Nonsynonymous Polymorphism in Guanine Monophosphate Synthetase Is a Risk Factor for Unfavorable Thiopurine Metabolite Ratios in Patients With Inflammatory Bowel Disease

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Nonsynonymous Polymorphism in Guanine Monophosphate Synthetase Is a Risk Factor for Unfavorable Thiopurine Metabolite Ratios in Patients With Inflammatory Bowel Disease

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Background: Up to 20% of patients with inflammatory bowel disease (IBD) who are refractory to thiopurine therapy preferentially produce 6-methylmercaptopurine (6-MMP) at the expense of 6-thioguanine nucleotides (6-TGN), resulting in a high 6-MMP:6-TGN ratio (>20). The objective of this study was to evaluate whether genetic variability in guanine monophosphate synthetase (*GMPS*) contributes to preferential 6-MMP metabolizer phenotype.

Methods: Exome sequencing was performed in a cohort of IBD patients with 6-MMP:6-TGN ratios of >100 to identify nonsynonymous single nucleotide polymorphisms (nsSNPs). In vitro assays were performed to measure GMPS activity associated with these nsSNPs. Frequency of the nsSNPs was measured in a cohort of 530 Caucasian IBD patients.

Results: Two nsSNPs in *GMPS* (rs747629729, rs61750370) were detected in 11 patients with very high 6-MMP:6-TGN ratios. The 2 nsSNPs were predicted to be damaging by in silico analysis. In vitro assays demonstrated that both nsSNPs resulted in a significant reduction in GMPS activity ($P < 0.05$). The SNP rs61750370 was significantly associated with 6-MMP:6-TGN ratios ≥100 (odds ratio, 5.64; 95% confidence interval, 1.01–25.12; $P < 0.031$) in a subset of 264 Caucasian IBD patients.

Conclusions: The *GMPS* SNP rs61750370 may be a reliable risk factor for extreme 6MMP preferential metabolism.

Key Words: azathioprine, 6-mercaptopurine, 6-thioguanine nucleotides (6-TGN), 6-methylmercaptopurine (6-MMP), guanosine monophosphate synthetase (GMPS)

INTRODUCTION

Azathioprine (AZA) and 6-mercaptopurine (6-MP) are prodrugs that undergo a complex metabolism resulting in the generation of several inactive products and the active 6-thioguanine nucleotide (6-TGN) metabolites and hepatotoxic metabolite 6-methylmercaptopurine (6-MMP) (Fig. 1).[1] Concentrations of 6-TGN >235 pmol/8 × 10^8 red blood cells (RBCs) have been associated with disease remission in IBD, whereas concentrations of 6-MMP exceeding 5700 pmol/8 × 10^8 have been associated with an increased risk of hepatotoxicity.[2] Poppe et al.[3] demonstrated that 1 of the 6-TGNs, 6-thioguanine triphosphate (6-TGTP) (Fig. 1), contributes markedly to the overall immunosuppressive effect of

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thiopurine therapy by binding to the small guanosine triphosphatase (GTPase) RAC1 (Ras-related C3 botulinum toxin substrate 1) on CD28 co-stimulation in $CD4^+$ T cells. Binding of 6-TGTP to RAC1 blocks Vav guanine nucleotide exchange factor (Vav) exchange activity, leading to the disruption of the Vav1-Rac1 signalling cascade and a therapeutic reduction in inflammation (Fig. 1).[4]

Up to 20% of patients on thiopurine therapy preferentially produce 6-MMP, at the expense of 6-TGN production, resulting in a high 6-MMP:6-TGN ratio (>20) and increased risk of hepatotoxicity and ineffectiveness of thiopurine therapy.[5] Several authors have proposed that this preferential 6-MMP producer phenotype is due to elevated thiopurine S-methyltransferase (TPMT) activity. However, the vast majority (>95%) of patients who exhibit this phenotype seem to have normal or intermediate TPMT activity. In 51 patients with IBD, Dubinsky et al.[2] found that the baseline RBC TPMT activity did not differ between patients who responded to 6-mercaptopurine and patients who preferentially metabolized 6-mercaptopurine to 6-MMP.[2] More recently, a survey of 1879 patients, of which 74% had a diagnosis of IBD, found that 19% (349) had a 6-MMP:6-TGN ratio >20, and the mean RBC TPMT activity of these patients was similar to the activity in patients with a ratio <20.[5] Only 3% of patients with a high 6-MMP:6-TGN ratio were found to have ultrahigh TPMT enzyme activity.[5] Taken together, these studies suggest strongly that high TPMT enzyme activity is not the primary cause of preferential 6-MMP production.

