Limitations of extensive TPMT genotyping in the management of azathioprine-induced myelosuppression in IBD patients

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Abstract
Background and aims: TPMT deficiency is associated with azathioprine (AZA)-induced myelosuppression (MS). However, in one previous study, only about ¼ of MS episodes in Crohn’s Disease patients under AZA can be attributed to TPMT deficiency. Recently, new TPMT mutations have been described and our aim is to investigate their clinical relevance before and after a first MS episode on thiopurine therapy.

Methods: Clinical data from 61 IBD patients having developed MS during AZA therapy were collected. Sequencing analysis was carried out on TPMT cDNA for the presence of all currently known mutations.

Results: Only TPMT *2, *3A and *3C mutations were found in this cohort. TPMT mutations were observed in 15 out of 61 patients (25%). Four out of 15 were homozygous for a TPMT mutation (low methylator, LM genotype) and 11 were heterozygous (intermediate methylator, IM genotype). Median delays of MS onset were 2, 2.75 and 6 months in the LM, IM and HM (high methylator, wild type TPMT) groups, respectively. After the first MS episode, 36 patients resumed thiopurine treatment of which 13 experienced a second MS episode. This second episode was also rarely associated with TPMT mutations.

Conclusions: One quarter of MS episodes during AZA were associated with TPMT deficient genotype. After a first leucopenia episode, thiopurine therapy may be resumed in a majority of patients independently of their TPMT genotype.

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Introduction
Thiopurine drugs, azathioprine (AZA) and its metabolite 6-mercaptopurine (6-MP), have been used in the treatment of inflammatory bowel disease (IBD) for more than 40 years [1]. Their use has considerably increased over the last decade as it may be steroid sparing [2], enhance closing of fistulas or induce mucosal healing.

In up to one third of patients, thiopurine drug efficacy is not obtained because of side effects leading to drug dose reduction or complete withdrawal [3,4]. One of the thiopurine major side effects is myelosuppression (MS).

In a review of thiopurine-induced myelotoxicity in patients with IBD, compiling data from 8302 patients from 66 trials [5], the cumulative incidence of AZA/MP-induced myelotoxicity was 7%. The incidence rate (per patient and year of treatment) was 3%. Bone marrow toxicity may occur any time after initiation of thiopurine therapy. In this review, the delay for the onset of MS ranged from 12 days [6] to 27 years [7] but most cases occurred within the first months of therapy.

While patients with myelosuppression have sometimes to be hospitalized, data about rate and duration of hospitalization are usually missing.

There is a dose-dependent effect of thiopurine [8] in IBD therapy. Recommended dosages are 2 to 2.5 mg/kg for AZA and 1 to 1.5 mg/kg
for 6MP. These recommendations do not take into account individual thiopurine metabolism variability. AZA is rapidly converted by both enzymatic and non-enzymatic conjugation via glutathione [9] into 6-mercaptopurine which, in turn, is enzymatically converted into an active and an inactive moiety. The enzymes involved are hypoxanthine phosphoribosyl transferase (HPRT), Xanthine oxidase (XO), inosine triphosphate pyrophosphatase (ITPA) and thiopurine methyltransferase (TPMT). TPMT is the most frequently studied enzyme of TP metabolism and the only one usually tested for in routine clinic. Both phenotype and genotype TPMT status tests are available.

TPMT genetic polymorphism was first described by Weinsilboum [10]. The expression of the enzyme is inherited in an autosomal codominant fashion, and consequently varies within the population. In Caucasians, 11% of the population harbour heterozygous and 0.3% homozygous TPMT mutations, leading to an intermediate or low TPMT activity, respectively. In these patients, thiopurine metabolism is shunted towards an increased production of active but also toxic compounds. The gene encoding TPMT is located on chromosome 6 and contains 10 exons. The wild type alleles responsible for a normal or high TPMT activity are TPMT 1, IA and 1S. Various mutant alleles, characterized by one or more single nucleotide polymorphisms, have been described leading to a decreased or unknown activity of the enzyme [11]. A high degree of concordance was demonstrated between TPMT genotype and phenotype in Caucasians [12,13]. Heterozygous patients have intermediate activity whereas homozygous patients have low activity, although variability may be seen between these groups. The use of these tests in clinical practice remains controversial: in contrast to European [14] guidelines, American guidelines suggest the use of TPMT determination before TP administration [3].

