Clinical implications of thiopurine methyltransferase genotyping in patients with inflammatory bowel disease

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Clinical implications of thiopurine methyltransferase genotyping in patients with inflammatory bowel disease

Directed by Professor Won Ho Kim

The Master’s Thesis submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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June 2002
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Jae Hak Kim
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ABSTRACT

Clinical implications of thiopurine methyltransferase genotyping in patients with inflammatory bowel disease

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The metabolic pathway catalyzed by thiopurine methyltransferase (TPMT) is in competition with a pathway that leads from 6-mercaptopurine to 6-thioguanine nucleotides. TPMT activity exhibits genetic polymorphism. Approximately 90% of Caucasian subjects was reported as homozygous for an allele for high RBC TPMT activity, about 10% was heterozygous and had intermediate activity, and about one of every 300 subjects was homozygous for an allele for low RBC TPMT and lacked detectable
enzyme activity. The correlations between phenotype and genotype of TPMT have been reported greater than 95%.

Using polymerase chain reaction (PCR) method, common TPMT genotypes in one hundred subjects with no disease and three hundred subjects with inflammatory bowel disease (IBD) were studied. The correlations between the genotype of TPMT and bone marrow suppression during the treatment with AZA or 6-MP were made.

G238C, G460A, and A719G polymorphism was not detected in normal subjects (0/200 alleles) and in the patients with IBD (0/600 alleles. But the frequency of bone marrow suppression during the treatment with AZA or 6-MP was considerably higher (31.3%) than what was reported in previous studies (5%). There was no correlation between thiopurine- induced bone marrow suppression and TPMT genotype.
Keywords: Inflammatory bowel disease, TPMT genotype,
Azathioprine, 6-mercaptopurine, Bone marrow suppression
Clinical implications of thiopurine methyltransferase genotyping in patients with inflammatory bowel disease

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I. Introduction

To treat inflammatory bowel disease (IBD), sulfasalazine, its analogs, and corticosteroids are frequently used.\textsuperscript{1,2} But, it is sometimes refractory to these drugs and adverse effects of corticosteroids must be taken into consideration. Immune modulating agents, such as azathioprine (AZA) and 6-mercaptopurine (6-MP), are indicated for treating active disease, steroid sparing, and remission maintenance.\textsuperscript{3-6} However, these thiopurine drugs also cause side effects such as allergic-type reactions (pancreatitis, fever, rash, arthralgias, malaise, nausea,
diarrhea) and non-allergic-type reactions (leukopenia, thrombocytopenia, infection, hepatitis, malignancy).\textsuperscript{7} Especially more profound bone marrow suppression requiring hospitalization was seen in about 2\%.\textsuperscript{8}

As illustrated in Figure 1, AZA is rapidly converted to 6-MP and the conversion of 6-MP to thioinosine 5'-monophosphate (TIMP) is catalyzed by hypoxanthine phosphoribosyltransferase and a series of kinases to give thioguanine nucleotides (TGN), which can be incorporated in DNA and cause cell death.\textsuperscript{9,10} Alternatively, 6-MP is inactivated by xanthine oxidase (XO) or thiopurine methyltransferase (TPMT). The methylation of 6-MP and TIMP is catalyzed by TPMT.\textsuperscript{11}

TPMT is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds.\textsuperscript{11} Human TPMT gene is approximately 34kb in length and consists of 10 exons, 8 of which encodes protein. The active gene was mapped to the short arm of chromosome 6 within band 6p22.3.\textsuperscript{12} TPMT activity exhibits genetic polymorphism. Approximately 90\% of Caucasian subjects was reported as homozygous for an allele for high RBC TPMT activity, about 10\% was heterozygous and had intermediate activity,
and about one of every 300 subjects was homozygous for an allele for low RBC TPMT and lacked detectable enzyme activity.\(^{13}\) But, this trimodal frequency distribution was not shown in studies on Korean population.\(^{14,15}\)