At present, only 3 studies have investigated the possibility that genetic variation other than in *TPMT* may contribute to high 6MMP:6TGN ratios. In the first study, 20 preferential 6-MMP metabolizers and 17 IBD patients with 6-MMP:6-TGN ratios of <20 were screened for sequence variants in inosine-5-monophosphate dehydrogenase 1 (*IMPDH1*) and *IMPDH2*. A 9-bp insertion identified in the promoter of *IMPDH1* abolished a cAMP response element (CRE) and was found to significantly reduce gene expression in vitro ($P < 0.001$).[6] However, this variant was only found in 1 of the 20 preferential 6-MMP producers that had a 6-MMP:6-TGN ratio of ≥100. Furthermore, no other genetic variants found in this study were associated with preferential

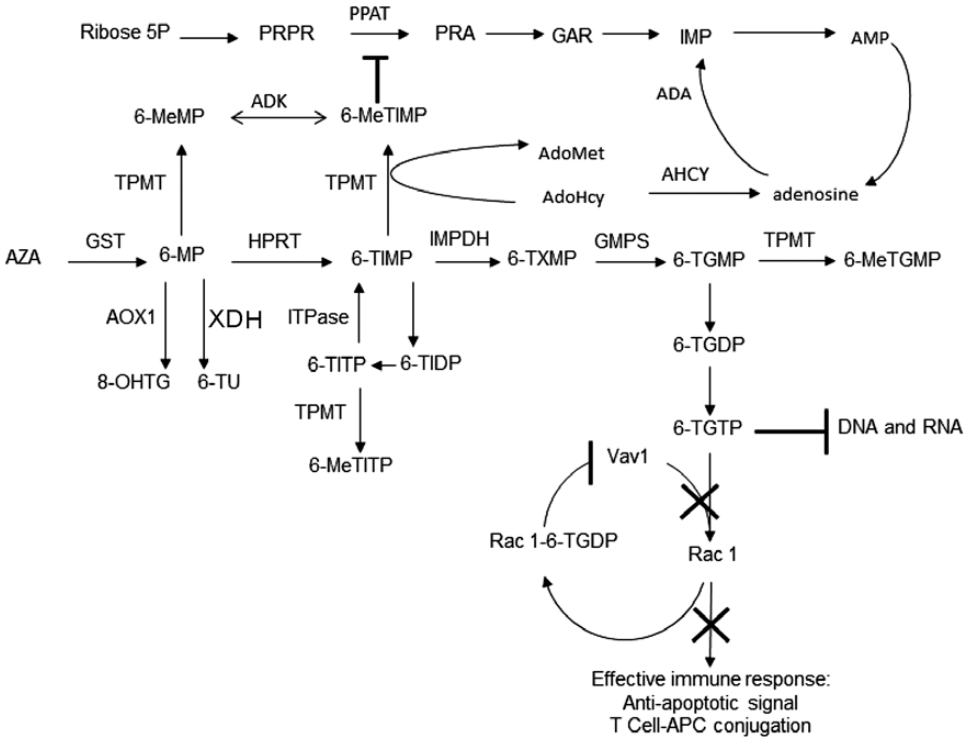

FIGURE 1. Thiopurine drug metabolism. The pro-drug AZA is quickly converted, via a nonenzymatic reaction, to 6-MP, which undergoes extensive metabolism via 3 competing pathways, mediated by xanthine dehydrogenase (XDH), TPMT, and HPRT, respectively. The pathway mediated by XDH produces the inactive metabolite 6-thiouric acid. TPMT catalyses the production of a second inactive metabolite (6-MMP). The HPRT pathway generates 6-TIMP. Once formed, 6-TIMP may be transformed into either 6-TGN by the rate-limiting IMPDH and GMPS, methylated into 6-MMP by TPMT, or phosphorylated to 6-thio-ITP. 6-MMP, 6-TGNs, and 6-thio-ITP are all active metabolites. In normal cells, the accumulation of 6-thio-ITP to toxic concentrations is prevented by ITPAse, which converts 6-thio-ITP back to 6-TIMP. The principal cytotoxic mechanism of thiopurines is the incorporation of 6-TGNs into DNA and RNA. Abbreviations: 6-MeMP, 6-methylmercaptopurine; 6-MeTGMP, 6-methylthioguanine monophosphate; 6-MeTITP, 6-methylthioinosine triphosphate; 6-TGDP, 6-thioguanine diphosphate; 6-TGMP, 6-thioguanine monophosphate; 6-TIDP, 6-thioinosine diphosphate; 6-TITP, 6-thioinosine triphosphate; 6-TU, 6-thiouric acid; 6-TXMP, 6-thioxanthosine monophosphate; 8-OHTG, 8-hydroxythioguanine; AOX1, aldehyde oxidase 1; GST, glutathione S-transferase; ITPAse, inosine triphosphate pyrophospatase.

