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RESEARCH ARTICLE

Genetic profiling of *NUDT15* in the Slovenian population

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ABSTRACT

Determining variant *TPMT* alleles to predict patient response to thiopurine therapy represents one of the first successful implementations of pharmacogenomics in clinical practice. However, despite the *TPMT*-adjusted thiopurine dosing, some *TPMT* wild-type patients still exhibit toxicity at standard doses. Over the past decade, the pharmacogene *NUDT15* has emerged as a significant co-modulator of thiopurine therapy. Initially, *NUDT15* was considered important predominantly in Asian populations, but recent studies have highlighted its relevance in European populations as well.

To evaluate the pharmacogenetic significance of *NUDT15* in the Slovenian population, we sequenced extended regions of exon 1 and exon 3 in 109 healthy individuals and 37 patients with acute lymphoblastic leukemia.

We identified eight variants, including one with established clinical significance (allele *3) and one extremely rare variant (Chr13 at 48045861; GRCh38, NC_000013.11). The frequencies of most previously described variants in both the general population and in the ALL cohort were consistent with those reported in other European populations, except for rs45465203, which was less frequent in the Slovenian population. None of the variants, except for *NUDT15*3*, were associated with cumulative thiopurine doses in ALL patients. However, these variants warrant further investigation in larger ALL cohorts.

PLAIN LANGUAGE SUMMARY

Pharmacogenes are genes coding for enzymes, transporters and drug targets that can affect an individual's response to drugs. Determining genetic variants in pharmacogenes prior to treatment enables more personalized and effective treatments. *NUDT15* is a gene that plays a crucial role in the metabolism of cytostatic and immunosuppressive drugs, specifically thiopurines, which are commonly used in the treatment of acute lymphoblastic leukemia (ALL). Certain genetic variants can result in lower enzyme activity and consequently a higher risk of severe toxicities from thiopurines. Our study reports the frequencies of *NUDT15* genetic variants in the Slovenian population. We discovered extremely rare genetic variant in the *NUDT15* gene, located on chromosome 13 at position 48045861 (GRCh38, NC_000013.11), which did not have a previously assigned rs number. Furthermore, we found that a patient with ALL who had a variant allele *NUDT15*3* received a lower dose of thiopurines compared with other patients with the wild-type genotype. This research may help to further understand genetic variations in different populations. Patients treated with thiopurines should have genetic variants in the *NUDT15* gene determined. This study further supports the guidelines for dose reduction in patients with variant *NUDT15*3* genotype.

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6-mercaptopurine; acute lymphoblastic leukemia; dose reduction; minor allele frequency; NUDT15; population genetics; thiopurines

1. Introduction

The main objective of the pharmacogenomic approach to any therapy is to predict the patient's response to a drug before its application, to reduce risks of side effects or ineffective treatment. The patient's response to treatment can be predicted by determining the clinically relevant variants in their DNA. The Dutch Pharmacogenetics Working Group (DPWG) and the Clinical Pharmacogenomic Implementation Consortium (CPIC) proposed that a genetic variant that is included in the pharmacogenomics (PGx) diagnostic panel has a well-documented association with the gene expression or protein function and minor allele frequency (MAF) equal to or higher than 1% in the general population. In case the MAF in the general population is lower, the variant should exert an extremely strong association with the

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phenotype or should have an MAF of at least 1% in a specific subpopulation of interest [1]. The Association for Molecular Pathology PGx Working Group has set the recommended MAF for the Tier 1 alleles at ≥0.1% and for Tier 2 alleles at ≥0.01% [2]. Since the function of many pharmacogenes is the detoxification of xenobiotics, they are not highly conserved and are very polymorphic with their mutation profiles exhibiting heterogeneity across different ethnic groups [3,4].

One of the most successful examples of the implementation of pharmacogenomics in clinical practice involves thiopurine drugs used in treatments for cancer, autoimmune disease and after organ transplantation [5,6]. Thiopurines are prodrugs with their active cytotoxic metabolites being 6-thioguanine nucleotides (TGNs) [7]. TPMT is the key enzyme in hematopoietic cells which deactivates thiopurines to 6-methylmercaptopurine (meMP) [7]. *TPMT* gene has been a major PGx marker in thiopurine therapy, as patients with variant alleles causing decreased enzyme activity more readily experience severe side effects [8].

