Association of Azathioprine Metabolite Levels and Adverse Reactions in Japanese Patients with Inflammatory Bowel Disease

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Abstract: Azathioprine (AZA) is administered for treatment of inflammatory bowel disease (IBD), though it frequently induces severe adverse reactions (ARs). AZA is converted to the active metabolite 6-thioguanine nucleotide (6-TGN) through 6-mercaptopurine (6-MP) and 6-thiosine phosphate (6-TIP) by various metabolic enzymes, with 6-MP and 6-TIP excreted after methylation. Prior studies have found that a 6-TGN level above the threshold of 450 pmol/8x10⁸ red blood cells is associated with increased risk of myelotoxicity. In the present study, we investigated the association of blood concentrations of AZA metabolites with ARs.

Blood samples were collected over a period of 52 weeks from 49 Japanese IBD patients prescribed with AZA. After removing protein, we measured the levels of dephosphorylated AZA metabolites using liquid chromatography and mass spectrometry in tandem, then categorized the metabolites into 6-MPs, 6-methylmercaptopurines (6-MMPs), and 6-TGNs, and compared their levels between patients with and without ARs. Statistical analysis was performed using a Mann-Whitney U-test.

ARs developed in 14 of the 49 patients (28.6%). The average concentrations of 6-MMPs and the 6-MMPs/6-TGNs ratio in patients without were significantly greater as compared to those with ARs (P<0.05). In addition, leukopenia was detected in 10 (20.4%) of the patients with ARs and 6-TGNs levels were significantly higher in leukopenia patients (P<0.01). Accordingly, we consider that monitoring is useful for predicting AR occurrence.

Keywords: Inflammatory bowel disease, Azathioprine, 6-Thioguanine, 6-Methylmerucaptoprine.

1. INTRODUCTION

Azathioprine (AZA) is used for treating inflammatory bowel disease (IBD), including ulcerative colitis and Crohn’s disease. However, it frequently induces myelosuppression, such as leukopenia and agranulocytosis, as well as pancreatitis, hepatitis, alopecia, rash, and flu-like symptoms [1, 2].

Following oral administration, AZA is absorbed into plasma and converted to 6-mercaptopurine (6-MP) or glutathionyl imidazole by glutathione transferase. Next, 6-MP is mainly metabolized to 6-thiouric acid by xanthine oxidase (XO) and excreted in urine, while which has escaped metabolism by XO is converted to an active metabolite as 6-thiosine triphosphosphate (6-TITP) or 6-thioguanine nucleotide (6-TGN) via 6-thiosine monophosphate (6-TIMP). These metabolites are then methylated for inactivation by thiopurine-S-methyltransferase (TPMT) through each pathway. Furthermore, conversion of 6-TITP to 6-TIMP is related to inosine triphosphate pyrophosphohydrolase (ITPA) (Figure 1) [3]. Individuals with lower activity levels of these enzymes show accumulation of 6-TGN and 6-TITP [4, 5].

Recently, many studies have investigated single nucleotide polymorphisms in nucleoside diphosphate-linked moiety X-type motif 15 (NDT15), which is strongly associated with thiopurine-induced myelosuppression [6-8]. Accordingly, analysis of those genetic polymorphisms provides helpful information for decision-making regarding administration and dosage of thiopurine. However, the cost of and time required for such analysis can be high, since many other enzymes participate in the AZA pathway. Furthermore, if the patient does not have mutations, the possibility of an adverse reaction remains. On the other hand, previous studies that focused on AZA metabolites have reported that 6-TGN levels above 450 pmol/8x10⁸ red blood cells (RBCs) were associated with myelotoxicity [9, 10].

The activity of TPMT in Japanese individuals is reported to be lower as compared to other ethnic groups [11, 12]. Thus, in Japan, the regular dosage of AZA (1.0 mg/kg/day) is about half of that used in other countries. In our previous investigation, we measured the concentration of 6-TGN over a period of 52 weeks in IBD patients, and also analyzed genotypes of TPMT.
However, those mutations were found in only some of the patients who had adverse reactions [13]. Additionally, determination of the concentration of 6-TGN with high performance liquid chromatography in that study was not effective to accurately estimate adverse reactions.

