Impact of *NUDT15* genetics on severe thiopurine-related hematotoxicity in patients with European ancestry

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Purpose: Severe hematotoxicity in patients with thiopurine therapy has been associated with genetic polymorphisms in the thiopurine S-methyltransferase (TPMT). While TPMT genetic testing is clinically implemented for dose individualization, alterations in the nudix hydrolase 15 (NUDT15) emerged as independent determinant of thiopurine-related hematotoxicity. Because data for European patients are limited, we investigated the relevance of *NUDT15* in Europeans.

Methods: Additionally to TPMT phenotyping/genotyping, we performed in-depth Sanger sequencing analyses of *NUDT15* coding region in 107 European patients who developed severe thiopurine-related hematotoxicity as extreme phenotype. Moreover, genotyping for *NUDT15* variants in 689 acute lymphoblastic leukemia (ALL) patients was performed.

Results: As expected TPMT was the main cause of severe hematotoxicity in 31% of patients, who were either TPMT deficient

(10%) or heterozygous carriers of *TPMT* variants (21%). By comparison, *NUDT15* genetic polymorphism was identified in 14 (13%) patients including one novel variant (p.Met1Ile). Six percent of patients with severe toxicity carried variants in both *TPMT* and *NUDT15*. Among patients who developed toxicity within 3 months of treatment, 13% were found to be carriers of *NUDT15* variants.

Conclusion: Taken together, *NUDT15* and *TPMT* genetics explain ~50% of severe thiopurine-related hematotoxicity, providing a compelling rationale for additional preemptive testing of *NUDT15* genetics not only in Asians, but also in Europeans.

INTRODUCTION

Thiopurine-related hematoxicity is clinically relevant and therapy-limiting in treatment of patients with acute lymphoblastic leukemia (ALL) or inflammatory bowel disease (IBD). In the past, retrospective and prospective clinical studies corroborated the clinical utility of genetic testing for thiopurine S-methyltransferase (TPMT) to avoid severe thiopurine-cytotoxicity.[1–4] For clinical implementation, the Clinical Pharmacogenetics Implementation Consortium (CPIC) published peer-reviewed and evidence-based gene/drug clinical practice guidelines[5] indicating preemptive *TPMT* genotype-guided thiopurine-dose individualization to mitigate drug toxicity. Recently loss-of-function germline variants in *NUDT15* have been identified as additional genetic determinants of thiopurine intolerance.[6,7] Laboratory studies demonstrated excessive DNA damage and subsequent bone marrow suppression by the active thiopurine metabolite

thioguanosine triphosphate (TGTP) in patients with NUDT15 deficiency. Inactivation of TGTP by converting TGTP to TGMP (thioguanosine monophosphate) is regulated by the nucleotide diphosphatase activity of NUDT15, thereby counteracting the cytotoxic effects of thiopurines. Consistently, thiopurine-treated *Nudt15* knock-out mice also develop severe leukopenia compared with wild-type mice.[8]

So far the impact of NUDT15 on thiopurine hematotoxicity as an extreme phenotype in populations other than Asians is limited because *NUDT15* variants are most prevalent in Asians.[6,9] However, first reports indicate that for instance *TPMT* and *NUDT15* genes are both related to thiopurine-related toxicity in patients from Uruguay.[10] In the present paper, we evaluate the importance of *NUDT15* genetics, in addition to *TPMT* variation, for development of severe hematotoxicity in thiopurine-treated patients with European ancestry.