However, in the last decade, the importance of other pharmacogenes associated with thiopurine therapy has emerged, most notably *NUDT15* [9]. The endogenous substrate for NUDT15 is 8-oxo-guanosine triphosphate, which is dephosphorylated to its monophosphate form by NUDT15 [10,11]. Due to structural similarity to endogenous nucleotides, NUDT15 can also hydrolyze active metabolites of 6-MP, 6-thioguanosine triphosphate (6-TGTP), into 6-thioguanosine diphosphate and 6-thioguanosine monophosphate. This prevents their incorporation into DNA and reduces the cytotoxic effects of thiopurines [10]. *NUDT15* gene is 10 kb long and is located on the long arm of chromosome 13 (13q14.2) [12]. Genetic variants in *NUDT15* can lead to loss of function. In patients treated with thiopurines, the presence of such genetic variants results in more 6-TGTP being available for incorporation into DNA, thus leading to excessive myelosuppression in an affected patient [13–16].

Currently, both the CPIC [9] and DPWG [1] guidelines recommend the assessment of *TPMT* and *NUDT15* variants before starting thiopurine therapy. Although *NUDT15* variants were initially considered as being clinically relevant only in Asian populations [14], their relevance is now becoming recognized in European populations as well [17]. The first *NUDT15* variant associated with overt toxicity of thiopurine drugs was a missense mutation rs116855232 (c.415C > T; p.R139C) [13]. It causes a complete loss of enzyme activity in the homozygous state *in vitro*, while homozygous patients receiving standard doses of thiopurines experience severe myelosuppression [14]. Homozygous pediatric ALL patients were able to tolerate 8% and heterozygous patients 63% of the standard 6-MP dose, respectively [16]. To date, 19 variant alleles in *NUDT15* have been identified, designated as *2-*20, while *1 is a wild-type allele [18]. The definition of *NUDT15* alleles, their functionality and population frequencies are described in Table 1.

The majority of variants described in Table 1 lie in exons 1 and 3 of the *NUDT15* gene, pinpointing exons 1 and 3 as *NUDT15* mutational hot spots. Variants *13, *17 and *18, located in exon 2, have not been detected in the European population. The most common *NUDT15* variant alleles in the European population are *3, *6 and *9, with the frequencies of diplotypes *1/*3, *1/*6 and *1/*9 being 0.5959%, 0.3973% and 0.3633%, respectively [20]. Individuals with such diplotypes are classified as intermediate metabolizers, who should use a decreased dose if the standard dose is 75 mg/m^2/day or higher, while in the case of the lower standard dose, dose adjustment might not be necessary in some cases. However, it must be noted that in the case where the patient has an intermediate metabolizer genotype at both *TPMT* and *NUDT15* loci, a substantial decrease in the standard dose is recommended [19]. Homozygotes or combined heterozygotes for two non-functional alleles are classified as poor metabolizers. In such patients, the recommended starting dose is 10 mg/m^2/day for treatment of malignant disease, while in cases of therapy for non-malignant disease, alternative non-thiopurine immunosuppressants should be considered. The most common poor metabolizer diplotype in Europeans is *3/*9 with a frequency of 7.3×10^{-6} [9].

While *TPMT* genotyping is already used in clinical practice in Slovenia, *NUDT15* genotyping has not yet been implemented in clinical settings. Therefore, this study aimed to explore the *NUDT15* polymorphisms in the mutational hot spots in exons 1 and 3 in the Slovenian cohort of ALL patients and in the Slovenian cohort of healthy individuals as well as to investigate the effect of the identified variants on the cumulative dose of 6-MP administered during the maintenance phase of ALL treatment.

2. Patients & methods

2.1. Study design & study population

The study was designed in two parts. The first part consisted of an exploratory observational study with the aim to explore the presence of *NUDT15* genetic variants in Slovenian pediatric patients with ALL and assess their effects on the cumulative dose of 6-MP administered during the maintenance phase of the treatment. The study population consisted of 37 patients who have completed the maintenance phase of therapy. Peripheral blood was collected for DNA extraction at the Depart-

Table 1. *NUDT15* alleles definition, functionality and frequencies in South Asian, East Asian and European populations.