In the present study, we measured the concentrations of 6-TGN and other AZA metabolites to investigate their relationship with adverse reactions. To the best of our knowledge, this is first study conducted in Japan for monitoring AZA metabolite levels over an extended period using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2. MATERIALS & METHODS

Subjects

We examined 49 Japanese IBD patients receiving treatment at Jikei University School of Medicine Kashiwa Hospital. This study was approved by the ethics committee of that hospital (27-323(8208)), and the committee of Niigata University of Pharmacy and Applied Life Sciences (H29-001). All participants received an explanation regarding the purpose of the study and methods involved prior to enrollment, and each provided individual consent.

From March 2008 to June 2011, whole blood samples were drawn at 0, 1, and 2 weeks, and then every 4 weeks thereafter after beginning AZA administration (0-52 weeks, mean 46.8 weeks). For the patients in this study, the initial AZA dosage was 1.0 mg/kg/day [14, 15].

AZA Metabolites

We categorized AZA metabolites into 3 groups, 6-TGNs, 6-MMPs, and 6-MPs (Figure 1), and then measured their concentrations using LC-MS/MS.
Deproteinization and hydrolyzation of blood samples were performed prior to LC-MS/MS analysis. Each sample was collected and placed into an EDTA-2K tube, then stored at -20°C before further processing. For testing, 250 µL of blood, 25 µL of dithiothreitol (100 mg/mL), 25 µL of 70% perchloric acid, and 250 µL of dichloromethane were added together into a new tube. After vortexing for 30 seconds, the tube was centrifuged for 15 minutes at 13,000 rpm, then 200 µL of the supernatant was transferred to another tube and hydrolyzation was performed for 120 minutes at 105°C. The mixture was then cooled to room temperature and an additional centrifugation was performed for 3 minutes at 3000 rpm. Next, it was vortexed for 30 seconds prior to addition of 50 µL of 2 M NaOH, then that mixture was centrifuged for 5 minutes at 13,000 rpm. Finally, 20 µL of supernatant was diluted with 1 mL of purified water and that was regarded as a sample for LC-MS/MS.

For analysis, 10 µL of each sample was analyzed at 300 µL/minute at 30°C. Isocratic analysis was performed using an A mobile phase, which was mixed with 2 mM of ammonium acetate and 0.05% formic acid, and a B phase, which was mixed with methanol, for an A:B ratio of 95:5. LC-MS/MS was performed with a UPLC I-class Xevo TQ-S (Waters Corp.) and ACQUITY UPLC® HSS T3 column (Waters Corp.). Diurnal and daily variations for this analysis were 3.0-3.5% (6-TGNs) and 4.4-6.2%, respectively.

### Statistical Analysis

For the present study, leukopenia was defined as a white blood cell count less than 2500/µL, while hepatitis was defined as elevated serum alanine aminotransferase and aspartate aminotransferase levels above the upper limit of the normal range (30 IU/L). We determined the average concentrations of AZA metabolites from weeks 4 to 52. Measurements obtained at weeks 0 to 2 were excluded, because the levels were unstable during that period. Differences in AZA metabolite levels between subjects with and without an adverse reaction were compared using a Mann-Whitney U test, and p values <0.05 were considered to indicate significance. All analyses were performed using BellCurve® for Excel (Social Survey Research Information Co., Ltd.).

### 3. RESULTS & DISCUSSION

Adverse reactions developed in 14 (28.6%) of the 49 patients, including leukopenia in 10 (20.4%), alopecia in 3 (6.1%), hepatitis in 1 (2.0%), and rash in 1 (2.1%). One patient with leukopenia developed agranulocytosis and alopecia (Table 1). Previous studies have suggested that a 6-TGNs level greater than 230 pmol/8x10^8 RBCs was related to effectiveness [9] and that above 450 pmol/8x10^8 RBCs was related to myelotoxicity [10]. Furthermore, a 6-

