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

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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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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

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I. Introduction

To treat inflammatory bowel disease (IBD), sulfasalazine, its analogs, and corticosteroids are frequently used.[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.[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).[7] Especially more profound bone marrow suppression requiring hospitalization was seen in about 2%.[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.[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.[11]

TPMT is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds.[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.[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.

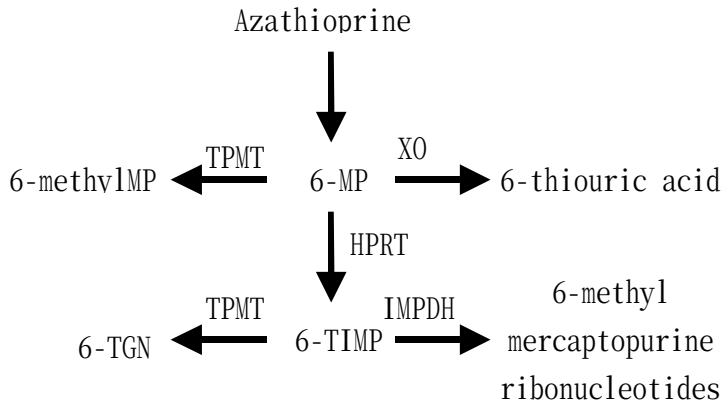

Figure 1. Azathioprine and 6-mercaptopurine metabolism.

MP, mercaptopurine;

TPMT, thiopurine methyltransferase;

XO, xanthine oxidase;

HPRT, hypoxanthinephosphoribosyl transferase;

TGN, thioguanine nucleotides;

TIMP, thioinosine monophosphate;

IMPDH, inosine monophosphate dehydrogenase

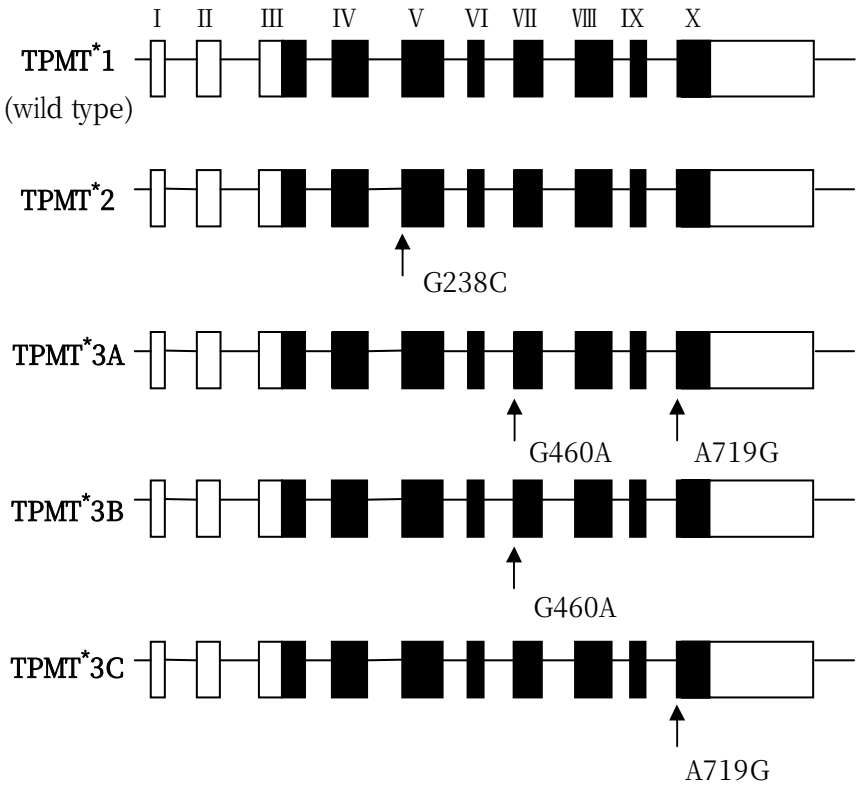

Figure 2. Human TPMT polymorphisms. The figure shows schematically the wild-type allele for TPMT*1 and four variant alleles for low enzyme activity. Exons are numbered and shown as rectangles, with black areas representing the open reading frame and white areas representing 3'-and 5'-untranslated regions.