NUDT15 allele	Allele definition (NM_018283.4)	Allele functionality	Allele frequency (%)		
			South Asian	East Asian	European
*1	Wild-type	Normal	93.0024	87.8742	99.313
*2	rs746071566 (c.38GAGTCG[4]; p.V18_V19insGV) + rs116855232 (c.415C > T; p.R139C)	No enzyme activity	0.0000	3.5000	0.0000
*3	rs116855232 (c.415C > T; p.R139C)	No enzyme activity	6.7000	6.0500	0.2000
*4	rs147390019 (c.416G > A; p.R139H)	Uncertain effect	0.0033	0.1113	0.0033
*5	rs186364861 (c.52G > A; p.V18I)	Uncertain effect	0.0448	1.1080	0.0008
*6	rs746071566 (c.38GAGTCG[4]; p.V18_V19insGV)	Uncertain effect	0.2000	1.3000	0.3000
*7	rs766023281 (c.101G > C; p.R34T)	Uncertain effect	0.0000	0.0565	0.0000
*8	(c.103A > G; p.K35E)	Uncertain effect	0.0000	0.0000	0.0000
*9	rs746071566 (c.38GAGTCG[2]; p.del17_18GV)	No enzyme activity	0.0495	0.0000	0.1829
*10	rs769369441 (c.2T > C; p.M1T)	Function not assigned	0.0000	0.0000	0.0027
*11	rs1950545307 (c.139G > A; p.G47R)	Function not assigned	0.0000	0.0100	0.0000
*12	rs149436418 (c.156C > G; p.F52L)	Function not assigned	0.0000	0.0000	0.0181
*13	rs761191455 (c.343dup; p.E115fs)	Function not assigned	0.0202	0.0259	0.0000
*14	rs777311140 (c. 80_81insCGGG; p.C28fs)	Likely no enzyme activity	0.0000	0.0000	0.0210
*15	rs139551410 (c.467T > A; p.L156Q)	Function not assigned	0.0036	0.0000	0.0016
*16	rs1202487323 (c.88C > T; p.L30V)	Function not assigned	0.0000	0.0000	0.0000
*17	rs1368252918 (c.352G > T; p.E118X)	Function not assigned	0.0000	0.0000	0.0000
*18	rs1457579126 (c.221del; p.N74fs)	Function not assigned	0.0000	0.0000	0.0000
*19	(c.3G > C; p.M1I)	Function not assigned	0.0000	0.0000	0.0000
*20	rs768324690 (c.386C > G; p.P129R)	Function not assigned	0.0000	0.0000	0.0016

Data taken from [20].

ment of Pediatric Hematology and Oncology, University Children's Hospital, University Medical Centre Ljubljana. Detailed data on dose intensities of 6-MP, including all dose changes and treatment interruptions throughout the whole period of the maintenance phase of therapy, was extracted from medical records of children with acute lymphoblastic leukemia (ALL). The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (KME 89/07/13). For patients under 18 years, informed consent to participate in the study was provided by their parents or legal guardians.

The second part of the study was a population-based study aiming to explore the presence and frequencies of *NUDT15* polymorphisms in the mutational hot spots in exons 1 and 3 in general Slovenian population. Peripheral blood from 109 unrelated healthy adult volunteers from Slovenia was randomly collected at the Student Health Centre of the University of Ljubljana as a part of a pharmacogenomic study, which was approved by the National Medical Ethics Committee (KME 85/06/15). All participating subjects provided their informed consent to participate in the study.

2.2. DNA extraction, polymerase chain reaction & sequencing

First, DNA was extracted from 200 μl of collected blood according to the manufacturer's instructions (QIAamp DNA Blood Mini Kit, Qiagene, Venlo, Netherlands). The region of interest was then amplified using the following primers (Merck, Sigma-Aldrich, USA): Exon 1 was amplified using forward primer with 5′-3′ sequence CAAAGCACAACTGTAAGCGAC and reverse primer with 5′-3′ sequence CACACCTCACAGACGAACTC (the size of the amplicon was 680 bp) and Exon 2 was amplified using forward primer with 5′-3′ sequence CAAGCAAATGCAAAGCATCAC and reverse primer with 5′-3′ sequence GGCTGAAAGAGTGGGGGATA (the size of the amplicon was 451 bp). Before Sanger sequencing, we performed separate polymerase chain reactions (PCRs) for exon 1 and exon 3 of *NUDT15*. The PCR reaction mix contained 75–150 ng of DNA per sample. For PCR, we used Hot Fire Pol Taq DNA Polymerase, buffer, MgCl (all Solis BioDyne, Tartu, Estonia) and dNTPs (Promega, USA) according to the manufacturer's instructions. The mixture for exon 1 contained 15% of G/C rich enhancer (Solis BioDyne, Tartu, Estonia). The following thermal cycling conditions were applied: 12 min 95°C, 45× (30 s 95°C, 30 s 58°C (exon 1) or 55 (exon 3), 1 min 72°C), 8 min 72°C and cooling. The presence of PCR fragments was confirmed by gel electrophoresis composed of 2% agarose with 1X SYBR safe DNA gel stain (Thermo Fisher Scientific, Invitrogen, Waltham, MA, USA). The separation of fragments was performed at 100 V and 400 A and lasted 20 min. Samples were sequenced in forward and reverse direction by McLab (San Francisco, CA, USA).

