

High-Resolution Melting Analysis of the *TPMT* Gene: A Study in the Polish Population

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The thiopurine *S*-methyltransferase (*TPMT*) gene encoding thiopurine methyltransferase is a crucial enzyme in metabolism of thiopurine drugs: azathioprine and 6-mercaptopurine, which are used in the treatment of leukemia or inflammatory bowel diseases. Genetic polymorphism of the *TPMT* gene correlates with activity of this enzyme, individual reaction, and dosing of thiopurines. Thirty-one variants of the *TPMT* gene with low enzymatic activity have been described with three major alleles: *TPMT**2 (c.238G>C), *3A (c.460 G>A, c.719A>G), and *3C (c.719A>G), accounting for 80% to 95% of inherited *TPMT* deficiency in different populations in the world. The aim of the study was to establish a rapid and highly sensitive method of analysis for the complete coding sequence of the *TPMT* gene and to determine the spectrum and prevalence of the *TPMT* gene sequence variations in the Polish population. Recently, high-resolution melting analysis (HRMA) has become a highly sensitive, automated, and economical technique for mutation screening or genotyping. We applied HRMA for the first time to *TPMT* gene scanning. In total, we analyzed 548 alleles of the Polish population. We found 11 different sequence variations, where two are novel changes: c.200T>C (p.P67S, *TPMT**30) and c.595G>A (p.V199I, *TPMT**31). Detection of these new rare alleles *TPMT**30 and *31 in the Polish population suggests the need to analyze the whole *TPMT* gene and maybe also the extension of routinely used tests containing three major alleles, *TPMT**2, *3A, and *3C. Identification of sequence variants using HRMA is highly sensitive and less time consuming compared to standard sequencing. We conclude that HRMA can be easily integrated into genetic testing of the *TPMT* gene in patients treated with thiopurines.

Introduction

THE THIOPURINE *S*-METHYLTRANSFERASE (*TPMT*) GENE (MIM 187680) is located on chromosome 6 (6p22.3). It encompasses 35 kb of genomic DNA and contains 10 exons, where 9 provide coding information for protein synthesis, that is, the enzyme *TPMT* (Szumlanski *et al.*, 1996). In 1980 Weinshilboum and Sladek (1980) reported that high, intermediate, and undetectable enzyme activity is correlated with the genetic variation and the autosomal codominant inheritance of the *TPMT* gene. *TPMT* (EC 2.1.1.67) catalyzes the *S*-methylation of thiopurines, including the immunosuppressant azathioprine and the anticancer agents 6-mercaptopurine (6MP) and 6-thioguanine (6TG) (Tai *et al.*, 1996). These drugs are currently used in the treatment of inflammatory bowel disease (IBD), hematological malignancies, and in transplantation (Sahasranaman *et al.*, 2008). However, intermediate and

low *TPMT* enzyme activity leads to toxicity of thiopurine drugs (Yates *et al.*, 1997; Pandya *et al.*, 2002; Formea *et al.*, 2004). Increased risk of adverse reactions of 6MP depends on the accumulation of thioguanine nucleotide (TGN) metabolites (6TGN), which concentration is inversely proportional to the activity of *TPMT* enzyme. Approximately 1 in 300 persons (0.3%) has low or undetectable *TPMT* enzyme activity, whereas about 11% are heterozygous for *TPMT* gene mutations resulting in intermediate *TPMT* activity (Weinshilboum, 2001). There are currently 31 known alleles responsible for *TPMT* deficiency (*TPMT**2, *3A, *3B, *3C, *3D, *4, *5, *6, *7, *8, *9, *10, *11, *12, *13, *14, *15, *16, *17, *18, *19, *20, *21, *22, *23, *24, *25, *26, *27, *28, and *29) and 3 alleles associated with normal *TPMT* activity (*TPMT**1 and two silent polymorphisms) (Derijks and Wong, 2010; Lee *et al.*, 2012). The *TPMT**2 (c.238G>C), *3A (c.460G>A, c.719A>G), and *3C (c.719A>G) alleles account for 80% to 95% of inherited *TPMT*