Colombel et al. [15] analyzed the distribution of 9 mutant alleles associated with TPMT deficiency in 41 patients with CD and MS during thiopurine therapy. A TPMT allele deficiency (homo or heterozygous) was found in 27% of patients experiencing myelosuppression vs. 10% in a European control population. This result suggests a modest relationship between the presence of these mutations and the occurrence of bone marrow suppression but does not explain occurrence of leuco-thrombocytopenia in all the reported cases. The authors concluded therefore that other causes like viral infections, use of drugs interfering with thiopurine metabolism or use of myelotoxic drug might have been considered. Alternatively the presence of yet unidentified TPMT mutations could explain numerous MS episodes.

Indeed, recently, additional TPMT mutations were identified and characterized. More than 25 mutations are now indexed but the clinical relevance of some of them remains unclear [11]. Nonetheless, very few studies have assessed the contribution of these new TPMT mutations on the occurrence of MS in IBD patients treated with AZA. We therefore retrospectively investigated the impact of genotyping extensively the TPMT exons, by use of TPMT mRNA in IBD patients who experienced MS while taking thiopurine therapy. We aimed at comparing the clinical characteristics of MS in deficient and normal TPMT groups. Finally, we studied the impact of TP re-administration on recurrence of MS.

Methods

Cohort of patients

Sixty-one patients (median age 39 years [15–75 years]), with IBD (48 CD and 13 UC, 33 men and 28 women) were retrospectively included after ethical committee approval and after obtaining an individual informed consent. Only patients having developed MS (defined as white blood cell count below 3000/mm³ and/or thrombocytopenia defined as a platelets count below 100,000/mm³) during thiopurine therapy were included. The median treatment dose was 100 mg/day (range 50–250) and 2 mg/kg (range 0.7–2.5). Delay of onset of MS, the list of concomitant medications at that time, MS characteristics and required therapy, as well as frequency and length of related hospitalizations were recorded. Follow-up data on the outcome of thiopurine treatment were also collected.

Results


Total RNA was isolated from venous blood using Trizol reagent (Roche) and retro-transcribed by Superscript™ II RNase H-reverse transcriptase (Invitrogen) according to the manufacturers’ instructions. TPMT cDNA was amplified by PCR using Ampli Taq DNA polymerase (Applied Biosystems) and a pair of primers that anneal to sequences within exon 4 and exon 10 of the published sequence of the TPMT cDNA (NCBI: accession number BC009596) as follows: Sense primer: 5′-GGGAACATATCAGTTGAGACA-3′; Anti-sense primer: 5′-AAAAACATCTCAGTGATTTATTTTT-3′. Primers were designed to avoid co-amplification of the highly similar processed pseudogene [16]. The anticipated size of the PCR product was 819 bp. The PCR protocol consisted of an initial denaturation step at 95 °C for 2 min, followed by 32 PCR cycles (94 °C for 50 s, 56 °C for 40 s and 72 °C for 2 min 15 s) and a final extension at 72 °C for 10 min. cDNA sequencing was performed on both strands with the Big Dye® terminator cycle sequencing kit (Applied Biosystems), using an automated AB3130 capillary sequencer. Sequences were compared with the wild type sequence using the SeqScap version 2.0 software, which identifies variant and sequence matches from an allele library. In addition, sequences identify variant and sequence matches from an allele library.

The control measurements for preventing carry-over were previously described [17]. Briefly, contamination at the DNA level was excluded by performing PCR analysis without reverse transcriptase, as well as by including a water control containing no cDNA template.