2.3. Data analysis & statistical methods

Sequences in *NUDT15* regions were determined using Finch TV software (Version 1.4.0; Geospiza, Inc, WA, USA; http://www.geospiza.com). Data analysis and data

presentation were performed using MS Excel (Version 2408, Microsoft 365 for enterprise), GraphPadPrism (Version 10.2.3) and R (Version, 2023.06.1). Where applicable, the Shapiro-Wilk test was used for assessing the normality of the distribution. The significance of Hardy-Weinberg equilibrium and comparison of allele frequencies between different populations was determined by Fisher's exact test. Relative cumulative dose intensities between groups with different genotypes were compared using the Student *t*-test. Prediction algorithms embedded in the Franklin (2024.2) platform (Aggregated Prediction, Splice AI, FATHMM, Revel, AlphaMissense, MUT Assessor, SIFT, MetaLR, DANN, MT, GenoCanyon and fitCons) were used to assume the consequences of identified variants.

3. Results

3.1. NUDT15 variants in patients with acute lymphoblastic leukemia

As an important pharmacogenetic marker, *NUDT15* is critical for predicting thiopurine response. Therefore, we first aimed to investigate its influence on thiopurine treatment. The study cohort comprised 23 female and 14 male pediatric patients with ALL in the maintenance phase of their treatment. The median age at diagnosis was 7 years, with interquartile range (IQR) of 5 years, indicating significant heterogeneity within the study population. All subjects were treated according to the ALL Intercontinental-Berlin-Frankfurt-Münster 2009 protocol (ALL IC BFM 2009) [21].

All DNA samples from patients were sequenced for *NUDT15* exon 1 and exon 3 with their vicinities, as most pharmacogenetically important variants lie within this region. All patients were of European descent. The identified variants in our ALL cohort were rs45465203, rs61973267, rs61746486 and rs116855232 with MAFs of 8.1%, 9.5%, 4.1% and 1.4%, respectively. We found one patient heterozygous for allele *NUDT15*3* (rs116855232).

3.2. Dosing regimen & NUDT15 genotypes

We next correlated the dosing of 6-MP with the presence of genetic variants in *NUDT15* found in ALL patients. The pediatric population is quite heterogeneous, especially in age, weight and height, which significantly affects the dosing of 6-MP. The dose of 6-MP is further adjusted throughout maintenance therapy based on hematological parameters, primarily leukocyte count and the severity of adverse events. To normalize the interindividual variability in dose regimens in patients in our cohort, we calculated the ratio between the actual cumulative dose received during the entire maintenance therapy and the theoretical cumulative dose an individual would receive based on body surface area calculation (Mosteller formula) and individual-specific duration of maintenance therapy with treatment interruption days excluded (relative cumulative dose intensity). The values of relative cumulative dose intensities among 37 studied patients ranged from 0.42 to 1.46, with a mean of 0.96 ± 0.24. This indicates that patients in our cohort received, on average, 96% of the theoretical cumulative dose.

We observed a trend, although statistically non-significant, that individuals with a heterozygous genotype for rs61746486 received higher relative cumulative doses of 6-MP as a part of their maintenance phase of ALL treatment compared with patients without this variant (1.17 vs. 0.94). Similarly, individuals with at least one variant allele for rs61973267 received higher cumulative doses of 6-MP compared with non-variant homozygotes (1.05 vs. 0.95, statistically non-significant). No difference in cumulative dose intensity of 6-MP according to rs45465203 genotype was observed (Table 2).