### Table 1: Patients with Adverse Reactions During AZA Therapy for IBD

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (years)</th>
<th>sex</th>
<th>Disease</th>
<th>Adverse Reaction</th>
<th>Duration (Days)</th>
<th>Continuation of Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>F</td>
<td>UC</td>
<td>Leukopenia</td>
<td>116</td>
<td>Continued’</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>M</td>
<td>UC</td>
<td>Leukopenia</td>
<td>117</td>
<td>Continued’</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>M</td>
<td>CD</td>
<td>Leukopenia</td>
<td>7</td>
<td>Continued’</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>F</td>
<td>UC</td>
<td>Leukopenia</td>
<td>40</td>
<td>Discontinued</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>F</td>
<td>UC</td>
<td>Leukopenia</td>
<td>135</td>
<td>Continued'</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>F</td>
<td>CD</td>
<td>Leukopenia</td>
<td>81</td>
<td>Continued’</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
<td>F</td>
<td>CD</td>
<td>Leukopenia</td>
<td>91</td>
<td>Discontinued</td>
</tr>
<tr>
<td>8</td>
<td>58</td>
<td>M</td>
<td>UC</td>
<td>Leukopenia</td>
<td>15</td>
<td>Discontinued</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
<td>F</td>
<td>UC</td>
<td>Leukopenia</td>
<td>364</td>
<td>Continued’</td>
</tr>
<tr>
<td>10</td>
<td>31</td>
<td>F</td>
<td>UC</td>
<td>Alopecia</td>
<td>59</td>
<td>Continued’</td>
</tr>
<tr>
<td>11</td>
<td>16</td>
<td>F</td>
<td>UC</td>
<td>Agranulocytosis, alopecia</td>
<td>21</td>
<td>Discontinued</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>F</td>
<td>UC</td>
<td>Alopecia</td>
<td>21</td>
<td>Discontinued</td>
</tr>
<tr>
<td>13</td>
<td>65</td>
<td>F</td>
<td>UC</td>
<td>Hepatitis</td>
<td>60</td>
<td>Discontinued</td>
</tr>
<tr>
<td>14</td>
<td>24</td>
<td>M</td>
<td>CD</td>
<td>Rash</td>
<td>147</td>
<td>Continued’</td>
</tr>
</tbody>
</table>

AZA, azathioprine; IBD, inflammatory bowel disease; F, female; M, male; UC, ulcerative colitis; CD, Crohn’s disease.

*Continued after temporary discontinuation.*’Continued with reduced dose.*’Continued at same dose.
MMPs level above 5700 pmol/8x10⁸ RBCs has been shown to be associated with increased risk of myelotoxicity and hepatotoxicity [9, 17].

Comparisons of the patients with leukopenia are presented in Table 2 and Figure 2. We found that leukopenia developed in 10 (20.4%), none of whom showed an average concentration of 6-TGNs above 450 pmol/8x10⁸ RBCs. Nevertheless, the average 6-TGNs level was higher in patients with as compared to those without leukopenia (p<0.01). In contrast, the level of 6-MPs and 6-MMPs/6-TGNs ratio in the without-leukopenia group showed a tendency to be higher as compared to the with-leukopenia group, though the differences were not significant. Accordingly, a high level of 6-TGNs is considered to be associated with an increased risk of myelotoxicity, which is not inconsistent with previous reports [9, 10].

Table 2: Characteristics of Leukopenia

<table>
<thead>
<tr>
<th></th>
<th>Leukopenia Patients n=10 (20.4%)</th>
<th>Without Leukopenia n=39 (79.6%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-TGNs (pmol/8x10⁸ RBCs)</td>
<td>209.2 ± 42.7 (218.7)</td>
<td>178.3 ± 12.9 (180.6)</td>
<td>0.0052*</td>
</tr>
<tr>
<td>6-TGNs &gt;450-&lt;450</td>
<td>0:10</td>
<td>1:38</td>
<td>-</td>
</tr>
<tr>
<td>6-MMPs (pmol/8x10⁸ RBCs)</td>
<td>281.5 ± 90.8 (270.9)</td>
<td>297.9 ± 28.5 (300.9)</td>
<td>0.2282</td>
</tr>
<tr>
<td>6-MPs (pmol/8x10⁸ RBCs)</td>
<td>19.9 ± 18.5 (14.7)</td>
<td>21.3 ± 5.3 (21.8)</td>
<td>0.0955</td>
</tr>
<tr>
<td>6-MMPs/6-TGNs</td>
<td>2.0 ± 0.6 (1.8)</td>
<td>2.2 ± 0.2 (2.2)</td>
<td>0.0648</td>
</tr>
</tbody>
</table>

Values are shown as the mean ± standard deviation (median). Mann-Whitney U test; *P<0.05, **P<0.01.