There are several relatively frequent mutations in the TPMT gene that are responsible for the low TPMT activities. The wild-type allele is designated as TPMT\(^*1\) and the four novel variant alleles are TPMT\(^*2\) (G238C; Ala80→Pro), TPMT\(^*3A\) (G460A and A719G; Ala154→Thr and Tyr240→Cys), TPMT\(^*3B\) (G460A; Ala154→Thr), and TPMT\(^*3C\) (A719G; Tyr240→Cys) (Figure 2). These account for more than 80% of the defective phenotypes. Additional rare variants, such as TPMT\(^*3D\), TPMT\(^*4\), TPMT\(^*5\), and TPMT\(^*6\), have been reported.\(^{16}\) The correlations between phenotype and genotype of TPMT have been reported to be greater than 95%.\(^{17,18}\)

The aim of this study was to find the frequency of TPMT genotype in one hundred subjects with no disease and three hundred patients with IBD. The correlations between the genotype of TPMT and bone marrow suppression during the treatment with AZA or 6-MP were made.
Figure 1. Azathioprine and 6-mercaptopurine metabolism

MP, mercaptopurine;
TPMT, thiopurine methyltransferase;
XO, xanthine oxidase;
HPRT, hypoxanthine phosphoribosyltransferase;
TGN, thioguanine nucleotides;
TIMP, thioinosine monophosphate;
IMPDH, inosine monophosphate dehydrogenase
Figure 2. Human TPMT polymorphisms. The figure shows schematically the wild-type allele for TPMT*1 and four variant alleles for low enzyme activity. Exons are numbered and shown as rectangles, with black areas representing the open reading frame and white areas representing 3’- and 5’-untranslated regions.
II. Materials and Methods

1. Normal subjects and patients

Blood samples were obtained from one hundred adult blood donors at the blood bank of Severance Hospital and from three hundred unrelated patients with IBD who visited Severance hospital between Oct. 2001 and Jan. 2002. All patients were asked to participate in the study, and patients agreed. Information about this study was presented to the patients prior to the study.

2. TPMT genotyping assay

Genotyping was performed with minor modifications to the use of PCR by Yates et al.\textsuperscript{17} and Kumagai et al.\textsuperscript{19}

DNA was extracted by genomic DNA isolation kit provided by Puregene (Minneapolis, MN, USA). All PCR was performed with thermocycler (Geneamp PCR system 9600, Perkin-Elmer, Norwalk, CT, USA). A commercially available AccuPower® PCR PreMix was provided by Bioneer (Taejon, Korea) and all primers as 20 pmole were also provided (Table 1). Acc I and Mwo I restriction enzymes and digestion buffers were provided by New England Biolabs.
(Beverly, MA, USA). QIA quick gel extraction kit by QIAGEN
(Chatsworth, CA, USA) was used for DNA illusion. PCR products
and digested fragments were analyzed on 1.5% agarose gel
electrophoresis by Metaphor (FMC bioproducts, Rockland, ME,
USA) in the presence of ethidium bromide by Sigma- Aldrich (Milan,
Italy).
Table 1. Sequences of oligonucleotides used in the experiment

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>G238C sense</td>
<td>5'- GTATGATTTTATGCAGGTTTG- 3'</td>
</tr>
<tr>
<td>G238C mutant</td>
<td>5'- GTATGATTTTATGCAGGTTTC- 3'</td>
</tr>
<tr>
<td>sense</td>
<td></td>
</tr>
<tr>
<td>G238C common</td>
<td>5'- TAAATAGGAACCATCGGACAC- 3'</td>
</tr>
<tr>
<td>antisense</td>
<td></td>
</tr>
<tr>
<td>G460A sense</td>
<td>5'- ATAACAGAGTGGGAGGCCTGC- 3'</td>
</tr>
<tr>
<td>G460A antisense</td>
<td>5'- CTAGAACCAGAAAAAGTAGATA- 3'</td>
</tr>
<tr>
<td>A719G sense</td>
<td>5'- GAGACAGAGTTTCACTACTGG- 3'</td>
</tr>
<tr>
<td>A719G antisense</td>
<td>5'- AGGCTTTAGCATAATTTCAATTCCCTC- 3'</td>
</tr>
</tbody>
</table>
3. Detection of G238C