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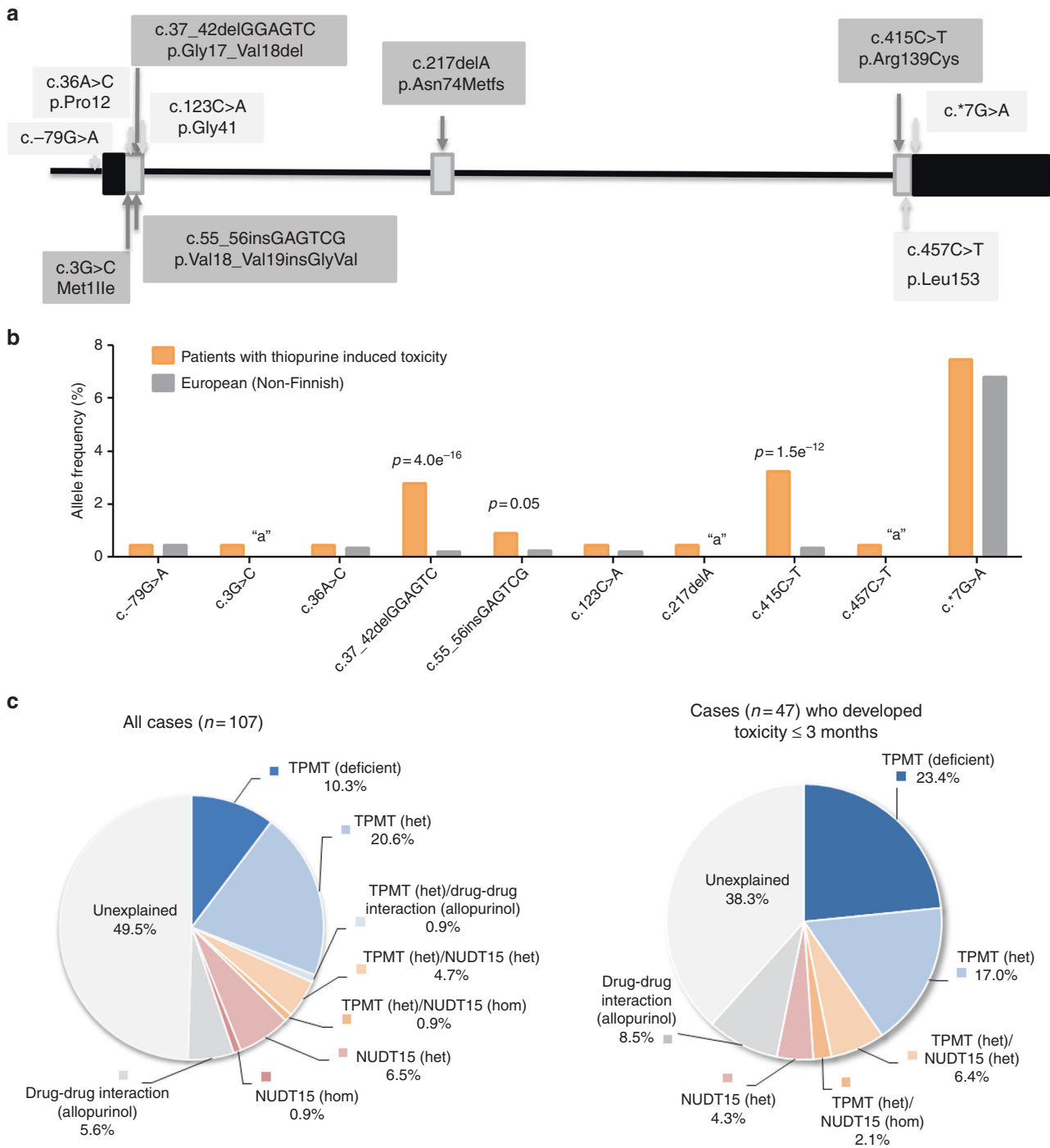
Fig. 1 *NUDT15* genetics and thiopurine-related hematotoxicity (**a**) Genetic variants detected in the *NUDT15* gene region in patients with severe thiopurine-related toxicity. Dark gray arrows indicate variants resulting in variation of the NUDT15 protein sequence. Boxes indicate exons 1–3, and untranslated regions are shown in black. (**b**) Allele frequency of *NUDT15* variants in patients with thiopurine-induced toxicity and individuals of European (non-Finnish) ancestry extracted from the Genome Aggregation Database (gnomAD)[14] (see Table S3). *P* values indicate significant differences in genotype frequency as tested by the Armitage's trend test. [a]Variants are not reported in exome or genome sequencing data of gnomAD.[14] (**c**) Pie chart illustrates percentage of patients with variation in *TPMT* or *NUDT15* genetics as well as percentage of patients with allopurinol treatment. Left part shows fractions in all patients (*n* = 107), whereas fractions in patients (*n* = 47) who developed toxicity within 3 months after the first administration of therapy are illustrated on the right.