Interestingly, the patient with the *NUDT15*3* allele received a relative cumulative dose of 6-MP that was 1.25-times lower than that of other non-variant patients (0.77 vs. 0.97 ± 0.24), which aligns with the reported lower enzyme activity in individuals carrying this allele and thus lower tolerability of standard 6-MP doses. Since there was only one patient with the *NUDT15*3* (rs116855232) allele in our cohort, the statistical significance of this observed difference could not be calculated.

3.3. Genetic variants in NUDT15 in the general Slovenian population

To further understand the distribution and impact of *NUDT15* genetic variants, we extended our analysis to a healthy population. The information on population genetics enables the determination of the frequency and thus rationalizes the clinical diagnostic significance of these polymorphisms in ALL patients.

Our study cohort comprised 109 healthy individuals, including 78 females and 31 males, all of European descent, with a median age of 19 years at the time of blood collection. All DNA samples were sequenced for *NUDT15* exon 1, exon 3 and their vicinities. We identified eight *NUDT15* SNPs in the Slovenian population. The identified polymorphisms included rs181638201, rs45465203, rs79687000, rs1249937565, rs61746486, a novel SNP at Ch13 48045861 A > G (Figure 1), rs61973267 and rs116855232, also known as *NUDT15*3* allele (Table 3).

Table 2. Relative cumulative dose of 6-MP according to *NUDT15* variants in pediatric patients with ALL.

Variant genotype		rs45465203			rs61746486			rs116855232			rs61973267		
		GG	GA	*p*-value	AA	AC	*p*-value	CC	CT	*p*-value	GG	GA/AA	*p*-value
Relative cumulative dose intensity	Mean	0.9526	0.9952		0.9423	1.168		0.9658	0.7743		0.9473	1.046	
	SD	0.2451	0.2034	0.6733	0.2362	0.09589	0.1179	0.2369		NA	0.2489	0.097665	0.3904
	N	30	7		34	3		36	1		32	4	