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TABLE 1. PRIMERS SEQUENCES AND AMPLICON SIZES USED IN HIGH-RESOLUTION MELTING ANALYSIS

Primer name	Product size (bp)	Sequence (5' → 3')
TPMT_exon2_For	197	CGGAAGACATATGCTTGT GAGA
TPMT_exon2_Rev		ACCAAATATTTCTTACTGAT GTCCTTG
TPMT_exon3_For	213	TGAATGAAAAGTGTTACCC TACC
TPMT_exon3_Rev		CATCCTGTAAATCACCCA AAGA
TPMT_exon4_For	249	GCAGGTTTGCAGACCG GGGA
TPMT_exon4_Rev		TGCGTGCTAAATAGGAACC ATCGGA
TPMT_exon5_For	122	TTCATTATTTTATTACAGA GTTCTTCG
TPMT_exon5_Rev		GCCTGGCAAGCATTCAAA
TPMT_exon6_For	173	TGTTGAAGTACCAGCATG CAC
TPMT_exon6_Rev		CTTACCATTTGCGATCAC CTG
TPMT_exon7_For	179	CTTCCTTCCCTGCCTTTTGT
TPMT_exon7_Rev		CCCAACAACCTTTACCTGG ATG
TPMT_exon8_For	191	GAGAAGAACATGCCACATC ATCACCTA
TPMT_exon8_Rev		TTTGTTTAAAAAGTTACAGC ATAAGT
TPMT_exon9_For	213	GAATCCCTGATGTCATTC TTCA
TPMT_exon9_Rev		CCTCAAAAACATGTCAGTG TGA

TPMT, thiopurine S-methyltransferase.

deficiency in different populations all over the world (Yates *et al.*, 1997). These results based on clinical and genetic study generate the need for efficient diagnostic tools for genotyping and screening of alleles in the *TPMT* gene. The guidelines developed by the Clinical Pharmacogenetics Implementation Consortium provide dosing recommendations (updates at www.pharmgkb.org) for azathioprine, MP, and TG based on the *TPMT* genotype (Relling *et al.*, 2011). Standard methods (e.g., RFLP, denaturing high-performance liquid chromatography [DHPLC], and Sanger sequencing) for mutation detection in the *TPMT* gene are time consuming, laborious, and expensive. The aim of our study was to establish a highly sensitive, automated, and economical molecular approach for the determination of *TPMT* alleles and to check the prevalence of genetic changes in this gene in the Polish population. We present the application of fast and efficient high-resolution melting analysis (HRMA) for genotyping and screening of the *TPMT* gene in the Polish population.

Materials and Methods

DNA samples

For estimation of frequency of the *TPMT* alleles in the Polish population, 274 DNA samples (548 alleles) obtained from 91 patients with diagnosed IBD and treated with thiopurines, as well as 183 unrelated healthy persons (including

91 men and 92 women), were subjected for analysis using HRMA. In this study, all DNA samples were considered as part of a Polish population group without association to the clinical reaction to thiopurine drugs. DNA samples were provided from the DNA Bank of the Institute of Human Genetics Polish Academy of Sciences in Poznan and from the Department of Gastroenterology, Human Nutrition and Internal Diseases, University School of Medical Sciences in Poznan.

Informed consent of the subjects was given in written form. Ethics approval for this study was obtained from the Local Ethics Committee of the University of Medical Sciences in Poznan, Poland. DNA was isolated from peripheral blood according to standard procedures using the method with guanidine isothiocyanate and stored in a TE buffer at 4°C.

High-resolution melting analysis

We designed sequences of primers to cover the complete coding region of the *TPMT* gene using Primer3 software. Primer sequences and amplicon sizes are shown Table 1.

A commercially available kit for amplification was used: High-Resolution Master Mix (Roche Diagnostics GmbH, Mannheim, Germany). Polymerase chain reaction (PCR) was carried out in a volume of 10 µL containing 5 µL Master Mix (include FastStart Taq DNA Polymerase, reaction buffer, dNTP mix, and High-Resolution Melting Dye), 1.2 µL MgCl₂ (25 mM), 0.5 µL of each primer (5 pmol), and 20 ng DNA. HRMA was performed using the Light Cycler[®] 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) equipment with 96-well block.

