**Objectives**

While the somatic mutation of Janus Kinase 2 (JAK2) and the thrombopoietin receptor (c-MPL) gene are thought to affect the pathogenesis of bcr/abl negative chronic myeloproliferative neoplasm (MPN), the relationship between the mutation and the clinical features remain obscure.

**Methods**

The mutation status of these genes in granulocytes, platelets, T-cells, and erythroid colonies (BFU-E) was obtained from 115 MPN patients, and then the clinical features of the MPN subtypes were compared.

**Results**

The JAK2-V617F mutation was observed in three lineages of granulocytes, platelets, and BFU-E in almost all polycythemia vera (PV) and primary myelofibrosis (PMF) patients. In contrast, 68% of essential thrombocythemia (ET) patients have the JAK2-V617F mutation in at least one of the lineages, of which 70% of these patients have the JAK2-V617F mutation in three lineages; the remaining ET patients with the JAK2-V617F mutation only exhibited the mutation in one or two lineages. Further, the ET patients that exhibited the JAK2-V617F mutation in three lineages had higher WBC and granulocyte counts as compared to the ET patients that did not have the JAK2-V617F mutation or only had the mutation in one or two lineages. Concerning the MPL gene, two ET patients had the MPL-W515L gene mutation in their platelets, although the lineage of the JAK2-V617F mutation involved differed from case to case.

**Conclusion**

The progenitor cells that are involved with the JAK2-V617F mutation in MPNs are different in each subtype and this difference may also affect the clinical features of MPNs.

**Key words:** myeloproliferative neoplasm, JAK2, clonality, progenitor cells
with regard to the conclusions that can be drawn from these findings (3, 4).

We have previously reported the advantage of studying the JAK2-V617F mutation in platelets in MPN patients, as we have found that approximately 10% of essential thrombocythemia (ET) patients only have the JAK2-V617F mutation in their platelets (5). Based on these results, we decided to investigate the JAK2-V617F mutation status in four cell lineages of granulocytes, platelets, T-cells, and erythroid colonies, and then determine whether there was a difference in the cell lineages that are involved with the JAK2-V617F mutation in each of the MPN subtypes. Therefore, we analyzed 115 MPN patients for the JAK2-V617F mutation status in these four cell lineages. We also examined the patients for the JAK2 exon12 mutation in granulocytes, and the MPL mutation status in granulocytes and platelets. These findings were then compared to the mutation status found for these lineages in each MPN subtype.

Materials and Methods

Patients

A total of 115 patients (25 patients with PV; 82 patients with ET; and 8 patients with PMF) were enrolled in this study. Patients were diagnosed according to the World Health Organization (WHO) classification (6). Study protocols were approved by the Institutional Review Board of Gunma University Hospital, with written informed consent obtained from all patients prior to entry into this study. Cyto-reductive therapy (basically hydroxyurea) was performed in 9 of the 25 patients with PV, 28 of 82 patients with ET, and 1 of the 8 patients with PMF prior to or at the time of sampling.

Isolation of platelets, granulocytes and T-cells, in vitro colony assays, and DNA and RNA preparation

Each heparinized peripheral blood sample (20 mL) was diluted 1 : 1 with Hank’s balanced salt solution and centrifuged at 170 g for 10 minutes, followed by the isolation of platelets from the upper half of the plasma layer. The remaining portion of the sample was subjected to Ficoll-Hypaque density gradient centrifugation at 400 g for 30 minutes. To obtain the erythroid colonies, mononuclear cells were suspended in RPMI 1,640 medium, with 5x10^5 cells then plated in duplicate in 1 mL of methylcellulose medium with or without 3 IU EPO/mL (H 4,330 and H 4,230, respectively, Stem Cell Technologies, Vancouver, Canada). Plates were cultured in 35-mm Petri dishes under a humidified 5% carbon dioxide atmosphere maintained at 37°C. Erythroid colonies were measured at 14 days. More than 100 colonies were analyzed. T-cells recovered from the remaining mononuclear cells were further positively selected using anti-CD3 immunoclonjugated magnetic beads (Miltenyi Biotec, Bergisch Gladbach Germany). Granulocytes were isolated from the pellet after the removal of red blood cells by hypotonic lysis (5).