The final volume for all PCR assays was 20 µl. By the use of 50 ng of genomic DNA (5 µl) as a template, PCR was performed with 0.2 µl of mixed G238C wild specific sense and 0.2 µl of G238C mutant specific sense with 0.2 µl of G238C common antisense in the PCR premixture. With thermocycler, after 94 °C for 5 minutes, amplification was done for 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 30 seconds. A final extension step at 72 °C for 7 minutes was also performed. PCR products were analyzed by electrophoresis in 1.5% Metaphor gels containing ethidium bromide. The DNA fragment can be amplified with a G238C wild specific sense primer in addition to a G238C common antisense primer in the wild-type allele, whereas it can be amplified with a G238C mutant specific sense primer in the TPMT*2 allele.

4. Detection of G460A

A PCR assay with G460A sense and G460A antisense, 0.2 µl of each primer per reaction tube, was done under conditions similar to those discussed above, except for 100 ng of genomic DNA as a
template and 61 °C for 45 seconds of annealing step. The PCR product was analyzed by electrophoresis and was extracted using QIA quick gel extraction kit. Mwo I was used to digest the products for 2 hour at 60 °C. Digested products were analyzed by gel electrophoresis. Mwo I digestion of wild-type DNA yields fragments of 267 and 98 base pairs, whereas DNA containing the G460A polymorphism was not digested and yields an uncleaved fragment of 365 base pairs.

5. Detection of A719G

A PCR assay with A719G sense and A719G antisense was conducted under the similar conditions as those used for the G238C polymorphism except for slight modifications. Modifications included 29 cycles of amplification and annealing temperature of 62 °C for 30 seconds. The PCR products were extracted by the same method as those used for the G460A polymorphism and analyzed using gel electrophoresis. A slight modifications to the existing protocols included digestion with Acc I for 2 hours at 37 degrees C. The A719G polymorphism can introduce an Acc I restriction site in the amplified fragment and yield fragments of 283
and 90 base pairs. Wild-type DNA yields an uncleaved fragment of 373 base pairs.

6. Data analysis

Statistical analysis was performed using SPSS 10.0 for Windows (SPSS Inc, Chicago, IL, USA).

III. Results

1. Patient characteristics

Table 2 summarizes the demographic characteristics of three hundred patients with IBD. Immune modulating agents, such as AZA and 6-MP were used in forty-eight patients. Among them, eighteen patients developed side effects during the treatment with AZA, or 6-MP including bone marrow suppression, fever, joint pain, and vomiting.

2. Detection of G238C

G238C polymorphism was not detected in normal subjects and in patients with IBD (0%, 0/200 alleles; 0%. 0/600 alleles, Figure 3).
3. Detection of G460A

G460A polymorphism was not detected in normal subjects (0%, 0/200 alleles) and in patients with IBD (0%, 0/600 alleles, Figure 4).

4. Detection of A719G

A719G polymorphism was not detected in normal subjects and in patients with IBD (0%, 0/200 alleles; 0%, 0/600 alleles, Figure 5).

5. Genotypes of TPMT

TPMT *2, TPMT *3A, *3B, and *3C were not observed in normal subjects and in patients with IBD.
## Table 2. Details of patients with inflammatory bowel disease