MATERIALS AND METHODS

The study cohort (*n* = 180) comprised of consecutively included patients who experienced severe clinical thiopurine-related cytopenia/pancytopenia and were tested for TPMT at the pharmacogenetic laboratory at our institute between January 2002 and September 2006. Thiopurine hematotoxicity was

documented by the responsible physician in a standardized questionnaire and/or if available on hematological laboratory criteria as previously published[11] and in line with accepted criteria (https://ctep.cancer.gov/protocolDevelopment/ electronic_applications/docs/ctcaev3.pdf). Only patients with a sufficient amount of remaining DNA ($n = 107$) and patient characteristics were selected for analyses of *NUDT15* genetics through Sanger sequencing (see supplementary materials and methods). Further information about patient characteristics and thiopurine treatment is given in Table S1. The study was approved by the local ethics committee of the University of Tübingen, Germany, and all analyses were done anonymously.

The second cohort included ALL children ($n = 689$) treated according to the Berlin–Frankfurt–Münster (BFM) trial AIEOP-BFM ALL 2009 (ref. [12]) and was used as an independent control group of 6-mercaptopurine (6-MP) treated patients with preemptive genotyping for *TPMT* and subsequent 6-MP dose adjustment only in case of TPMT deficiency to avoid thiopurine-related hematotoxicity.[11,13] For details, see Table S2. The study was approved by the ethical review board and written informed consent was provided. Further details about the method used for *NUDT15* and *TPMT* genotyping are provided in supplementary materials and methods.

Detailed information about statistical methods used to compare frequency distributions with the general European population extracted from the Genome Aggregation Database (gnomAD)[14] is given in supplementary materials and methods.

RESULTS

Although previous studies suggested that *NUDT15* genetics is associated with thiopurine-related hematotoxicity especially in Asians, only limited data are available for patients of European ancestry. Therefore, we performed the first analysis of genetic variability of *NUDT15* in 107 European patients who developed severe thiopurine-associated hematotoxicity. The study cohort consisted mainly of patients with inflammatory bowel diseases ($n = 68$) or other autoimmune diseases. The median dosage of azathioprine and 6-MP was 100 mg (range 50 to 300 mg) and 50 mg (50 to 125 mg), respectively (Table S1). In total, ten different genetic variants were found within *NUDT15* by Sanger sequencing covering all exonic and flanking intronic regions (Fig. **1a**). All ten genetic variants and their frequency distribution in 107 individuals are listed in Table S3. In the exonic regions, eight variants were found, of which five were missense variants (see Table S3). Three of these missense variants have previously been associated with thiopurine-related toxicity (p.Gly17_-Val18del, p.Val18_Val19insGlyVal, p.Arg139Cys).[6,7] To further investigate an association of these variants with toxicity, we compared their frequencies in our study cohort with the frequency present in the general European population. Here, two of these variants were strongly (p.Gly17_-Val18del: $p = 4.0e^{-16}$ and p.Arg139Cys: $p = 1.5e^{-12}$) and one