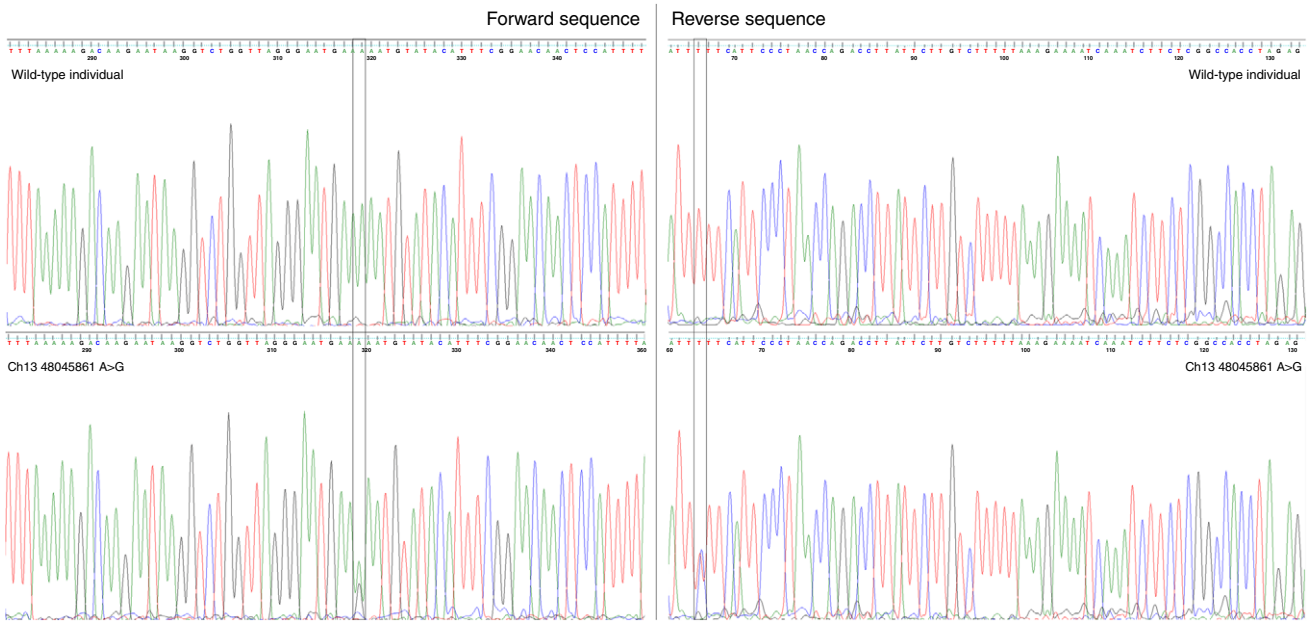

Figure 1. Part of a sequence of *NUDT15* for a non-variant homozygous individual (above) and the heterozygous individual (below) for genetic polymorphism at Chr13 48045861. Electropherograms were obtained after forward (left) and reverse (right) Sanger sequencing of a PCR fragment, corresponding to exon 3 of *NUDT15*.

Table 3. Genetic polymorphisms identified in the Slovenian population and their allele and genotype frequencies.

rs number	Wild-type allele Variant allele	Allele frequency [%]	Genotype	Genotype frequency [%] (N)	Hardy-Weinberg[a]
rs181638201	G	99.5	GG	99.1 (108)	>0.9999
	A	0.50	GA	0.9 (1)	
			AA	0.0	
rs45465203	G	91.3	GG	82.6 (90)	0.8555
	A	8.7	GA	17.4 (19)	
			AA	0.0	
rs79687000	C	97.2	CC	94.5 (103)	>0.9999
	T	2.8	CT	5.5 (6)	
			TT	0.0	
rs1249937565	A	95.9	AA	91.7 (100)	>0.9999
	G	4.1	AG	8.3 (9)	
			GG	0.0	
rs61746486	A	99.1	AA	98.2 (107)	>0.9999
	C	0.9	AC	1.8 (2)	
			CC	0.00	
Chr13 48045861	A	99.5	AA	99.1 (108)	>0.9999
	G	0.5	AG	0.9 (1)	
			GG	0.0	
rs61973267	G	96.8	GG	94.5 (103)	0.7680
	A	3.2	GA	4.6 (5)	
			AA	0.9 (1)	
rs116855232	C	99.5	CC	99.1 (108)	>0.9999
	T	0.5	CT	0.9 (1)	
			TT	0.0	

[a]*p*-value after Fisher's exact test.

All genetic polymorphisms were in Hardy-Weinberg equilibrium. The minor allele frequencies (MAF) of identified SNPs in Slovenian population were 0.5% for rs181638201, 8.7% for rs45465203, 2.8% for rs79687000, 4.1% for rs1249937565, 0.9% for rs61746486, 0.5% for Chr13 48045861 A > G, 3.2% for rs61973267 and 0.5% for rs116855232. Compared with data on population of general European origin, we observed significantly lower MAF for rs45465203 (Fisher's exact test, $p = 0.0115$) and lower (but not significantly) MAF for rs61973267 (Fisher's exact test, $p = 0.1124$) [22]. However, compared with global frequencies, MAFs for both alleles were in line with the published data. For rs181638201, rs79687000, rs61746486 and rs116855232, the allele frequencies were

similar to those previously reported for the European population in the 1000 Genome project [22].

4. Discussion

The present study is the first to investigate variants in the *NUDT15*, an important pharmacogene related to thiopurine treatment, in patients with ALL as well as in the general population in Slovenia. Altogether, eight *NUDT15* variants located in or near exons 1 and 3 were identified in our study. Four variants were detected in both study cohorts, pediatric patients with ALL and in the general Slovenian population. Allele frequencies of identified variants in the ALL cohort were higher compared with those reported for the European population (i.e., 5.6, 6.1, 0.4 and 0.28%, respectively) [22]; however, this discrepancy may be ascribed to the small sample size. Due to the influence on thiopurine toxicities, pharmacogenomic guidelines recommend adjusting the thiopurine dose according to the *NUDT15* genotype. Individuals carrying *NUTD15*2*, *NUDT15*3*, *NUDT15*6,* or *NUDT15*9* allele are assigned as intermediate (heterozygotes) or poor metabolizers (variant homozygotes or compound heterozygotes), who have to receive 30–80% or 10% of the standard thiopurine dose, respectively. Of the variants, rs116855232 (*NUDT15*3* allele) has a well-established clinical impact on thiopurine treatment-related tolerability. It is one of the first *NUDT15* variants to be associated with intolerance to thiopurines (i.e., requiring a dose reduction) or thiopurine-related adverse drug effects such as leukopenia in patients with ALL as well as in patients with inflammatory bowel disease (IBD) [18,23]. The association has been confirmed later by several independent studies which were mostly conducted in Asian patients [14,18,23–27] and also in the recently published systematic review [28]. When we analyzed the genetic background and predicted phenotypes by using advanced platforms for variant classification and interpretation (Franklin by Genoox) – Table 4 [29], this variant was classified as deleterious by several prediction models, although some categorized it as benign. Based on strong evidence from clinical studies, the PharmGKB database assigns a level 1A association (the highest level) to this variant concerning thiopurine therapy.