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Count (Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (M:F)</strong></td>
<td>138:162</td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
<td>40.8 (range: 15-79)</td>
</tr>
<tr>
<td><strong>Type of disease</strong></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>78 (26.0%)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>150 (50.0%)</td>
</tr>
<tr>
<td>Behcet’s disease</td>
<td>70 (23.3%)</td>
</tr>
<tr>
<td>Etc</td>
<td>2 (0.7%)</td>
</tr>
<tr>
<td><strong>Disease and location</strong></td>
<td></td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td></td>
</tr>
<tr>
<td>small bowel</td>
<td>12 (15.4%)</td>
</tr>
<tr>
<td>small bowel with perianal lesion</td>
<td>1 (1.3%)</td>
</tr>
<tr>
<td>ileocolitis</td>
<td>19 (24.4%)</td>
</tr>
<tr>
<td>ileocolitis with perianal lesion</td>
<td>16 (20.5%)</td>
</tr>
<tr>
<td>colitis</td>
<td>19 (24.4%)</td>
</tr>
<tr>
<td>colitis with perianal lesion</td>
<td>5 (6.4%)</td>
</tr>
<tr>
<td>remission</td>
<td>4 (5.1%)</td>
</tr>
<tr>
<td>anastomosis</td>
<td>2 (2.5%)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td></td>
</tr>
<tr>
<td>left-sided</td>
<td>89 (59.4%)</td>
</tr>
<tr>
<td>pancolitis</td>
<td>58 (38.6%)</td>
</tr>
<tr>
<td>remission</td>
<td>2 (1.3%)</td>
</tr>
<tr>
<td>anastomosis</td>
<td>1 (0.7%)</td>
</tr>
<tr>
<td>Behcet’s disease</td>
<td></td>
</tr>
<tr>
<td>ileocecal</td>
<td>53 (75.7%)</td>
</tr>
<tr>
<td>other part of colon</td>
<td>6 (8.6%)</td>
</tr>
<tr>
<td>remission</td>
<td>3 (4.3%)</td>
</tr>
<tr>
<td>anastomosis</td>
<td>8 (11.4%)</td>
</tr>
<tr>
<td>Details of patients with inflammatory bowel disease</td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Immune modulating agents use 48 (16%)</td>
<td></td>
</tr>
<tr>
<td>Azathioprine 41 (85.4%)</td>
<td></td>
</tr>
<tr>
<td>6-mercaptopurine 7 (14.6%)</td>
<td></td>
</tr>
<tr>
<td>Indications</td>
<td></td>
</tr>
<tr>
<td>Steroid dependent 34 (70.8%)</td>
<td></td>
</tr>
<tr>
<td>Steroid refractory 8 (16.7%)</td>
<td></td>
</tr>
<tr>
<td>Fistulizing disease 4 (8.3%)</td>
<td></td>
</tr>
<tr>
<td>Maintenance of remission 2 (4.2%)</td>
<td></td>
</tr>
<tr>
<td>Previous intestinal resection 48 (16%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Detection of G238C polymorphism. The DNA fragment was amplified using a G238C wild specific sense primer in addition to a G238C common antisense primer in the wild-type allele. G238C polymorphism was not detected. W, wild-type; M, mutant type.

Figure 4. Detection of G460A polymorphism. Mwo I digestion produced 267 and 98 base pair fragments in the wild-type allele. G460A polymorphism was not detected. W, wild type.
Figure 5. Detection of A719G polymorphism. Acc I digestion produced an uncleaved 373 base pair fragment in the wild-type allele. A719G polymorphism was not detected. W, wild-type.
IV. Discussion

Correlation between TPMT genotype and phenotype has been reported to be more than 95%.\textsuperscript{17,18} Prior to administering AZA or 6-MP, the investigation of TPMT polymorphism may provide clinically useful therapeutic information, such as exact tailored dosing regimens, and reduce thiopurine-induced complications. Patients with low or absent TPMT activity can be treated with AZA or 6-MP, provided that the dosage be reduced to one tenth to one fifteenth the standard dose. Even at a such reduced dosage, these patients must be observed carefully for signs of toxicity.\textsuperscript{20,21} Also, the patients with very high levels of TPMT activity might be at risk for undertreatment with standard doses of AZA or 6-MP.\textsuperscript{22}

For Korean population, Park-Hah et al.\textsuperscript{14} and Jang et al.\textsuperscript{15} measured TPMT activity. It revealed that detectable TPMT activity was 12.0 to 12.4 U/ml RBC and the frequency distribution was of normal, unimodal (range 3.2- 5.2 U/ml RBC to 21.0- 22.9 U/ml RBC) distribution. Four of 10 Korean children subjects with low TPMT activity group were heterozygous at nucleotide A719G only. That is, their genotype was TPMT *1/*3C.\textsuperscript{16} Otterness et al.\textsuperscript{16} conducted the study only with twenty-one samples who had extremes of frequency distribution of TPMT activity. Therefore, the exact frequency of TPMT *1/*3C in the population could not be
estimated.