6-MMP production, suggesting that if genetic variability in *IMPDH* does contribute to this phenotype, it does so only infrequently. The second study compared gene expression in 10 IBD patients who had 6-MMP:6-TGN ratios of >20 with expression profiles in 11 patients with ratios <20.[7] Microarray and quantitative real-time polymerase chain reaction (RT qPCR) analyses identified 18 genes that were significantly associated with high ratios. Nine of the identified genes play no known role in the purine pathway. Of the genes known to be related to the purine pathway, patients with 6-MMP:6-TGN ratios of >20 showed lower expression levels of microsomal glutathione S-transferase 2 (MGST2), TPMT, and NME6, but higher expression levels of IMPDH2 and NT5E compared with patients with 6-MMP:6-TGN ratios of ≤4.[7] This study did not explore whether these differences in expression were due to genetic variants. The third and most recent study employed exome sequencing and comparative genomic hybridization to identify genetic variants that may contribute to extreme 6-MMP metabolism. Despite detailed analysis, the authors were unable to identify any convincing candidate single nucleotide polymorphisms (SNPs) or copy variants that could explain the 6-MMP preferential metabolizer phenotype.[8]

Azathioprine undergoes extensive metabolism via 3 competing pathways (Fig. 1). The hypoxanthine guanine phosphoribosyl transferase (HPRT) pathway generates 6-thioinosine monophosphate (6-TIMP). Once formed, 6-TIMP may be transformed into either thioguanine nucleotides (6-TGN) by the rate-limiting IMPDH and guanine monophosphate synthetase (GMPS), 6-MMP by TPMT, or phosphorylated to 6-thio-inosine triphosphate (6-thio-ITP) (Fig. 1). As GMPS catalyses the final step in the generation of 6-TGNs (Fig. 1), any genetic variation that impairs GMPS function would reduce 6-TGN production, thereby skewing AZA metabolism and potentially resulting in a preferential 6-MMP metabolizer phenotype.

The overall aim of our study was to screen patients with IBD for the potential existence of loss of function nonsynonymous single nucleotide polymorphisms (nsSNPs) in *GMPS*, which could contribute to preferential 6-MMP metabolism. Our a priori hypothesis was that such polymorphisms were more likely to exist in patients who had an extreme preferential 6-MMP metabolizer phenotype. For the purpose of this study, we defined extreme preferential 6-MMP metabolizers as individuals with a 6-MMP:6-TGN ratio (>100).[9]