Polymerase chain reaction (PCR) procedure started with preincubation at 95°C for 10 min, followed by 45 cycles of denaturation (95°C for 10 s), annealing (60°C for 15 s) and extension (72°C for 15 s), and a final extension step at 72°C for 5 min. After amplification, PCR products were denatured at 95°C for 1 min (transition rate, 4.4°C/s) and cooled to 40°C (transition rate, 2.2°C/s) to form heteroduplexes. Melting analysis of the amplicon was carried out at the temperature interval from 60°C to 95°C (ramping rate 1.0°C/s). The final stage of the experiment was to cool the PCR product to 4°C for storage. Melting curve analysis was performed on Light Cycler[®] 480 using Light Cycler[®] 480 Scanning software (Roche Diagnostics Ltd., Rotkreuz, Switzerland).

Sequencing of PCR products

The PCR products from the LightCycler PCR amplification were directly sequenced. Selected samples with different melting profiles were treated with the QIA quick[™] PCR purification kit (Qiagen, Hilden, Germany) and sequenced bidirectionally on a MegaBACE[™]1000 DNA Analysis System (Amersham Biosciences, Buckinghamshire, United Kingdom) using the DYEnamic[™] ET Dye Terminator technology.

Results

The Figure 1 presents an example of PCR product melting analysis for exons 2 and 3. A heterozygous substitution c.30T > A (p.Ile10Ile) was detected in exon 2 (Fig. 1A). Results for exon 3 show three repetitive, different melting profiles of the analyzed amplicons (Fig. 1B), which correspond to the alteration in the third intron in the position c.233+35C > T.