Figure 6. Samples from Hanyang University. For G460A polymorphism, Mwo I digestion produced an uncleaved fragment of 365 base pair fragment from TPMT*3B and a cleaved fragment of 267 and 98 base pair fragment from the wild-type allele (lane 1; sample from Hanyang University, 2; our sample). For A719G polymorphism, Acc I digestion yielded an uncleaved 373 base pair fragment from the wild type allele (lane 4-6; samples from Hanyang university, 7; our sample).
Because there was no positive control for G460A polymorphism, we obtained three samples from Hanyang University with known G460A polymorphism. Using our genotyping method, we analyzed the samples (Figure 6). Same results were obtained from them, indicating that our methods were reliable.

In this study, total 48 patients received immune modulating agents. Primary indications for these drugs were steroid dependent (n=34, 70.8%), steroid refractory (n=8, 16.7%), fistulizing diseases (n=4, 8.3%), and remission maintained (n=2, 4.2%). The median delay from diagnosis to start of drugs was 145.7 weeks (range 1-994).

Side effects were developed after 12.9 (range 4.3-167.1) weeks after application of the drug in eighteen patients (37.5%). Bone marrow suppression was the most common side effect (n=15, 31.3%). Fever, joint pain, and vomiting were also noted (n=1, 2.1%, respectively).

The risk of bone marrow suppression during treatment of IBD with AZA was reported as 5% and that bone marrow toxicity could occur anytime from 2 weeks to 11 years. Therefore it is recommended that complete blood count be monitored every 2
week for 3 months followed by every 3 months indefinitely.\textsuperscript{23}

We could not find common TPMT polymorphism such as \textsuperscript{*}2, \textsuperscript{*}3A, \textsuperscript{*}3B, and \textsuperscript{*}3C in patients who developed side effects, including bone marrow suppression, during the treatment with thiopurines. Such finding indicates that future studies on identification of rare TPMT polymorphisms and TPMT activity, concentrations of TGN are needed.

\textbf{Table 3. Details of patients with IBD receiving AZA or 6-MP}

<table>
<thead>
<tr>
<th>Details</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>29:19</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>45.3 (range 16-75)</td>
</tr>
<tr>
<td>Diseases</td>
<td></td>
</tr>
<tr>
<td>Crohn\’s disease</td>
<td>24 (50.0%)</td>
</tr>
<tr>
<td>Ulcerative colitis</td>
<td>15 (31.3%)</td>
</tr>
<tr>
<td>Behcet\’s disease</td>
<td>9 (18.8%)</td>
</tr>
<tr>
<td>Side effects of AZA or 6-MP</td>
<td>18 (37.5%)</td>
</tr>
<tr>
<td>Bone marrow suppression</td>
<td>15 (31.3%)</td>
</tr>
<tr>
<td>Fever</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>Joint pain</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>Delay from diagnosis to start of AZA or 6-MP (wk)</td>
<td>median 145.7 (range 1-994)</td>
</tr>
<tr>
<td>Development of side effects after the treatment with AZA or 6-MP (wk)</td>
<td>median 12.9 (range 4.3-167.1)</td>
</tr>
</tbody>
</table>
V. Conclusion

Results of this study demonstrated that common TPMT polymorphism was very rare but the frequency of bone marrow suppression during the treatment with AZA or 6-MP was considerably higher than what was reported in previous studies. There was no correlation between thiopurine-induced side effects and TPMT genotype.
References


20. Evans WE, Horner M, Chu YQ, Kalwinsky D, Roberts WM.


thiopurine methyltransferase

azathioprine (AZA) 6-mercaptopurine (6-MP) Thiopurine methyltransferase (TPMT) 6-MP 6-thioguanine nucleotides (TGN) TPMT 90% 10% 300 1 TMPT 95% polymerase chain reaction (PCR) 100 300 TPMT
G238C, G460A, and A719G are found in the 0/200 alleles, 0/600 alleles, and 0/200 alleles, respectively.

TPMT, Azathioprine, and 6-mercaptopurine are included.