METHODS

Study Participants

Participants included 530 IBD patients on current or previous thiopurine treatment, under the care of the IBD services at the Royal Adelaide Hospital and Flinders Medical Centre (South Australia), Royal Brisbane and Women's Hospital (Queensland, Australia), Fremantle Hospital (Western Australia), St. Vincent's Hospital (Melbourne, Victoria, Australia), and the VU University Medical Center (Amsterdam, the Netherlands). Patients were considered to fit the definition of preferential 6-MMP producers if they exhibited normal or low TPMT activity and had a ratio of 6-MMP to 6-TGN concentrations within red blood cells (RBCs) ≥20.[10] Controls (n = 735) were older than age 18 years and had no history of any inflammatory disease. The control sample set was recruited from the Otago region of New Zealand, as described in detail elsewhere.[11] All study participants were unrelated and of Caucasian ethnicity. Each participant was recruited with informed consent, and ethical approval for this study was obtained from the relevant Ethics Committee associated with each recruiting center.

Measurement of 6-TGN and 6-MMP in the Peripheral Blood of IBD Patients

Blood (5 mL) was collected in a heparinized tube and centrifuged to isolate RBCs. The RBCs were washed twice in 2 volumes of saline, counted to allow normalization of 6-TGN and 6-MMP concentrations to 8×10^8 RBCs, and then frozen. The concentrations of 6-TGN and 6-MMP in the peripheral blood of each IBD patient were determined using HPLC, as previously described.[12]

DNA Extraction and Exome Sequencing

DNA samples were collected from the peripheral blood of IBD patients and controls using guanidium isothiocyanate extraction, resuspended in Tris-EDTA buffer (pH 8.0), and stored at –20ºC.[13] Exome sequencing was performed on the DNA of 11 IBD patients using an Illumina HiSeq 2000. As per our a priori hypothesis, all patients selected for exome sequencing had 6-MMP:6-TGN ratios of >100 and a *TPMT*1/*1* genotype. Only these patients with a very high metabolite ratio were selected for exome sequencing to maximize the chance of detecting relevant new SNPs. Sequences were aligned using BWA to the human GRCh37 reference.[14] The Genome Analysis Toolkit (GATK)[15] was applied for base quality score recalibration, insertion/deletion (Indel) realignment, duplicate removal, and SNP and Indel discovery, and genotyping across all samples was performed simultaneously using standard hard filtering parameters or variant quality score recalibration.[16] The web-based genome analysis tool Galaxy[17–19] was used to extract exons of *GMPS* to identify SNPs discovered by the GATK.

PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/) were used to predict the functional impact of nsSNPs identified by GATK. nsSNPs predicted to be damaging by both these programs were confirmed by direct sequencing.

In Vitro Analysis of the Functional Impact of Selected GMPS nsSNPs on 6-TGN Production

A full-length *GMPS* (NM_003875) human cDNA open reading frame clone in a pCMV6 vector (OriGene

Technologies, MD) was used as the wild-type *GMPS* and as a template to generate mutant isoforms. Site-directed mutagenesis was used to introduce point mutations at +1583 bp and +1660 bp upstream of the *GMPS* start codon. PWO polymerase (Roche) was used with the following oligonucleotides in PCR reactions, as per previously described[20]: rs61750370 (+1583bp): Forward, 5'-GTCGTTCCTACAGTT***C***CGTGTGTGGA-3'; Reverse, 5'-GAGATTCCACACACG***G***AACTGTAGGA-3'; rs747629729 (+1660bp): Forward, 5'-CTTATACCTCGCATG***G***GTCACAACGT - 3'; Reverse, 5'-TGTTAACGTTGTGAC***C***CATGCGAGGT-3'. Mutated sites are shown in bold and underlined. Thermal cycling conditions were as follows: 2 minutes at 94°C (initial denaturation); 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C–65°C, 40 seconds at 72°C; and a final extension of 2 minutes at 72°C.