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.[17] and Kumagai et al.[19]

DNA was extracted by genomic DNA isolation kit provided by Puregene (Minneapolis, MN, USA). All PCR was performed with thermocylcer (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

(Berverly, 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

Name	DNA sequences
G238C sense	5'-GTATGATTTTATGCAGGTTTG-3'
G238C mutant sense	5'-GTATGATTTTATGCAGGTTTC-3'
G238C common antisense	5'-TAAATAGGAACCATCGGACAC-3'
G460A sense	5'-ATAACAGAGTGGGGAGGCTGC-3'
G460A antisense	5'-CTAGAACCCAGAAAAAGTATAG-3'
A719G sense	5'-GAGACAGAGTTTCACCATCTTGG-3'
A719G antisense	5'-AGGCTTTAGCATAATTTTCAATTCCTC-3'

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 ℃ for 5 minutes, amplification was done for 35 cycles consisting of denaturation at 94 ℃ for 30 seconds, annealing at 55 ℃ for 45 seconds, and extension at 72 ℃ for 30 seconds. A final extension step at 72 ℃ 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 l 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 ℃ 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 ℃. 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 amplication and annealing temperature of 62 ℃ 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

Characteristics	
Sex (M:F)	138:162
Age (yr)	40.8 (range: 15-79)
Type of disease	
Crohn's disease	78 (26.0%)
Ulcerative colitis	150 (50.0%)
Behcet's disease	70 (23.3%)
Etc	2 (0.7%)
Disease and location	
Crohn's disease	
small bowel	12 (15.4%)
small bowel with perianal lesion	1 (1.3%)
ileocolitis	19 (24.4%)
Ileocolitis with perianal lesion	16 (20.5%)
colitis	19 (24.4%)
colitis with perianal lesion	5 (6.4%)
remission	4 (5.1%)
anastomosis	2 (2.5%)
Ulcerative colitis	
left-sided	89 (59.4%)
pancolitis	58 (38.6%)
remission	2 (1.3%)
anastomosis	1 (0.7%)
Behcet's disease	
ileocecal	53 (75.7%)
other part of colon	6 (8.6%)
remission	3 (4.3%)
anastomosis	8 (11.4%)

Table 2. (continued)
Details of patients with inflammatory bowel disease

Immune modulating agents use	48 (16%)
Azathioprine	41 (85.4%)
6 -mercaptopurine	7 (14.6%)
Indications	
Steroid dependent	34 (70.8%)
Steroid refractory	8 (16.7%)
Fistulizing disease	4 (8.3%)
Maintenance of remission	2 (4.2%)
Previous intestinal resection	48 (16%)

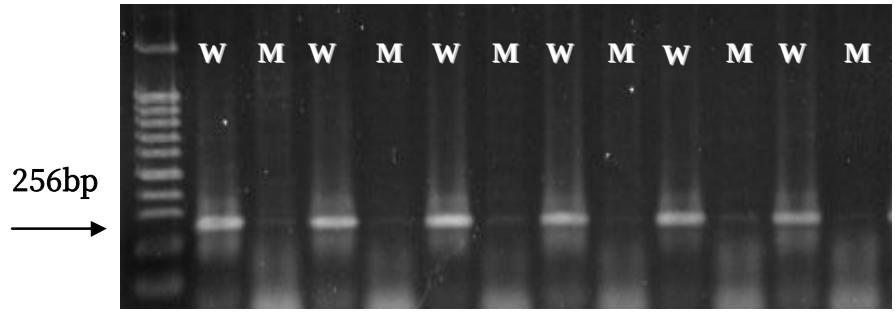

Figure 3. Detection of G238C polymorphism. The DNA fragment was amplified using a G238C wild specific sense primer in addition to a G238C common antisense primer in the wild-type allele. G238C polymorphism was not detected. W, wild-type; M, mutant type.

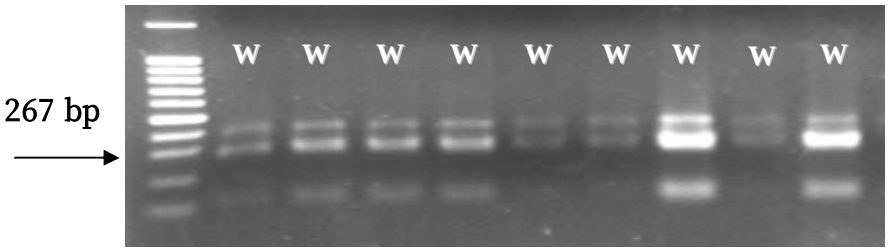

Figure 4. Detection of G460A polymorphism. Mwo *I* digestion produced 267 and 98 base pair fragments in the wild-type allele. G460A polymorphism was not detected. W, wild type.

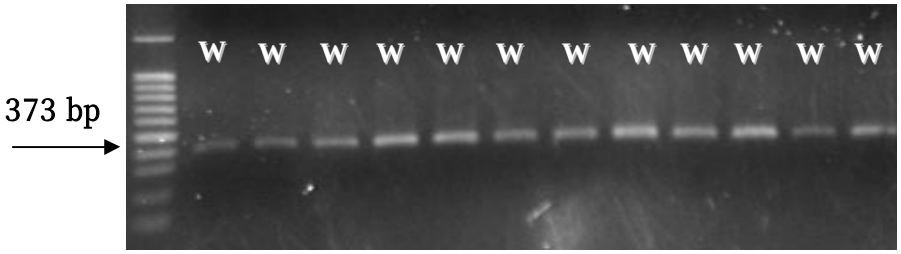

Figure 5. Detection of A719G polymorphism. Acc *I* digestion produced an uncleaved 373 base pair fragment in the wild-type allele. A719G polymorphism was not detected. W, wild-type.