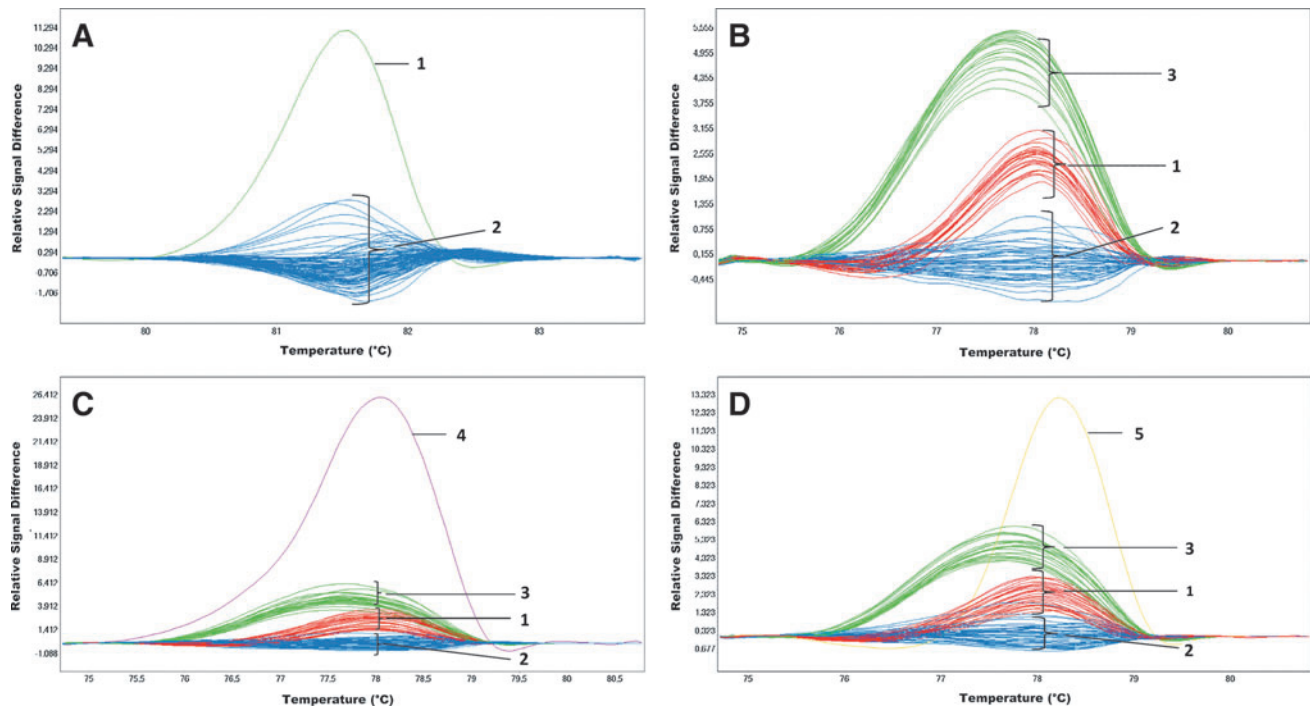


FIG. 1. High-resolution melting analysis (HRMA) detection of thiopurine S-methyltransferase (TPMT) changes in exons 2 and 3. **(A)** exon 2: c.30T>A; line 1—genotype TA, and lines 2—wild type; **(B)** exon 3: c.233+35C>T; lines 1—genotype CC; lines 2—genotype CT; lines 3—genotype TT; **(C)** exon 3: c.200T>C; genotype TC—line 4; other lines like in **(B)**; **(D)** exon 3: c.219G>A; genotype GC—line 5; other lines like in **(B)**. Color images available online at www.liebertpub.com/gtmb

Additionally, the melting analysis of exon 3 revealed the presence of 2 additional samples with a different profile compared to the remaining samples. Sequence analysis indicated two changes, c.219G>A (p.Ala73Ala) (Fig. 1C) and c.200T>C (p.Phe67Ser) (Fig. 1D), for individual samples (Table 2).

On the basis of the HRMA of exon 4, three repetitive melting profiles and additionally two different curves were observed within the entire group (Fig. 2A). Sequencing of the samples corresponding to each of the three profiles revealed the presence of a polymorphic site in intron c.366+58T>C (rs2518463) within the amplified fragment. There are three genotypes in the investigated group, which correspond to three melting curves, that is, T/T, T/C, and C/C. Two additional different melting curves were observed in the analysis

of exon 4 resulting from a polymorphic change c.399C>T in this fragment (Fig. 2A and Table 2).

HRMA conducted for exon 5 has not revealed melting curves of different shape, suggesting polymorphic changes or mutation within the sequences of the examined fragments.

The HRM results for exon 6 revealed the presence of five different melting profiles of the analyzed fragment (Fig. 2B). Having conducted the sequencing of samples representing each of the melting profiles, it was concluded that the reason for such differentiation is the occurrence of two polymorphic sites within the analyzed sequence c.474C>T (p.Ile158Ile) and c.460G>A (p.Ala154Thr) (Fig. 2B). The frequency of c.460G>A was 3.28% (Table 2).

In the case of exon 7, a single case of a different melting profile was observed within the investigated group (Fig. 3A),

TABLE 2. OBSERVED SEQUENCE VARIATIONS OF THE TPMT GENE

Exon	Nucleotide change	Amino acid change	Ref SNP number	Number of alleles	Observed frequency (%)	Name of allele (reference)
2	30T>A	Ile10Ile	rs200780293	1	0.18	
3	233+35C>T	Intron	rs4449636	292	53.28	
3	219G>A	Ala73Ala	—	1	0.18	
3	200T>C	p.Phe67Ser	Novel	1	0.18	*30 (Present study)
4	366+58T>C	Intron	rs2518463	201	36.68	
4	399C>T	Cys133Cys	—	2	0.36	
6	460G>A	Ala154Thr	rs1800460	18	3.28	*3A (Tai <i>et al.</i> , 1996)
9	719A>G	Tyr240Cys	rs1142345	18	3.28	
6	474C>T	Ile158Ile	rs2842934	431	78.65	
7	495-1G>A	Splice site	rs9333570	1	0.18	*15 (Lindqvist <i>et al.</i> , 2004)
8	595G>A	Val199Ile	Novel	1	0.18	*31 (Present study)