HEK-293 cells were maintained in DMEM containing 10% fetal calf serum in 75-cm^2 flasks at 37°C (5% CO2). *GMPS* isoforms were transfected into HEK-293 cells in 6-well plates seeded at 5 × 55 cells/well 24 hours before transfection. Transfections were performed using FuGENE6, as previously described,[21] and a control transfection was performed using empty pCMV6 vector. After 24-hour transfection, the cells were exposed to 50 µM 6-Mercaptopurine monohydrate (6-MP; Sigma-Aldrich Company Ltd, Poole, UK) for 6 hours. The cells were subsequently washed with 2 mL ice-cold PBS and resuspended by adding 750 µL H_20 containing 1mM DTT through mechanical scarping. The resuspended cells were transferred into the –80°C freezer for 20 minutes and then thawed at RT for 20 minutes for 2 cycles. The cells were then acid-hydrolyzed by adding 150 µL 60% perchloric acid, vortexed for 20 seconds, placed in an ice bath for 10 minutes, and spun down at 13,000 g for 15 minutes. The supernatant was then carefully removed from the cell debris and placed into a 1.5-mL tube, which was then boiled for 45 minutes and stored at –80°C.

6-TGN concentrations from cell lysates were each determined separately by high-performance liquid chromatography (Beckman Coulter) with UV detection; 6-TGN concentrations for each sample were expressed as µM and were calculated through a standard calibration curve using 6-TGN (Sigma). The mobile phase for 6-TGN included 98% H_2O, 2% acetonitrile, 20 mM potassium phosphate buffer, and 100 mM triethylamine. The retention time for 6-TGN was determined at ~6–7 minutes.

Genotyping of GMPS nsSNPs

Study participants were genotyped for *GMPS* nsSNPs rs61750370 and rs747629729 using TaqMan SNP genotyping assays following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Briefly, the assays were performed in a total reaction volume of 5 µL containing 2.5 µL of 2× TaqMan Universal Master Mix (Roche Molecular Systems Inc, Branchburg, NJ, USA), 0.25 µL of 20× SNP genotyping assay, and 12 ng of genomic DNA. The PCR was performed in 384-well format on a Roche LightCycler 480 real-time PCR system, with an activation step of 10 minutes at 95°C, followed by 40 cycles of denaturation (15 seconds at 92°C) and annealing/extension (1 minute at 60°C). Genotypes were assigned using the end point genotyping analysis software. A sequence-validated control was included in each batch of genotyping. To check the accuracy of the genotype assignment, genotyping was repeated on 10% of samples. The concordance between original and repeat genotypes was 100%. The web-based analysis program SHEsis (http://analysis2.bio-x.cn/myAnalysis.php)[22] was used to check for deviations in Hardy-Weinberg Equilibrium (HWE).

RESULTS

Measurement of 6-TGN and 6-MMP Concentrations in IBD Patients

Of the 530 IBD patients enrolled in the study, RBC 6-TGN and 6-MMP concentrations were measured in 275 IBD patients. A total of 172 patients had 6-MMP:6-TGN ratios ≥20, of whom 34 had ratios of ≥100. Eleven of the patients who had ratios of ≥100, representing all recruitment centers, underwent exome sequencing. Resource constraints limited further sequencing (see Table 1 for demographic details).

Identification and Characterization of nsSNPs

Exome sequencing was successfully completed in all 11 patients. A total of 18 intronic SNPs, 1 synonymous SNP, and 2 nsSNPs (rs61750370 and rs747629729) were found in *GMPS*. Both nsSNPs were predicted to be damaging by PolyPhen-2 and SIFT. These nsSNPs were validated by Sanger sequencing. Figure 2 shows how GMPS protein sequences are highly conserved across species.

In Vitro Analysis of 6-TGN Production by GMPS Variants Using HPLC

The functional effect of the *GMPS* nsSNPs on 6-TGN production was evaluated using a HEK-293 cell model and

TABLE 1: Demographics of 11 Exome-Sequenced Patients With 6-MMP/6-TGN Ratio >100

	No. Unless Otherwise Stated
Age, median (range), y	37.5 (24–51)
IBD type	9 CD, 2 UC
Thiopurine type	8 on 6-MP, 3 on AZA
Hepatotoxicity[a]	6
Severe nausea[a]	2
Allopurinol used successfully[a]	6

[a]Hepatotoxicity: liver function test results >3× normal; severe nausea: sufficient to withdraw therapy; allopurinol used successfully: resulting in correction of the 6-MMP/6-TGN ratio.