In our cohort of ALL patients, we identified one patient with the rs116855232 variant in the heterozygous state. Although statistical analysis could not be performed due to the small sample size, we observed that the patient's actual cumulative dose intensity was only 77% of the intended cumulative dose of 6-MP received as a part of maintenance therapy. During this period, several dose adjustments and treatment interruptions were required. The observed lower tolerability of 6-MP by this patient having one *NUDT15*3* allele is in line with this variant's well-established clinical impact on thiopurine treatment-related tolerability.

No association with the calculated ratio between actual and theoretical 6-MP cumulative dose was demonstrated for the other three variants detected among patients with ALL in this study, namely, rs61973267, rs45465203 and rs61746486. Among those, only rs61973267, a variant located in the 5′-untranslated region of the *NUDT15*, has been previously associated with the response to the thiopurine treatment. A letter to the editor reported that patients with chronic IBD carrying one variant allele and undergoing azathioprine therapy have an increased risk of myelosuppression compared with patients with the reference rs61973267 GG genotype [30]. However, we did not detect significant deviations from intended standard cumulative 6-MP dose in ALL patients carrying variant allele, nor did *in silico* prediction models suggest any functional or pathological associations for this variant. Prediction models do not suggest any functional or pathological associations for other variants detected in the ALL cohort and we did not find an association between them and the cumulative 6-MP dose.

Additional four variants were identified in a cohort of the general Slovenian population. Of these, one was intronic (rs79687000), two were upstream gene variants (rs181638201 and rs1249937565) and one was a downstream gene variant (Chr13 48045861 A > G, NC_000013.11), which has not been reported in either Ensembl variation database, 1000 Genomes Project or NCBI dbSNP. However, during the paper revision process, we have become aware that the latest release of the gnomAD database (v4.1.0) does list one among 807,162 individuals carrying this variant, making it extremely rare in European populations. For rs1249937565, this is the first report on population genetics. Variant rs79687000 is located 117 base pairs upstream of the first exon in *NUDT15* and although no clinical data have linked this variant to a response to thiopurine therapy, functional predictions suggest it may alter the splicing of the exon and therefore affect enzyme function, classifying it as potentially deleterious. Variant rs1249937565 is located 53 bp upstream of exon 1, suggesting it might be located in a splicing region. However, prediction models do not associate it with any pathology or functional effects. Detailed information on genetic variants identified in the general Slovenian population and ALL cohort are summarized in Table 4.

The guidelines suggest four basic criteria for the inclusion of a particular variant into the pharmacogenomics diagnostic panel [1]. According to population databases, the most frequent *NUDT15* haplotypes in populations

Table 4. Description and prediction scores for *NUDT15* variants identified in the Slovenian population.

rs number	Genomic location[a]	(Near) exon	Ref > Var	Amino acid change	Variant type	Prediction scores (Franklin Genoox)											Clinical evidence	PharmGKB annotations
						Splice AI	Aggr Pred	Revel	AlphaMissense	MUT Assesor	SIFT	MetaLR	DANN	MT	GenoCanyon	fitCons		
rs181638201	chr13:48037668	1	G>A		Upstream gene variant	0	0.01	/	/	/	/	/	/	/	/	/	No	None
rs45465203	chr13:48038078	1	G>A		Intron variant	0.09	0.14	/	/	/	/	/	/	/	/	/	No	None
rs79687000	chr13:48038021	1	C>T		Intron variant	0.69	0.74	/	/	/	/	/	/	/	/	/	No	None
rs1249937565	chr13:48037957	1	A>G		Intron variant	0	0.01	/	/	/	/	/	/	/	/	/	No	None
rs61746486	chr13:48037782	1	A>C	Pro12Pro	Synonymous	0	0.01	/	/	/	/	/	/	/	/	/	No	None
None assigned	chr13:48045861	3	A>G		D.stream gene variant	0	0.01	/	/	/	/	/	/	/	/	/	No	None
rs61973267	chr13:48045806	3	G>A		D.stream gene variant	0	0.01	/	/	/	/	/	/	/	/	/	[30]	
rs116855232	chr13:48045719	3	C>T	Arg139Cys	Missense variant	0	0.3	0.17	0.159	2.02	0.161	0	0.94	1	0.99	0.73	[14,18,23–25]	Level 1A