A formal comparative statistical analysis was not attempted because of the absence of comparator group and the relatively low number of TPMT deficient patients.

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Results

Fourty-six patients (75%) were wild type homozygous (high methylator: HM) for all known mutations, 11 were heterozygous for at least 1 non functional mutation (intermediate methylator: IM) and 4 were homozygous (low methylator: LM) (Table 1). The TPMT variants identified were TPMT*2, *3A and *3C. In homozygous deficient patients, mutations were *2A/*2A in 1 patient, *3A/*3A in 1 patient and *3C/*3C in 2 patients. In heterozygous patients, 10 were *3A/*1 and 1 was *2A/*1.

Median delay between azathioprine initiation and myelosuppression was 2 months (range, 5 weeks to 5 months) in LM (low methylator phenotype) patients 2.75 months (range, 4 weeks to 6 years) in IM (intermediate methylator phenotype) patients; and 6 months (range, 11 days to 7 years) in patients with HM (high methylator genotype or wild type) (Fig. 1). Leucopenia was found in 58/61 patients (including 14/15 LM + IM patients), thrombocytopenia in 12 patients (including 4/15 LM + IM patients) and leuco-thrombocytopenia in 9 patients (including 3/15 LM + IM patients). Anemia defined as a hemoglobin level below 10 g/dl was found in 27 patients. Pancytopenia was found in 8 patients (including 3/15 LM + IM patients).
Concomitant medications at the time of MS were: aminosalicylates (n=40); methylprednisolone (n=10); budesonide (n=4); infliximab (n=4); allopurinol (n=3); spironolactone (n=2) and NSAID (n=2). No other medication was given in 11/61 patients. Of these, ASA, allopurinol, diuretics, infliximab and NSAID’s are known to have a drug–drug interaction with TP.

The most prevalent complication was the occurrence of an infectious syndrome (20/61 pts). Infections were considered severe in 5 patients, including 2 patients with pneumonia, 2 with Staphylococcus aureus septicemia and 1 Listeria meningitidis. No death was reported.

Three patients developed bleeding episodes related to thrombocytopenia.

Transfusions were given in 17 patients.

Hospitalization was required in 19/61 patients (median duration, 15 days; range, 2 to 42 days).

With respect to the MS episode treatment, it is tempting to speculate that LM + IM patients might be prone to consume more frequently medical resources. Hospitalizations were more frequent (7/15 vs. 12/46) in LM + IM patients, and they needed proportionally more medical interventions (transfusions and antibiotics) (8/15 vs. 14/46) than HM patients. Azathioprine was definitively stopped in 25 patients, stopped and re-introduced progressively at similar dose in 9 patients (2 IM, 7 HM) or lower dose in 27 patients (1 LM, 4 IM, 22 HM) (Fig. 2).

Six of the 9 patients in whom TP was re-introduced at similar dose developed a second episode of MS which then led to a definitive withdrawal of TP therapy. Among these 6 patients, 2 were IM whereas the 4 others were HM (see Table 2). Seven of the 27 patients receiving a decreased dose of AZA dose developed a second episode of leucopenia. Only one was IM, the 6 others were HM.

Discussion

This study shows that IBD patients developing MS under AZA therapy are only mildly enriched in TPMT mutants. These findings are in line with the observations from Colombel et al. In our hands, extensive TPMT cDNA genotyping did not yield any significant additional mutation as compared to the genotyping looking only for the TPMT*2, *3A, *3B, *3C, *3D mutations. In the present study, the population was mainly Caucasians. The identified TPMT mutations were mainly *3A, *3C and *2, which reproduces findings in similar populations [18]. Our results confirm that focusing genotyping, at least in Caucasian populations on the presence of TPMT3A/3C/2 variants may be sufficient. More importantly, our findings emphasize the limitations of performing TPMT genotyping in the management of AZA induced MS.

Table 1

Summary of results.