moderately (p.Val18_Val19insGlyVal: $p = 0.05$) significantly more frequent in our patient cohort compared with the general European population (Fig. **1b**), which further supports an association of these variants with thiopurine toxicity and functional consequences. In addition, we identified one novel variant resulting in the loss of the start codon (c.3G>C, *NUDT15*19*) and one missense frameshift variant (c.217delA, *NUDT15*18*), both with consequences on *NUDT15* function and activity due to severe alterations in the NUDT15 protein sequence. Moreover, both of these variants seem to be rare and were not detected in exome or genome sequencing data in the European (non-Finnish) population (gnomAD,[14] see Table S3). Genotyping of the most frequent functional relevant variants, p.Gly17_Val18del and p. Arg139Cys, in a cohort of ALL patients revealed that both the p.Gly17_Val18del and p.Arg139Cys variant were absent in this cohort of ALL patients, indicating their very low frequency in patients of European ancestry (Table S3). Because the frequencies of the genetic variants detected in the 5'UTR or 3'UTR were not significantly different between patients who developed toxicity and the European population, it is unlikely that they are linked to functional consequences resulting in thiopurine toxicity (Fig. **1b**). Moreover, synonymous variants resulting in no alterations of *NUDT15* protein sequence most likely do not impair *NUDT15* function. Thus only the five missense variants described above were considered as candidate variants for the explanation of thiopurine-associated toxicity in our patient cohort. In total, 14 (13%) of the 107 patients carry at least one of these variants. The characteristics of these patients together with their *NUDT15* genotypes/haplotypes as well as detailed information about thiopurine treatment are summarized in Table **1**. Because it is well established that TPMT pheno- or genotype is associated with thiopurine-related hematotoxicity, all patients of our study were phenotyped or genotyped for the most frequent inactivating *TPMT* alleles (see supplementary materials and methods). Of note, 6 of 14 patients who carry at least one *NUDT15* missense variant are also carriers of one *TPMT* allele responsible for reduced TPMT activity (Table **1**). As shown in Fig. **1c** (left panel), among all 107 patients, 11 (10%) were TPMT deficient and 22 (21%) carried one *TPMT* variant allele. To evaluate the impact of comedication on the development of toxicity, we solely investigated cotreatment with allopurinol. Allopurinol is the only drug for which dose adjustment to avoid hematotoxicity is recommended by the prescription information of thiopurines.[15,16] In our cohort, seven patients received allopurinol as comedication without adequate dose adjustment of azathioprine. One of these patients was also *TPMT*1/*3A*. In 50% of patients the occurrence of toxicity could not be explained by both *TPMT* and *NUDT15* genetics, or by allopurinol medication. Considering only patients ($n = 47$) who developed hematotoxicity within 3 months after onset of thiopurines indicated that 13% of these patients carry at least one *NUDT15* variant and 53% of these patients are carriers of at least one *NUDT15* or *TPMT* variant (Fig. **1c**, right panel).

Table 1 Patient characteristic of *NUDT15* variant carriers

Patient	*NUDT15* alleles	Sex	Age	TPMT activity	TPMT genotype	Duration of therapy until pancytopenia occurred (months)	Therapy	Dose	Weight (kg)	Diagnosis
1	*1/*6	F	64	38	*1/*1	10	AZA	75	52	IBD
2	*1/*3	F	80	48	*1/*1	0.5	AZA	100	80	IBD
3	*3/*3	F	34	48	*1/*1	NA	AZA	100	62	IBD
4	*1/*9	M	39	78	*1/*1	16	AZA	200	86	IBD
5	*1/*6	F	19	18	*1/*3C	11 (intermittent)	AZA	150	58	IBD
6	*1/*3	M	58	22	*1/*3A	3	AZA	100	80	IBD
7	*1/*9	F	36	48	*1/*1	18	AZA	50	54	IBD
8	*9/*9	F	51	17	*1/*3A	0.75	AZA	75	75	RA
9	*1/*3	M	14	31	*1/*1	1	AZA	75	39	IBD
10	*1/*18	M	63	25	*1/*3A	3	6-MP	50	78	IBD
11	c.37_42delGGAGTC and c.415C>T	M	14	51	*1/*1	1.2	AZA	100 mg for 22 days/50 mg for 14 days	62	IBD
12	*1/*3	F	40	55	*1/*3A	6	AZA	150	58	Multiple sclerosis
13	*1/*19	M	54	23	*1/*3A	0.25	AZA	150	75	Pemphigus vulgaris
14	*1/*9	M	68	62	*1/*1	12	AZA	200	88	Myasthenia gravis

Definition of *NUDT15* haplotypes adheres to the proposed nomenclature of the Pharmacogene Variation (PharmVar) Consortium[19] at https://www.pharmvar.org/gene/NUDT15 (details see supplementary data): *1 allele represents the *NUDT15* reference haplotype. For patient 11, who carries two *NUDT15* variants (c.37_42delGGAGTC and c.415C>T) haplotypes could not be assigned with certainty (either *3/*9 or *1/*new).
AZA azathioprine, *IBD* inflammatory bowel disease, *NA* not available, *RA* rheumatoid arthritis, *6-MP* 6-mercaptopurine.

Of note, all 11 TPMT deficient patients in our cohort developed toxicity within 3 months. Taken together, toxicity in 38% of patients remained unexplained.