HPLC analysis. Analysis of variance revealed a significant difference in 6-TGN production between wild-type and variants of the *GMPS* enzyme ($P < 0.0001$; F = 48.77; RSq = 0.8667). A paired *t* test comparing mean 6-TGN production found a significant reduction in 6-TGN production by the SNP rs61750370 compared with wild-type ($P < 0.0001$) and the SNP rs747629729 compared with wild-type ($P < 0.05$) (Fig. 3).

Frequency of GMPS nsSNPs in Controls and IBD Patients

Seven hundred thirty-five healthy Caucasian controls were genotyped for *GMPS* nsSNPs. The minor allele frequency of *GMPS* SNP rs61750370 was 0.01 in this control set. The *GMPS* SNP rs747629729 was not detected in any of the controls.

Excluding the 11 patients who underwent exome sequencing, 519 IBD patients were genotyped for the 2 GMPS nsSNPs. A total of 12 IBD patients were heterozygous for the *GMPS* SNP rs61750370 (minor allele frequency, 0.023). No patient was homozygous or heterozygous for the second *GMPS* SNP rs747629729.

Metabolite ratios were available for 264 patients who had not been exome-sequenced. In this subset, 6 patients were heterozygous for *GMPS* rs61750370. Association testing found that the *GMPS* nsSNP rs61750370 was significantly associated with 6-MMP:6-TGN ratios ≥100 (odds ratio, 5.64; 95% confidence interval, 1.01–25.12; $P = 0.031$), but not with ratios 6-MMP:6-TGN ≥20 ($P = 0.579$) (Table 2).

DISCUSSION

To our knowledge, the current study is only the fourth to explore the possibility that variation in genes other than *TPMT* may contribute to the preferential 6-MMP metabolizer phenotype. The major finding of our study is the identification of nsSNPs rs61750370, 1583A>C, and Y528S in exon 13 of the *GMPS* gene, which was shown to significantly reduce enzyme activity in vitro and was significantly associated with extreme preferential 6-MMP production (6-MMP:6-TGN ratios ≥100).

Guanine monophosphate synthetase (EC 6.3.5.2) is a glutamine-dependent amidotransferase consisting of a single structural domain formed by the C-terminus and 2 catalytic domains, 1 required for glutaminase activity and 1 for pyrophosphate-ATPase (PP-ATPase) activity.[23, 24] In addition to mediating the final step in the generation of 6-TGNs (Fig. 1), GMPS also plays a key role in de novo purine synthesis. As an ample supply of purines is essential for many life processes, it is not surprising that much of the amino acid sequence of *GMPS* is highly conserved across prokaryotic and eukaryotic species.[25, 26] The SNP rs61750370 results in the substitution of a tyrosine residue for a serine at position 528 in the GMPS protein. Alignment of the human *GMPS* sequence against *GMPS* sequences from other species demonstrates that tyrosine 528 is conserved across mammals (Fig. 2). Tesmer et al.[24] elucidated the crystal structure of *Escherichia coli* GMPS. This bacterial GMPS exists as a tetramer and is hypothesized to coordinate the activity of its 2 catalytic domains through a series of conformational changes that bring the active sites of the 2 domains into close juxtaposition. Integral to these movements are 2 dimerization domains that are formed from the C termini of the 4 GMPS subunits that make up the tetrameric protein. The dimerization domains assist in forming the ATP-binding site of the ATP-PPase domain.[24] It is possible that amino acid changes within the dimerization domain, such as the 1 described in this study, may reduce the catalytic functioning of GMPS by altering the conformation of the PP-ATPase domain and thereby the binding and turnover of ATP. In contrast to *E. coli* GMPS, there is some evidence to suggest that the human enzyme exists as a monomer, rather than a tetramer.[23] However, given the degree of amino acid conservation observed across species (Fig. 2),