DNA and RNA preparation and JAK2-V617F mutation analysis

DNA was extracted from granulocytes, T-cells and BFU-E using the RNeasy Mini Kit (QIAGEN, Germantown, MD, USA). Total RNA was reverse transcribed into cDNA in a 20-μL reaction mixture using Superscript III RNase H-Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s recommendations. Using the direct sequencing method previously discussed (5), the JAK2-V617F mutation analyses were performed on the granulocytes and platelets. Due to the limitation of sample numbers, we were unfortunately unable to investigate RNA amounts from BFU-E. The sensitivity of the detection of JAK2-V617F mutation between DNA and RNA samples by sequencing method was almost identical.

JAK2 exon 12 mutation analysis

Using DNA as a template, the JAK2 exon 12 mutation in granulocytes was analyzed by a direct sequencing method in all PV patients (7). Furthermore, the mutation status was analyzed in BFU-E in 3 PV patients who had a mutation in the JAK2 exon 12.

c-MPL gene mutation analysis

RNA from the granulocytes and platelets was used to analyze the thrombopoietin receptor (c-MPL) gene mutation (MPL-W515L/K) in all patients using the following procedure. cDNA (1 μL) was amplified using forward and reverse primers (5’-TCGTTGGCGGACCCAATCTGGGT-3’ and 5’-AGTGTCCTAAAGGTACTGGCTA-3’, respectively). Primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was performed at an annealing temperature of 65°C for 35 cycles, followed by direct sequencing that was performed using the same primers. Three patients were found to have the c-MPL mutation in their granulocytes or platelets. In these patients we performed an additional analysis of the c-MPL gene in BFU-E using a method that has been described elsewhere (2). The sensitivity of the detection of c-MPL gene mutation by RNA was same as that by DNA.

Statistical analysis

All statistical analysis was performed using R software (http://www.r-project.org/). Differences in positive ratios were analyzed using Fisher’s exact test. Differences between
two different groups were analyzed using the Mann-Whitney U test. A probability value of p<0.05 was considered to indicate statistical significance. The population proved to be too small to perform statistical analysis in the PMF group.

Results

For the 25 PV patients, 22 (88.0%) had the JAK2-V617F mutation in granulocytes, with 21 (95.5%) of these patients having the mutation in three lineages of granulocytes, platelets and BFU-E. In the remaining 1 patient, the mutation was only noted in BFU-E (Fig. 1A). Of the 3 PV patients that did not have the JAK2-V617F mutation, 1 patient had the N542-E543del mutation in the JAK2 exon 12 in the granulocytes. Therefore, a total of 23 patients (92.0%) had a mutation in the JAK2 gene in the granulocytes. In the other 2 patients, no genetic aberrations were noted in spite of finding endogenous erythroid colony formation.

In the 82 ET patients, 56 (68.3%) had the JAK2-V617F mutation in at least one of the lineages (Fig. 1B), with 39 of the patients (69.6%) exhibiting the mutation in three lineages of granulocytes, platelets and BFU-E, a value that was significantly lower than that noted for the PV patients (69.6% vs. 95.5%; p<0.05). In the remaining 17 cases, 1 patient (1.8%) exhibited the mutation in the granulocytes. Therefore, a total of 23 patients (92.0%) had a mutation in the JAK2 gene in the granulocytes. In the other 2 patients, no genetic aberrations were noted in spite of finding endogenous erythroid colony formation.