DISCUSSION

Thiopurine-associated hematotoxicity has so far been mainly attributed to alterations in TPMT, but the role of NUDT15 especially in Europeans remains elusive. Therefore, we performed for the first time a comprehensive analysis of genetic variability of NUDT15 in European patients ($n = 107$) with different diseases, who developed severe thiopurine-related hematotoxicity documented by a standardized questionnaire and/or hematological laboratory.[11] In summary, we identified through comprehensive sequencing ten different genetic variants within NUDT15, including five missense variants (see Table S3). Three of these missense variants have already been associated with thiopurine-related toxicity (p. Gly17_Val18del, p.Val18_Val19insGlyVal, p.Arg139Cys), which was corroborated by studies investigating their functional consequences.[6,7] These findings are further supported by our study, indicating a significantly higher frequency of these variants in our patient cohort representing an extreme phenotype (severe thiopurine-related hematotoxicity) compared with the general European population (Fig. 1b). In addition, two rare missense variants in NUDT15 were detected, which have not been associated with thiopurine-related hematotoxicity so far. Of note, both variants have functional consequences resulting either in the loss of the start codon (c.3G>C, NUDT15*19) or in a frameshift (c.217delA, NUDT15*18). Thus our findings provide first evidence that rare functional variants within NUDT15 exist in Europeans, which supports the relevance for in-depth sequencing in patients with European ancestry prior to thiopurine therapy to avoid thiopurine-related hematotoxicity.

Because severe hematotoxicity in patients with thiopurine therapy (e.g., azathioprine, 6-mercaptopurine) is associated with genetic polymorphisms in TPMT, all patients in our study were not only phenotyped, but also genotyped for relevant TPMT alleles as well. Thereby we confirmed that TPMT was the main cause of severe hematotoxicity in 31% of patients, who were either TPMT deficient (10%) or heterozygous carriers of TPMT variants (21%). Regarding NUDT15, functionally relevant missense variants were identified in 14 (13%) patients, of whom 6 patients with severe toxicity carried variants in both TPMT and NUDT15 (Fig. 1c, left panel). Thus the combination of impaired NUDT15 and TPMT activity might further increase the probability for developing thiopurine-related toxicity compared with deficiency in only one of the two genes. Generally, toxicity occurred at median within 4 months, but a broad range up to 120 months was observed. This long period was observed in one patient with IBD and TPMT heterozygosity receiving 60% of the recommended standard dose. In spite of dose reduction, over the long term 60% of dosage appears to be still too high, corroborating individual thiopurine dosing

(40% up to 70% of standard dose) in TPMT heterozygous patients. Because severe toxicity due to genetic reasons most likely occurs within a few months,[2] we performed a subanalysis for the association of TPMT and NUDT15 genetics and thiopurine-related toxicity including only those patients who developed toxicity within 3 months. In total, among patients who developed toxicity within 3 months of treatment, 53% could be explained by TPMT and/or NUDT15 genetics, but 38% remained unexplained, also considering comedication (Fig. 1c, right panel). Because of their normal TPMT activity (median: 42.0 [24–72]) (refs. [13,17]), rare genetic variants within TPMT that were not tested in the present study can be excluded to explain toxicity. Because thiopurine-related hematotoxicity is multifactorial, additional risk factors (e.g., viral infection) or novel risk genes might be responsible for toxicity in these patients. Our study has some limitations. First, it is a retrospective analysis. Second, a potential bias of the study results may be the selection of only 107 of 180 patients reported with severe thiopurine-related hematotoxicity. However, because all patients for pharmacogenetic diagnostics at our center were consecutively collected and the frequency distribution of heterozygous and homozygous TPMT variant carriers is comparable in both groups of patients ($p = 0.32$; see Table S4), we believe the bias is negligible.

In summary, we provide evidence that NUDT15 genetics in addition to TPMT is a critical factor contributing to thiopurine-induced hematotoxicity in patients with European ancestry. Although prospective studies and health economic evaluations are currently missing, the process to implement NUDT15 genetics in clinical practice will most likely be facilitated by the upcoming guideline of the CPIC.[18] Our data indicate that NUDT15 variants are worth implementing in preemptive pharmacogenetic testing in clinical practice for genotype-guided thiopurine therapy.

DISCLOSURE
The authors declare no conflicts of interest.

BRIEF COMMUNICATION

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