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Human                481 tllqrvkact teedqeklmq itslhslnaf llpiktvgvq gdcrsysyvc gisskdepdw
Human variant        481 tllqrvkact teedqeklmq itslhslnaf llpiktvgvq gdcrssyvc gisskdepdw
Pan troglodytes      481 tllqrvkacs teedqeklmq itslhslnaf llpiktvgvq gdcrsysyvc gisskdepdw
Mouse                481 tllqrvkact teedqeklmq itslhslnaf llpiktvgvq gdcrsysyvc gisskdepdw
Rat                  481 tllqrvkact teedqeklme itsqhslnaf llpiktvgvq gdcrsysyvc gisskdepdw
Chicken              481 tllqrvkact teedqeklmq itslhslnaf llpiktvgvq gdcrsysyvc gisskdaphw
Chinese alligator    483 tllqrvkact teedqeklmq itslhslnaf llpiktvgvq gdcrsysyvc gisskdaphw
W. painted turtle    480 tllqrvkact teedqeklmq itslhslnaf llpiktvgvq gdcrsysyvc gisskdaphw
Zebrafish            480 tllqrvksci sdeeeeklmq itslhslnaf llpiktvgvq gdsrsysyvc gvsskeaphw
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FIGURE 2. Alignment of *GMPS* protein sequences. The amino acid residue altered by *GMPS* SNP rs61750370 (Y528S) is highlighted in the human variant and human reference protein sequence. The alignment of the corresponding protein sequences from other species shows the degree to which the affected amino acid residues are conserved.

it is likely that the C-terminal domain still plays an important role in the functioning of human GMPS. This assertion is supported by data related to C-terminal mutations in arginiosuccinate synthetase (ASS). These mutations cause the rare autosomal recessive disorder classical citrullinemia, which results in accumulation of citrulline in the blood and urine.[27] ASS is a monomeric amidotransferase that is closely related to GMPS and catalyzes the third step in the urea cycle. Mutations located in the C-terminal domain are believed to impair enzyme activity by compromising the tertiary and quaternary structure of the ASS.[27] Further evidence that the *GMPS* nsSNP rs61750370 may impact enzyme function comes from a recent exome array study of the plasma metabolome. This study found that rs61750370 was significantly associated with xanthosine levels in both the Framingham Heart Study cohort ($P = 2.8 \times 10^{-7}$) and the Atherosclerosis Risk in Communities Study cohort ($P = 6 \times 10^{-4}$).[28]

Although our study has found a significant association of the *GMPS* nsSNP rs61750370 with extreme preferential 6-MMP metabolism, this nsSNP is a rare polymorphism that was only found in 6 of the 264 IBD patients in whom metabolite ratios were determined and who were not included in the initial exome sequencing. Of these 6 patients, 3 had 6-MMP:6-TGN ratios <20. This finding indicates that although *GMPS* nsSNP rs61750370 seems to be a risk factor for extreme preferential 6-MMP metabolism, it is insufficient alone to cause this phenotype, and other factors contribute to determining 6-MMP metabolizer phenotype. These factors are likely to be a combination of genetic and environmental. It is conceivable that other rare nsSNPs and SNPs in noncoding regions of *GMPS*, and genetic variants in other enzymes such as IMPDH, interact with environmental factors to determine whether an individual is at risk of being a preferential 6-MMP metabolizer.

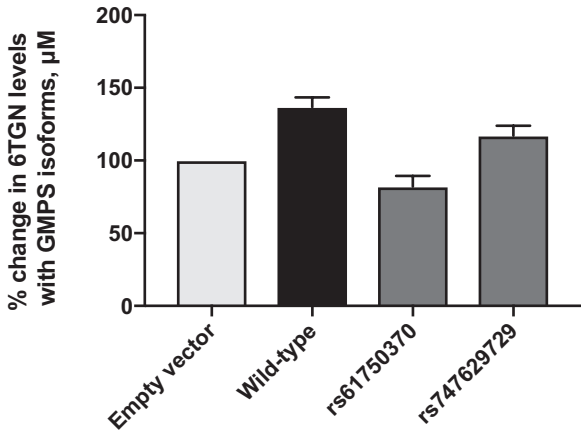

FIGURE 3. HPLC analysis of 6-TGN production by wild-type and variant *GMPS* isoforms. HEK293 cells were transfected with pCMV6 vector containing the human wild-type *GMPS* gene, or the variants rs61750370 and rs747629729, and then exposed to 50 µM 6-MP. Production of 6-TGN by the 3 *GMPS* isoforms was measured by HPLC, compared with the empty pCMV6 vector (EV), which was set at 100%. 6-TGN production was reduced significantly for both gene variants compared with wild-type.

