 9 (47.4%)            | 9 (30.1%)           | 14 (64.6%) | 13 (37.1%)  |           |
| Splenomegaly         | 11/16 (68.8%)        | 4/22 (18.2%)        | 5/20 (25%) | 4/33 (12.1%)| 0.0448*   |
| Thrombosis           | 0/11 (0%)            | 5/21 (23.8%)        | 6/17 (35.3%)| 7/30 (23.3%)| 0.0446*   |
| Therapy requirement  | 10/16 (90%)          | 15/22 (68.2%)       | 7/21 (33.3%)| 25/34 (73.5%)| 0.7408    |
| Cytogenetic abnormality | 1/8(0%)              | 0/16 (0%)           | 2/9 (22.2%)| 4/21(19%)   | 0.3618    |
| MF evolution         | 0/16 (0%)            | 1/22 (4.5%)         | 0/15 (0%)  | 1/30 (3.3%) | 0.3787    |
| AML evolution        | 0/11 (0%)            | 1/22 (4.5%)         | 0/15 (0%)  | 0/30 (0%)   | 0.7343    |
| Survival, mean (SD)  | 2036 (2267)          | 1883 (1643)         | 1706 (1847)| 1717 (292)  | 0.6528    |

Statistical analysis was done among four groups. P value is obtained from multivariate analysis. Asterisks indicate statistically significant values.
Table 6 Demographic and clinical characteristics of PV patients

| GCC genotype | No (n = 10) | Yes (n = 23) | P value |
|--------------|------------|-------------|---------|
| Age, mean (SD) | 63.2 (12.4) | 60.7 (14.0) | 0.5779  |
| Sex          |            |             |         |
| Female       | 4 (40%)    | 13 (56.7%)  | 0.4646  |
| Male         | 6 (60%)    | 10 (43.5%)  |         |
| Splenomegaly | 5/10 (50%) | 12/23 (52.2%) | 0.7413 |
| Thrombosis   | 3/10 (30%) | 4/20 (20%)  | 0.4786  |
| Therapy requirement | 9/10 (90%) | 22/23 (95.7%) | 0.2585 |
| Cytogenetic abnormality | 0/10 (0%) | 3/23 (13%) | 0.1781 |
| MF evolution | 3/10 (30%) | 3/23 (13%)  | 0.0924  |
| AML evolution | 1/10 (10%) | 3/23 (13%)  | 0.7614  |
| Survival, median (SD) | 3427 (2336) | 2840 (2822) | 0.7583  |

P value is obtained from multivariate analysis.

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Authors’ contributions
JHO participated in the design and interpretation of the analysis, statistical analysis, and writing of the article. KO planned and coordinated the research. KO, TL, and UT collected samples from patients, and MY performed DNA sequencing and PCR analysis. KO helped to write the article. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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