[a]*GRCh38, NC_000013.11* Aggr Pred – Aggregated Prediction, D. stream – down stream, PharmGKB – pharmacogenomic database.

– benign, – uncertain – deleterious, - splice-altering.

Data taken from [29].

of European descent, as listed by the Pharmacogene Variation Consortium, are *NUDT15*6* (0.3%), followed by *NUDT15*3* (0.2%) and *NUDT15*9* (0.18%). We found the *NUDT15*3* variant in one ALL patient (MAF 1.4%) and in one individual from the healthy cohort (MAF 0.5%). Although the variant does not completely meet the criterion of a minor allele frequency (MAF) equal to or higher than 1% in the general population [1], the substantial 6-MP dose reduction in the heterozygous patient suggests that it would be rational to sequence patients for this variant prior to thiopurine treatment in Slovenia.

The most abundant *NUTD15* variant in the Slovenian population is rs45465203, which was found in 17.4% of healthy individuals (MAF 8.7%) and in 18.9% of ALL patients, in all individuals in the heterozygous state. The frequency of variant alleles in individuals from Slovenia is significantly lower compared with some populations of European origin and higher than in populations from African and East Asian countries [22].

Since this was an exploratory study, it has some limitations. First, the number of patients in the ALL cohort was too low to enable statistical evaluation of the observed association of the rs116855232 variant with a lower cumulative dose of 6-MP during maintenance therapy. Further research is required to evaluate this association. Second, the sample size of the cohort of the general population was too small to detect rare variants, which might have important functional consequences on the protein, therefore we could not detect their frequencies.

5. Conclusion

This study is the first to investigate the presence of genetic variants in clinically important regions of the *NUDT15* gene in the Slovenian general population and in the small cohort of Slovenian ALL patients on thiopurine therapy. We identified extremely rare variant in the *NUDT15* gene in the downstream region of the gene, which the *in silico* prediction tools classified as being benign. We were not able to evaluate its significance in the clinical setting, since it was not present in the ALL cohort. Frequencies of most of the previously described variants found in the general population and in the ALL cohort were in concordance with those found in the other European populations. The exception was rs45465203, which was less frequent in the Slovenian population. We found only one variant allele included in the CPIC recommendations in the ALL cohort, namely *NUDT15*3*. The patient heterozygous for this allele was able to tolerate lower than standard doses of 6-MP, which is in concordance with the established CPIC recommendations. We conclude, that despite the relatively low frequencies in Slovenian population, *NUDT15* variants might be important in clinical settings when evaluating patients with 6-MP toxicity and absent variant *TPMT* alleles.

Article highlights

- An important pharmacogene, *NUDT15* was sequenced for the extended regions of exon 1 and exon 3 in 109 healthy individuals from Slovenia and 37 patients with acute lymphoblastic leukemia.
- This study systematically investigates the minor allele frequencies (MAF) of identified *NUDT15* genetic variants in the Slovenian population. When compared with the general European population, we observed significantly lower MAF for rs45465203.
- Within the cohort of healthy individuals, we identified extremely rare genetic variant on chromosome 13 at position 48045861 (GRCh38, NC_000013.11) without an assigned rs number.
- A patient with acute lymphoblastic leukemia and a heterozygous genotype for *NUDT15*3* received a significantly lower relative cumulative dose of 6-mercaptopurine compared with other patients without this variant.
- Determining genetic variants that impact enzyme functionality prior to thiopurine treatment can improve the dosing of 6-mercaptopurine and the safety of the treatment.

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Competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The study was carried out according to the requirements of the Declaration of Helsinki. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (KME 89/07/13 and KME 85/06/15). All participating subjects provided their informed consent to participate in the study. In the case of minor individuals, the informed consent was given by parents or legal guardians of the minor.

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