Our study has a number of important limitations. First, the study participants were all of Caucasian ethnicity. Therefore, it is unknown whether the *GMPS* nsSNP rs61750370 is a potential risk factor for preferential 6-MMP metabolism in other races. However, dbSNP genotyping data, albeit limited for rs61750370, suggest that the minor allele of this nsSNP occurs at a comparable frequency in South East Asian, Africa, and American populations, raising the possibility of a similar association between this SNP and thiopurine metabolism in other races. Second, only 11 patients underwent exome sequencing of *GMPS*. We cannot rule out the possibility that further rare nsSNPs with functional relevance to thiopurine metabolism exist and would have been identified if more patients were sequenced. Additionally, as we opted for exome sequencing, our study was unable to detect functional SNPs in noncoding regions. Finally, metabolite ratios were only measured in 275 of the 530 IBD patients, thereby limiting the power of our study. Taking into account the limitations of the current study, we recommend that future research into preferential 6-MMP metabolism is conducted on larger cohorts using full genome sequencing to maximize the ability to detect rare but potentially significant SNPs in both coding and noncoding regions of *GMPS*.

On average, there is a delay of 8–12 weeks between an IBD patient commencing thiopurine medication and receiving some therapeutic benefit,[22] and some patients may not receive

TABLE 2: Allele and Genotype Frequencies of *GMPS* SNP rs61750370 in Patients With Inflammatory Bowel Disease Stratified According to 6-MMP:6-TGN Ratios[a]

	Genotype			MAF		
6-MMP:6-TGN Ratio	T,T	T,G	G,G	G	Allelic *P*	OR (95% CI)
≥20	158 (0.98)	3 (0.02)	0 (0.00)	3 (0.01)	0.579	0.64 (0.13–3.18)
<20	100 (0.60)	3 (0.03)	0 (0.00)	3 (0.02)		
≥100	21 (0.91)	2 (0.09)	0 (0.00)	2 (0.04)	0.031	5.64 (1.01–25.12)
<100	237 (0.60)	4 (0.02)	0 (0.00)	4 (0.01)		

[a]Patients who were exom- sequenced were excluded from this analysis.

benefit for up to 6 months.[29] Early prediction of treatment failure or hepatotoxicity would therefore save valuable time in establishing effective treatment. Furthermore, in anti-TNF therapy, it has been shown that thiopurine co-medication has a beneficial synergistic effect,[30] but there remains a risk of hepatotoxicity in patients with preferential 6-MMP production. As it becomes clearer over time which SNPs significantly alter thiopurine metabolism, there may be merit in prospective genotyping of patients and including *GMPS* SNP rs61750370 (1583A>C) to identify a small but noteworthy percentage of patients who are at risk of reduced efficacy or hepatotoxicity from thiopurine therapy, leading to untimely and avoidable discontinuation of this therapy. A number of studies have shown that patients with IBD who have a 6-MMP:6-TGN ratio ≥20 on thiopurine treatment have had correction of the ratio and improved clinical outcome with co-prescription of allopurinol.[31–34] Favorable change in the ratio with co-prescribed allopurinol has occurred in every patient studied so far, suggesting that co-prescription of allopurinol overcomes preferential 6-MMP production in the patients with *GMPS* loss-of-function SNPs by causing indirect inhibition of TPMT, thus forcing the metabolism toward 6-TGN production,[35] although this is unproven as yet. Similarly, some European experts suggest that the use of a rescue thiopurine derivative, thioguanine, may be an alternative strategy in patients with this unfavorable thiopurine profile.[36] Although only IBD patients were included in this study, it is likely that other disease cohorts will display similar thiopurine metabolite profiles related to the newly identified SNPs.

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