In ET, we divided the patients into three groups: Group 1, patients with the JAK2-V617F mutation in three lineages of granulocytes, platelets and BFU-E; Group 2, patients with the mutation in one or two of the lineages; Group 3, patients who did not have the mutation at all. In a comparison of the clinical features at diagnosis between Group 1 and Group 2, the former had a significantly higher white blood cell (WBC) count as compared to the latter (mean; 13.2×10^9/L vs. 9.5×10^9/L; p<0.05). Group 1 also had higher WBC count than Group 3 (mean; 13.2×10^9/L vs. 8.7×10^9/L; p<0.05), but there was no difference in WBC count between Group 2 and Group 3. Likewise, the granulocyte count in Group 1 was higher than that of Group 2 and Group 3 (mean; 10.0×10^9/L vs. 5.6×10^9/L; p<0.05). No statistically significant differences were seen for either the hemoglobin level or the platelet count in these groups. As for clinical data at sampling, WBC and granulocyte count of Group 1 were higher than that of Group 2 and Group 3 (WBC count mean; 12.0×10^9/L vs. 9.1×10^9/L vs 5.2×10^9/L; p<0.05, respectively). Neither hemoglobin level nor platelet count was statistically different in three groups. In comparing the number of patients receiving therapies, the frequency was 11/39 in Group 1, 8/17 in Group 2, and 9/26 in Group 3, respectively, of which one case each in Group 2 and Group 3 did not receive hydroxyurea (MCNU and anagrelide, respectively). Thus, the frequency
Table 1. The Number of JAK2V617F Positive Cell Lineages and Clinical Data in ET Patients

<table>
<thead>
<tr>
<th>number of JAK2V617F positive lineages</th>
<th>3</th>
<th>1 or 2</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>white cell count (× 10^9/L)</td>
<td>13.2±5.3 *</td>
<td>9.5±2.6</td>
<td>8.7±2.1</td>
</tr>
<tr>
<td>granulocyte count (× 10^9/L)</td>
<td>10.0±5.2 *</td>
<td>6.3±1.9</td>
<td>5.6±1.7</td>
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<tr>
<td>Hemoglobin (g/L)</td>
<td>135±16</td>
<td>135±15</td>
<td>134±21</td>
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<tr>
<td>Platelet count (× 10^9/L)</td>
<td>998±296</td>
<td>1011±484</td>
<td>1089±529</td>
</tr>
<tr>
<td>at sampling</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>white cell count (× 10^9/L)</td>
<td>12.0±4.4 *</td>
<td>9.1±3.6</td>
<td>7.8±2.5</td>
</tr>
<tr>
<td>granulocyte count (× 10^9/L)</td>
<td>9.2±4.2 *</td>
<td>5.7±2.4</td>
<td>5.2±1.8</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>135±22</td>
<td>128±20</td>
<td>126±22</td>
</tr>
<tr>
<td>Platelet count (× 10^9/L)</td>
<td>906±344</td>
<td>947±491</td>
<td>783±300</td>
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</tbody>
</table>

*p < 0.05

of receiving cytoreductive therapy did not differ in the three groups (Table 1).

In PMF, 6 (75.0%) out of 8 patients had the JAK2-V617F mutation in at least one lineage. In 5 (83%) out of these 6 patients, the JAK2-V617F mutation was observed in all three lineages, with the remaining patient having the mutation only in the platelets (Fig. 1C). Although our study was limited by the small number of enrolled subjects, PMF patients having the JAK2-V617F mutation tended to have the mutation in all three of the lineages.

For the c-MPL gene, 1 PMF patient and 2 ET patients exhibited the MPL gene mutation. In the PMF patient, the MPL gene mutation was detected in the granulocytes, platelets and BFU-E. On the other hand, the MPL-W515L mutation was only detected in the platelets in both of the ET cases. Interestingly, one of the ET patients exhibited no JAK2-V617F mutation in any of the three lineages, while the other ET patient had the JAK2-V617F mutation in all three lineages. Thus, there was a discrepancy in that the involved cell lineages differed between those patients with the JAK2-V617F mutation and those with the MPL-W515L mutation. As for the PV patients, the MPL gene mutation was not detected in any of the cases. In T-cells, neither JAK-2 gene nor MPL gene had a mutation in all cases.