Figure 2: Changes in concentrations of AZA metabolites over time in patients with and without leukopenia.

a. The average 6-TGNs level was higher in patients with as compared to those without leukopenia (p<0.01).
b. The mean level of 6-MMPs was not significantly different between the groups.
c. The average concentration of 6-MPs and the 6-MMPs/6-TGNs ratio in the without-leukopenia group showed a tendency to be higher as compared to the with-leukopenia group, though the differences were not significant.
Adverse reactions in the present cohort are presented in Table 3 and Figure 3. One patient (2.0%) had an average 6-TGNs concentration above 450 pmol/8x10^8 RBCs, though did not show any adverse reaction, whereas 6 of 14 patients with adverse reactions exhibited a low 6-TGNs level (<100 pmol/8x10^8 RBCs). The average concentrations of 6-TGNs, 6-MPs, and 6-MMPs in patients without adverse reactions were higher as compared to those with adverse reactions, with the concentration of 6-MMPs significantly different between those groups (p<0.05). Additionally, the 6-MMPs/6-TGNs ratio in patients without adverse reactions was significantly higher as compared to the group with adverse reactions (p<0.05). Accordingly, there is a possibility that adverse reactions will occur in patients with a low level of 6-TGNs. Therefore, we recommend that measurement of the concentrations of several AZA metabolites, not only 6-TGNs, is important for predicting the possibility of an adverse reaction.

Table 3: Characteristics of Adverse Reactions (ARs)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>With ARs n=14 (28.6%)</th>
<th>Without ARs n=35 (71.4%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-TGNs (pmol/8x10^8 RBCs)</td>
<td>178.6 ± 35.8 (183.4)</td>
<td>184.3 ± 15.9 (184.4)</td>
<td>0.7779</td>
</tr>
<tr>
<td>6-TGNs &gt;450:&lt;450</td>
<td>0:14</td>
<td>1:34</td>
<td>-</td>
</tr>
<tr>
<td>6-MMPs (pmol/8x10^8 RBCs)</td>
<td>260.7 ± 79.5 (243.2)</td>
<td>306.1 ± 33.1 (307.1)</td>
<td>0.0333*</td>
</tr>
<tr>
<td>6-MPs (pmol/8x10^8 RBCs)</td>
<td>19.2 ± 12.2 (16.3)</td>
<td>21.9 ± 5.1 (23.6)</td>
<td>0.0857</td>
</tr>
<tr>
<td>6-MMPs/6-TGNs</td>
<td>1.9 ± 0.4 (1.9)</td>
<td>2.3 ± 0.2 (2.2)</td>
<td>0.021*</td>
</tr>
</tbody>
</table>

Values are shown as the mean ± standard deviation (median). Mann-Whitney U test; *P<0.05.

Figure 3: Changes in concentrations of AZA metabolites over time in patients with and without adverse reactions.

The average concentrations of 6-TGNs, 6-MPs, and 6-MMPs, and the 6-MMPs/6-TGNs ratio in patients without adverse reactions were higher as compared to those with adverse reactions. Furthermore, the concentration of 6-MMPs and the 6-MMPs/6-TGNs ratio were significantly different between those groups (p<0.05).
considered that the AZA metabolite measurement levels were reduced because the blood samples were collected more than 5 years prior to analysis. A comparison of 6-TGNs levels between the present and our previous study [13], which used HPLC, is presented in Figure 4. We found that the levels in the present samples were generally reduced by approximately half, thus we judged the results of this study to be justified because a correlation was found ($R^2 = 0.4217$).

Genetic polymorphisms of various metabolic enzymes are considered to induce individual differences in regard to AZA therapeutics and those relationships are gradually becoming elucidated. However, to the best of our knowledge, no report has been presented in regard to clinical application. Investigations of genetic polymorphisms, including NUDT15, are also important for revealing the mechanisms related to drug-induced individual differences. Genotyping and metabolite analysis may have complementary roles for clarifying the multiple processes involved in drug reactions. Prediction of an adverse reaction after starting administration is difficult when using only gene test results, while that can be more effectively performed by monitoring AZA metabolite levels, as shown by the present results. Additionally, when possible, determination of individual metabolites, e.g., 6-TGTP, can be useful for therapeutic drug monitoring.

An NUDT15 gene test prior to starting AZA administration will soon be approved for coverage by national insurance in Japan. In the future, we plan to perform an investigation to determine whether an examination with only NUDT15 is adequate and will also continue other analyses related to monitoring patients receiving AZA for prediction of adverse reactions. In addition, a collaborative study with a larger number of patients will be necessary.

**CONFLICTS OF INTEREST STATEMENT**

The authors have no conflicts of interest to declare in association with this study.

**REFERENCE**


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