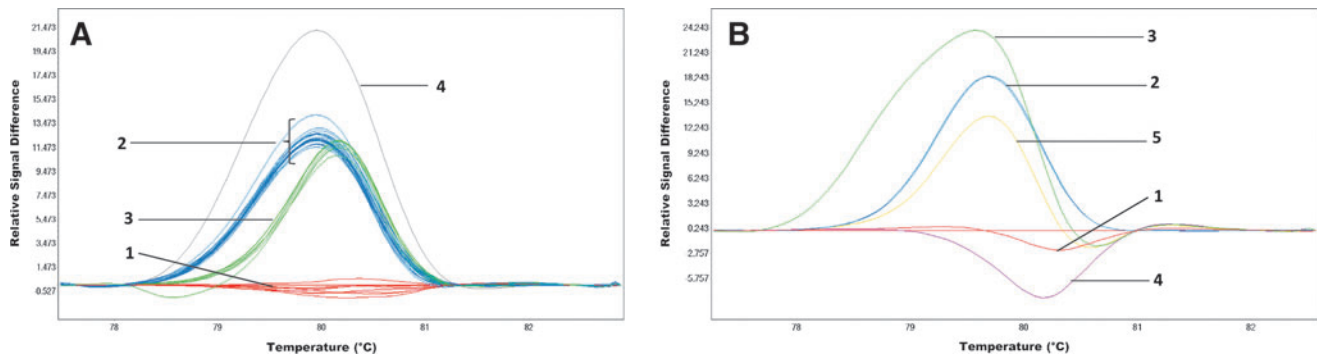


FIG. 2. HRMA detection of *TPMT* changes in exons 4 and 6. **(A)** exon 4: c.366+58T>C; lines 1—genotype CC, lines 2—genotype TC, lines 3—genotype TT, c.399C>T; line 4—genotype TC. **(B)** exon 6: c.460G>A and c.474C>T; line 1—genotype CT(c.474) and GG(c.460), line 2—genotype TT, (c.474) and GA (c.460), line 3—genotype CT (c.474) and GA (c.460), line 4—genotype CC (c.474) and GG (c.460), line 5—genotype TT (c.474) and GG (c.460). Color images available online at www.liebertpub.com/gtmb

while sequencing of this DNA fragment exposed an alteration within the splicing site c.495-1G>A.

On the basis of HRMA of exon 8, one sample with a different melting profile was selected. The profile resulted from a change in the sequence c.595G>A (p.Val199Ile) (Fig. 3B).

Figure 3C presents the results of the melting analysis for exon 9, with two groups of samples representing different melting profiles. As a result of sequencing, a change in sequence was identified, c.719A>G (p.Tyr240Cys), with a frequency of 3.28% (Table 2).

Discussion

Routine diagnostics of *TPMT*, the main enzyme participating in thiopurine biotransformation, aim at optimal adjustment of drug dose, to reduce the number of undesired reactions to treatment with thiopurine drugs in patients with low *TPMT* activity, and at improving the treatment in patients with overproduction of this enzyme. However, the results of phenotyping through evaluating the enzyme's activity in erythrocytes can be false (Ford and Berg, 2010). It is known that the activity of thiopurine methyltransferase is determined by the genotype of the *TPMT* gene. The Clinical Pharmacogenetics Implementation Consortium offers recommendations regarding drug dosing based on the *TPMT* genotype (Relling *et al.*, 2011). Until now, various methods have been used for that purpose, including restriction digest analysis (Yates *et al.*, 1997), single-strand conformational polymorphism (Spire-Vayron de la Moureyre *et al.*, 1998), amplification refractory mutation system (Roberts *et al.*, 2004), DHPLC (Schaeffeler *et al.*, 2001), pyrosequencing (Haglund *et al.*, 2004; Okada *et al.*, 2005), and fluorescence hybridization probe assays (Schütz *et al.*, 2000). Many of these methods are very time consuming, and except for the real time PCR analyses, they require further processing of the amplification product. To improve and optimize the diagnostics of *TPMT*, we used HRMA for the first time to examine the entire coding area of the *TPMT* gene, including the splicing sites.