Discussion

Numerous MPN patients have been found to have the JAK2-V617F mutation, which is a mutation that may hold the key to understanding the pathogenesis of this disorder. However, the mutation status within various cell lineages in MPN patients has yet to be clarified. In the present study, approximately 90% of the PV patients had the JAK2-V617F mutation, with most exhibiting the mutation in three lineages of granulocytes, platelets and BFU-E. But T-cells did not have the JAK2-V617F mutation in all cases. This implies that the JAK2-V617F mutation occurs in common progenitor cells of the granulocytes, platelets and erythrocytes in almost all PV patients. As for PMF, most of the patients with the JAK2-V617F mutation also had the mutation in three lineages, suggesting that it occurs in pluripotent stem cells as well.

In contrast, in the present study approximately 70% of ET patients were found to have the JAK2-V617F mutation in at least one of the lineages, of which one-third of these ET patients only had the mutation in one or two lineages. When compared to the findings for PV and PMF, the results imply that the JAK2-V617F mutation occurs in the more mature progenitor cells in ET. Our results are coincident with the fact that the JAK2-V617F CD34+ allele burden was low in ET compared with PV or MF (8). As BFU-E was obtained by the addition of erythropoietin, there is a possibility that selection bias might exist and miss the low burden of JAK2-V617F positive cells. However, AS-PCR method can detect 1% of JAK2-V617F positive cells, so this possibility is very low.

In comparing the clinical data in ET, when the JAK2-V617F mutation occurs in three lineages of granulocytes, platelets and BFU-E, there is also a higher WBC and granulocyte count at diagnosis as compared to when the mutation does not occur or it only occurs in one or two lineages. As hydroxyurea has been reported to reduce JAK2-V617F positive clone (9-11), cytoreductive therapies might affect these results. However, recent studies have demonstrated that the reduction of the allele burden was relatively low in ET patients (12, 13). Thus, the effect of hydroxyurea on the JAK2-V617F allele burden is controversial. The reason for this discrepancy might in part be due to whether patients received sufficient hydroxyurea aiming for complete response (CR) in the European Leukemia Net consensus or not (12-14). In this respect, in the present study the platelet count in Group 1, Group 2 and Group 3 at the time of sampling was 906×10^9/L, 947×10^9/L, and 783×10^9/L, respectively. This implies that hydroxyurea administration in our cases was mild so that reduction of JAK2-V617F allele burden might be low. Further, the frequency of receiving cy-
toreductive therapy was not different in three groups suggesting that the influence of therapies on our results seems to be limited. Some investigators have reported that the amount of the JAK2-V617F allele burden gene contributes to the clinical phenotype (15). In addition, our results also seem to suggest that the differential level of progenitor cells having the JAK2-V617F mutation may also lead to variations of the clinical features that are seen in ET.

The present study examined two ET patients and found that the cell lineages involved with the JAK2-V617F mutation were different from those with the MPL-W515L mutation (3, 4). Whether acquisition of the JAK2-V617F mutation is an early event or not in the pathogenesis of MPNs is one of the important issues to be resolved. The results of an X-chromosome based clonality assay and chromosome analysis performed in MPN patients suggested that the JAK2-V617F mutation is not a primary event in the progression of the disease (16-21). In addition, chromosomal abnormalities or other gene mutations have been reported to precede acquisition of JAK2-V617F in hematopoietic progenitor cells (18-21), suggesting that JAK2-V617F might be associated with clonal hematopoeisis that is caused by an unknown mechanism. Our observations reflect this model.

When taken together, we speculate that the preceding mutation is followed by the occurrence of JAK2 and/or MPL gene mutations at various levels of the progenitor cells, which can then lead to a variety of MPN subtypes. However, further studies will need to be undertaken in order to definitively elucidate the precise pathogenesis of these MPNs.

The authors state that they have no Conflict of Interest (COI).

References