<table>
<thead>
<tr>
<th></th>
<th>Wild type/HM</th>
<th>Heterozygos/IM</th>
<th>Homozygos/LM</th>
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</thead>
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<tr>
<td>N patients</td>
<td>46</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>Sex M/F</td>
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<td>4/7</td>
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<tr>
<td>Median age (years)</td>
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<td>42</td>
<td>23.5</td>
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<tr>
<td>Type of IBD</td>
<td>38 CD/8 UC</td>
<td>8 CD/3 UC</td>
<td>2 CD/2 UC</td>
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<tr>
<td>Median AZA dose (mg)</td>
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<td>100</td>
<td>100</td>
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<td>Median AZA dose (mg/kg)</td>
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<tr>
<td>Delay (months)</td>
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<td>Median white blood cells count (WBC/mm³)</td>
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<td>2260</td>
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<tr>
<td>Presence of thrombocytopenia</td>
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<td>Presence of anemia</td>
<td>16/46</td>
<td>7/11</td>
<td>4/4</td>
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<td>Presence of pancytopenia</td>
<td>4/46</td>
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<td>1/4</td>
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<td>Concomitant medications</td>
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<td>5-asa</td>
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<td>3/4</td>
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<td>3/4</td>
</tr>
<tr>
<td>Hospitalizations</td>
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<td>3/4</td>
</tr>
<tr>
<td>Median stay of hospitalization (days)</td>
<td>16.5</td>
<td>10.5</td>
<td>15</td>
</tr>
<tr>
<td>Medical intervention (AB, transfusion)</td>
<td>14/46</td>
<td>5/11</td>
<td>3/4</td>
</tr>
<tr>
<td>Re-introduction of azathioprine</td>
<td>29/46</td>
<td>6/11</td>
<td>1/4</td>
</tr>
<tr>
<td>Second episode of leucopenia</td>
<td>10/29</td>
<td>3/6</td>
<td>0/1</td>
</tr>
</tbody>
</table>

Fig. 1. Delay between administration of azathioprine and onset of myelosuppression.
Aza definitely stopped
N = 25
LM N = 3
IM N = 6
TPMT N = 17

Aza re-introduced to reach the
same dose
N = 9
IM N = 2
TPMT N = 7

Aza re-introduced to reach a
deeper dose
N = 27
LM N = 1
IM N = 4
TPMT N = 22

New episode of MS
N = 6/9

IM N = 2
TPMT N = 4

New episode of MS
N = 7/27

IM N = 1
TPMT N = 1

HM N = 6

Fig. 2. N = number of patient in each group, LM: low methylator, IM: Intermediate methylator, HM: high methylator, MS: Myelosuppression, TPMT: thiopurine Methyltransferase.

The median delay before MS onset was shorter in the LM + IM group (2 and 2.75 months for homozygous and heterozygous variants, respectively) vs. 6 months in the HM group. These differences were more pronounced than those in the Colombel’s study, where the median delays of MS onset were 1 month in LM, 4 months in IM and 3 months in HM (24). The longer delay before MS in HM patients support the hypothesis that other factors may be involved.

In our cohort, the median azathioprine dosage was slightly lower than the recommended 2–2.5 mg/kg (1.5 in LM, 1.9 in IM and 2 in HM). This reflects the well-known observation that patients are not all treated with the optimal AZA dose and confirms the discrepancy between recommendation and real life.

Infectious syndrome was observed in 20/61 (33%) patients including 5/15 LM + IM and 15/46 HM patients. This is much higher than the 6.5% infection rate reported in the literature [5]. Infections were severe but not lethal in 5/20 (25%) patients including 2 in the LM + IM and 3 in the HM subgroups. The retrospective nature of our study and the potential influence of steroid administration in several cases may hamper identification of a potential relationship between azathioprine therapy, MS and the occurrence of infection.

Twenty seven (27/61) patients had anemia with or without related symptoms such as asthenia, dyspnea and/or angina pectoris. As anemia is rather common in the course of IBD (due to iron deficiency and/or inflammation), it is not possible to identify the genuine impact of MS episode on the hemoglobin value in those cases.