HRMA was developed in 2004 as a precise and fast alternative for standard screening methods for investigating polymorphisms and mutations (Willmore *et al.*, 2004; Zhou *et al.*, 2005). The researchers conducting the studies of large genes (e.g., *BRCA1*, *BRCA2*, and *CFTR*) also confirmed high

sensitivity of HRMA, which amounted to over 96% (Chou *et al.*, 2005; De Leener *et al.*, 2008). These data combined with other advantages of the method: low labor input, fast results, omitting the gel technique stage, and make the method optimal for analyzing the *TPMT* gene, especially in the case of people who were not found to have the most common alleles and whose genetic analysis should be extended. In the HRMA, each fragment amplifies in real time and is analyzed for unknown sequence variants using a single, high-resolution melt dye. Allele-specific fluorescent probes are not necessary, which significantly reduces the costs of the analysis (Schütz *et al.*, 2000). HRM amplification and analysis are performed on one device. It decreases the duration of the analysis as well as the risk of accidental swap of the samples. Additional advantage of the HRMA, as opposed to other automated DHPLC-screening methods, is detecting differences in homozygous sequences (Chou *et al.*, 2005).

The optimization of the HRM method was conducted in accordance with the recommendations of the manufacturers and guidelines provided by other researchers (Vossen *et al.*, 2009; Wittwer, 2009). The highest differentiation of changes in sequences is obtained for short fragments below 300 bp. We designed primers for all exons of the *TPMT* gene, which amplify fragments between 122 and 249 bp (Table 1). It is critical for the HRMA to obtain a strong, single amplification product without nonspecific reaction products, such as dimers of primers. This way, multiple complex melting domains difficult to interpret can be avoided. An example of optimal graph of the melting temperature (T_m)-calling analysis obtained from exon 4 of the *TPMT* gene has been presented below (Fig. 4). An additional advantage of the developed HRM method for the *TPMT* gene analysis is a simple PCR protocol that all fragments were amplified in the same conditions (annealing temperature and $MgCl_2$ concentration). After PCR, the amplicons were analyzed in a wide spectrum of temperatures, between 60°C and 95°C, not to miss the melting point of the amplicon. Additionally, to verify the sensitivity of the optimized HRMA method, all samples were examined with the use of pyrosequencing for three most commonly observed changes in the Caucasian population *TPMT**2 (c.238G>C), *3A (c.460G>A, c.719A>G), and *3C (c.719A>G) (data unpublished). Results of the analyses by both methods are consistent in 100%, which confirms high