Ⅳ. Discussion

Correlation between TPMT genotype and phenotype has been reported to be more than 95%. [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.[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.[22]

For Korean population, Park-Hah et al.[14] and Jang et al.[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 $^{*}1/^{*}3C$.[16] Otterness et al.[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.

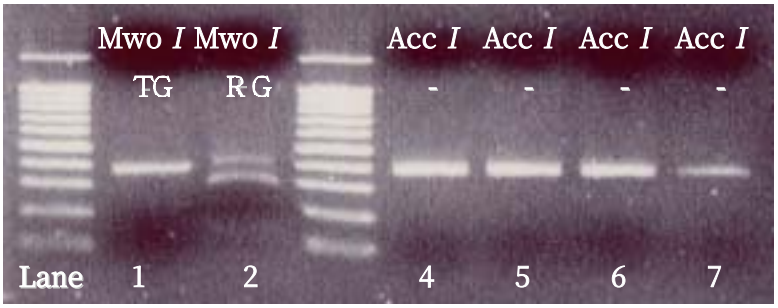

Figure 6. Samples from Hanyang University. For G460A polymorphism, Mwo *I* digestion produced an uncleaved fragment of 365 base pair fragment from TPMT*3B and a cleaved fragment of 267 and 98 base pair fragment from the wild-type allele (lane 1; sample from Hanyang University, 2; our sample). For A719G 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.[23] Therefore it is recommended that complete blood count be monitored every 2

week for 3 months followed by every 3 months indefinitely.[23]

We could not find common TPMT polymorphism such as *2, *3A, *3B, and *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.

Table 3. Details of patients with IBD receiving AZA or 6-MP

Sex (M:F)	29:19
Age (yr)	45.3 (range 16-75)
Diseases	
Crohn's disease	24 (50.0%)
Ulcerative colitis	15 (31.3%)
Behcet's disease	9 (18.8%)
Side effects of AZA or 6-MP	18 (37.5%)
Bone marrow suppression	15 (31.3%)
Fever	1 (2.1%)
Joint pain	1 (2.1%)
Vomiting	1 (2.1%)
Delay from diagnosis to start of AZA or 6-MP (wk)	median 145.7 (range 1-994)
Development of side effects after the treatment with AZA or 6-MP (wk)	median 12.9 (range 4.3-167.1)

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.

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국문요약

염증성 장질환 환자에서

thiopurine methyltransferase 유전자 검사의 임상적 유용성

염증성 장질환 환자의 치료에서 스테로이드는 매우 중요한 치료제이다. 그러나, 부작용이 나타나거나 치료에 불응하는 경우가 나타나므로 azathioprine (AZA)이나 6-mercaptopurine (6-MP)과 같은 면역 조절제를 사용하게 된다. Thiopurine methyltransferase (TPMT)는 이들 약제의 대사에 중요한 효소로 6-MP가 6-thioguanine nucleotides (TGN)로 대사되는데 경쟁적으로 작용하게 된다. TPMT 활성도는 유전적 다양성과 연관되는데 대략 90%의 서양인에서 고활성도를 갖는 동종 접합체를 갖고, 약 10% 는 중등도의 활성도를 보이는 이형 접합체를 보이며, 약 300명 중 1명이 저활성도를 갖는 동형 접합체를 갖는 것으로 알려져 있다. TMPT의 유전형과 표현형은 95% 이상 일치하는 것으로 보고 된다.

이에 연구자는 polymerase chain reaction (PCR)을 이용하여 정상인 100명과 염증성 장질환 환자 300명을 대상으로 TPMT 유전자

검사를 통하여 면역 조절제에 의한 골수 기능 억제와의 상관 관계를 알아보고자 하였다. G238C 다형성, G460A 다형성, 그리고 A719G 다형성은 정상인(0/200 alleles)과 환자군(0/600 alleles) 모두에서 발견되지 않았다 (0/200 alleles).

본 연구에서 흔한 TPMT 다형성은 극히 드무나, AZA 또는 6-MP사용에 따른 골수 기능 억제의 빈도가 이전의 연구 결과 보다 높은 것으로 보아 향후 드문 TPMT 다형성에 관한 연구와 TPMT 효소의 활성도 및 TGN의 농도 측정이 필요할 것으로 생각한다.

핵심되는 말: 염증성 장질환, TPMT 유전자 검사, Azathioprine, 6-mercaptopurine, 골수 기능 억제