Bleeding was infrequently observed (3/61 patients). This matches the low incidence of thrombocytopenia (12/61 patients), concordantly to the literature [5].

Concomitants drugs may interfere with thiopurine metabolism thereby precipitating MS. In clinical practice, assessing such interactions is often difficult.

The toxicity of drug interaction is well known for allopurinol [19], which inhibits xanthine-oxydase activity and shifts azathioprine metabolism towards active but potentially toxic moiety. Despite this well-known drug–drug interaction, in our series, 3 patients developed MS while treated simultaneously with both drugs (1 in IM group, 2 in HM group). The patients developed MS respectively 4, 5 and 10 weeks after initiation of allopurinol given at full dose (300 mg/day). These cases outline the toxicity of such an association and the need for reminding those related risks to health professionals and to patients.

A majority of patients received aminosalicylates (40/61) concurrently to azathioprine. An interaction between AZA and aminosalicylates has been reported in several clinical studies [20–22]. Aminosalicylates have been shown to increase the level of antipurine metabolites but the clinical relevance of such an interaction is not yet clinically demonstrated. Its mechanism remains unknown. To date no clinical study identified a change in the TPMT activity subsequent to aminosalicylates administration.

Diuretics, infliximab (IFX) and NSAIDs administration has also been associated with thiopurine drug–drug interaction [23] and these drugs were found in some of our patients.

In contrast to the Colombel study, where TP were only reintroduced in 7/41 (17%) patients, in the present study, AZA was readministered in 36/61 (59%) of the patients, to reach a similar dose in 9 or a lower dose in 27/36 patients. Such TP administration after the first MS episode was performed without knowing the TPMT genotype which was performed several months later (i.e. at the time of initiation of this retrospective study). As only 13/36 (36%) patients experienced a second episode of MS, it can be concluded that re-administration of thiopurine therapy may be relatively safely attempted in patients who have previously developed MS during AZA therapy. In addition, the finding that 23/36 patients did not experience any subsequent MS episode, pleads against an underlying genetic mechanism in those patients.

However, when thiopurine re-introduction is considered after a first MS episode, it has to be done with caution. From this study, we can advice to re-treat patient with a lower dose than the one initially given. Indeed, 6/9 patients treated with the same TP dose experienced a second MS episode, in contrast to only 7/27 that have been treated with a lower dose than initially.

The delay of onset of the 2nd MS event was shorter in 5 patients and longer in 6 patients suggesting that white blood cells monitoring should not be skipped.

There were fewer concomitant medications at the time of 2nd MS, except for IFX which was added 2 weeks before MS in 2 cases and 2.5 months in a third case. IFX-AZA has been reported as a possible drug–drug interaction inducing leucopenia [23].

It is interesting to note that AZA dose at the time of a second MS episode was similar to the dose used at the time of the 1st MS episode in two of the three LM + IM patients, which pinpoints a direct relationship between AZA dosage and a TPMT deficient genotype. In the third case, the second MS episode occurred at a lower thiopurine dose.

The occurrence of a second MS episode in HM patients however raises the hypothesis that other enzyme polymorphisms or deficiencies may also interfere and contribute to enhance the thiopurine toxicity.
In conclusion, an extensive TPMT genotyping explains only 25% of all MS event in IBD patients taking purine analogs. The TPMT variants found in these patients are the most common ones (*3A,*3C,*2). A TPMT genetic determination, if prescribed in a Caucasian population, might focus on those variants as the other mutations do not appear to be relevant. TPMT genotyping is a useful tool but does not seem sufficient to direct TP treatment. No differences were found between TPMT deficient genotype and TPMT wild type genotype in terms of severity of MS and frequency of complications. Resuming AZA therapy after a first MS episode may be considered, even without TPMT determination. However the administration of a lower thiopurine dose should be advocated in such cases.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.clinbiochem.2011.06.079.

References