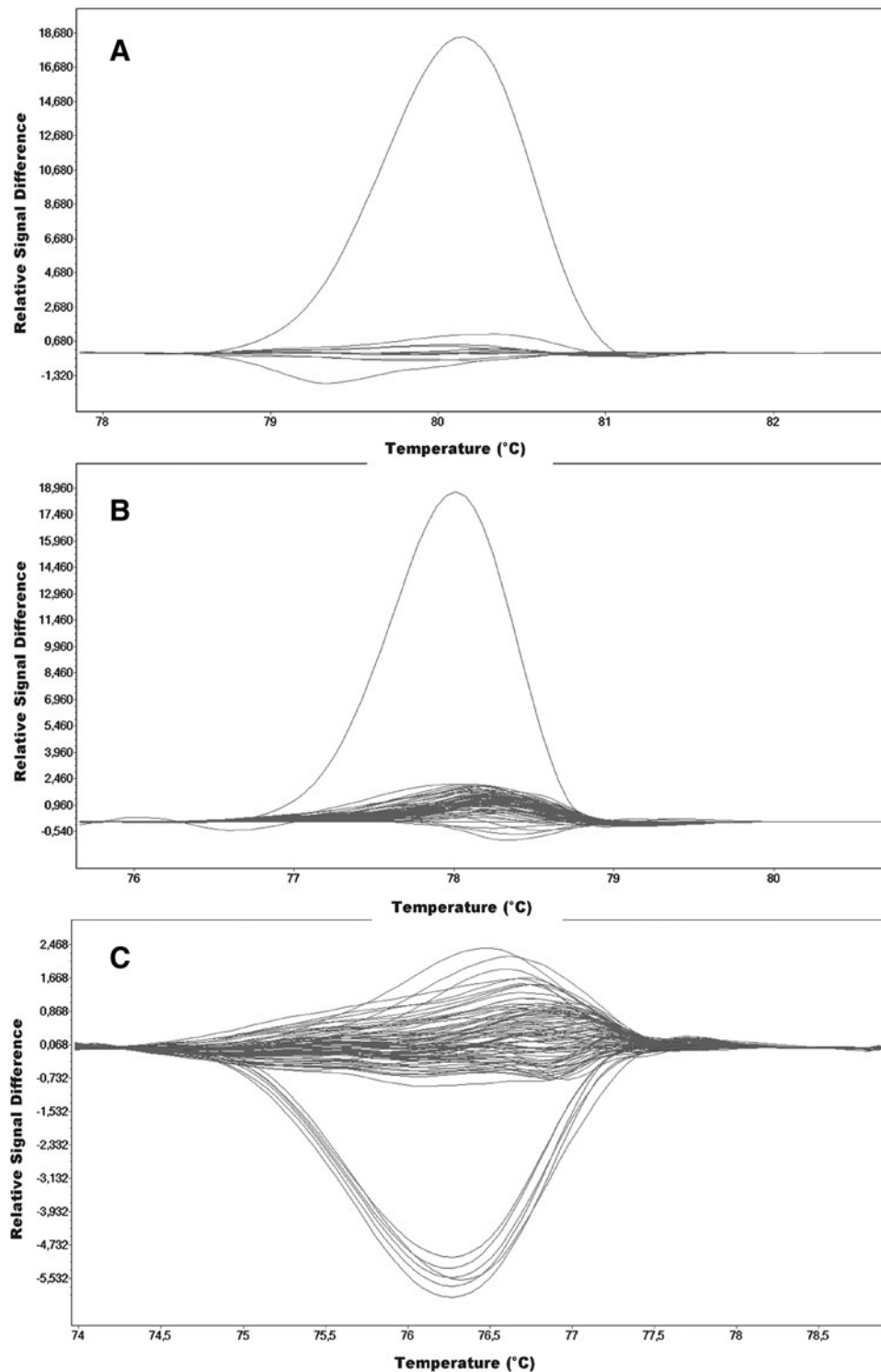


FIG. 3. HRMA detection of *TPMT* changes in exons 7, 8, and 9. **(A)** exon 7: c.495-1G>A; curved upper line—genotype GA and horizontal lines—wild type. **(B)** exon 8: c.595G>A; curved upper line—genotype GA and horizontal lines—wild type. **(C)** exon 9: c.719A>G; curved lower lines—genotype GA and horizontal lines—wild type.

precision and sensitivity of the HRMA method optimized for the *TPMT* gene.

The second aim of the study was to determine the spectrum and the frequency of the *TPMT* alleles for the Polish population. Usually, the genetic examination of the *TPMT* gene involved defining of five most common alleles *TPMT**1, *2, *3A, *3B, and *3C. Based on the HRMA and sequencing in the investigated group, two most frequent alleles for the Caucasian

population were found: *TPMT**1 with the frequency of 96.72% and *TPMT**3A with the frequency of 3.28%. Slightly lower frequency of the *3A allele was observed in the Polish population in earlier studies on this gene by Kurzawski *et al.* (2004) with the use of RFLP and ASO PCR, where in the group of 350 subjects (716 alleles), the wild-type allele *TPMT**1 was observed with the frequency of 96.8%, *TPMT**3A in 2.7%, *TPMT**2 in 0.4%, and *TPMT**3C in 0.1% of the subjects. The

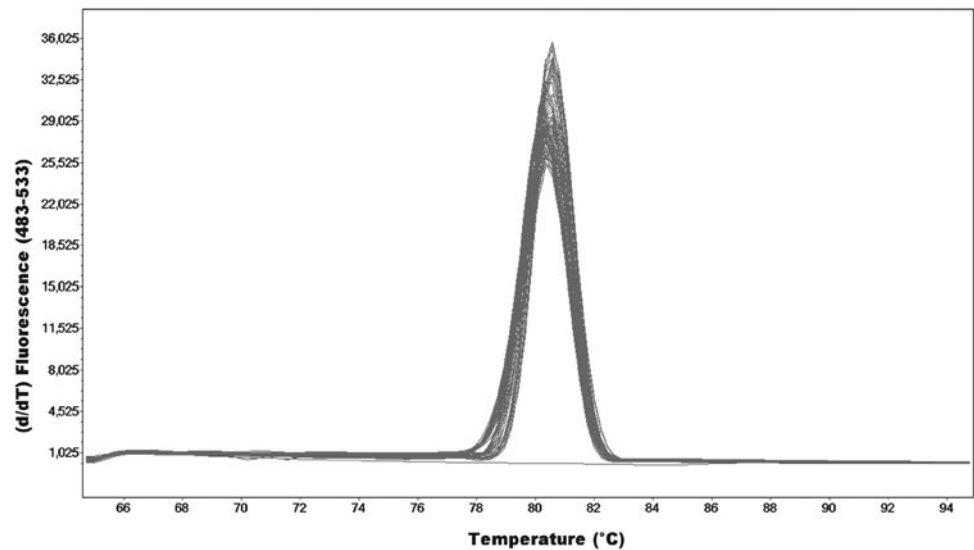


FIG. 4. Melting peaks for exon 4 amplicons of the *TPMT* gene.

research team of Peregud-Pogorzelski *et al.* (2011), based on the study of 203 Polish children suffering from acute lymphoblastic leukemia and subjected to thiopurine drug treatment, managed to determine the wild-allele frequency of 95.81%, *TPMT**3A of 4.2%, and *TPMT**3C of 0.25%. Similarly to our study, the rare *TPMT**2 allele was not observed. Generally, the distribution of the most common alleles *3A, *2, and *3C of the *TPMT* gene corresponds with the general pattern for other White populations: the French population (5.7%, 0.5%, and 0.8% respectively), the British population (4.5%, 0.4%, and .1%), the Italian population (3.9%, 0.5%, and 1.0%), the Russian population (2.6%, 0.14%, and 0.36%), and White Americans (3.2%, 0.17%, and 0.17%) (Spire-Vayron De la Moureyre *et al.*, 1998; Hon *et al.*, 1999; McLeod *et al.*, 1999; Rossi *et al.*, 2001; Nasedkina *et al.*, 2006). The HRMA method allowed us to determine the most frequent, routinely examined alleles *TPMT**2, *3A, and *3C, and extend the analysis to find sequence variations in the entire coding section of the *TPMT* gene along with the splicing sites. Therefore, we discovered 11 substitutions (Table 2), including 6, which did not cause alterations of the amino acid sequence, leaving the *TPMT* enzyme activity unchanged. Apart from two known most popular variants in Caucasians (c.460G>A, c.719A>G, and *TPMT**3A), one rare known variant in the acceptor splice site c.495-1G>A was found, which lead to *TPMT* deficiency. This change was first described in Sweden and named the *TPMT**15 allele (Lindqvist *et al.*, 2004). Moreover, the other two substitution, which so far remained unknown, were identified: in exon 3 c.200T>C (p.Phe67Ser) and in exon 8 c.595G>A (p.Val199Ile). These 2 novel *TPMT* variants were named *TPMT**30 and *31, following to the last-described *TPMT**29 allele (Lee *et al.*, 2012). Both changes are located in the conservative region of the *TPMT* gene (Salavaggione *et al.*, 2005) and cause the amino acid exchange, which indicates possible influence on lowered enzyme activity and is a subject for further investigation. In the current study, 2 new rare alleles *TPMT**30 and *31 were also detected, but the rare *TPMT**2 allele, which is included in the routine pharmacogenetic tests, was not observed among 274 subjects. To develop a more specific and sensitive *TPMT* test dedicated for

the Polish population, it would be reasonable to screen larger cohort of Polish samples in the future to determine the rare alleles, if that are recurrent.

Finally, the HRM technique proved to be highly effective and sensitive in the analysis of the entire coding part of the *TPMT* gene. It can be successfully used for *TPMT* genetic testing in patients for whom treatment with thiopurine drugs is planned.

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Author Disclosure Statement

No competing financial interests exist.

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