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ORIGINAL RESEARCH ARTICLE

A Simple Method for *TPMT* and *ITPA* Genotyping Using Multiplex HRMA for Patients Treated with Thiopurine Drugs

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Abstract Thiopurine methyltransferase (TPMT) and inosine triphosphatase (ITPA) are crucial enzymes involved in the metabolism of thiopurine drugs: azathioprine and 6-mercaptopurine, used in the treatment of leukemia or inflammatory bowel diseases (IBD). The activity in these enzymes correlates with the genetic polymorphism of the *TPMT* and *ITPA* genes, respectively, which determines an individual reaction and dosing of thiopurines. Three main *TPMT* alleles: *TPMT*2* (c.238G>C), *TPMT*3A* (c.460G>A, c.719A>G) and *TPMT*3C* (c.719A>G) account for 80–95 % of inherited TPMT deficiency in different populations in the world. In the *ITPA* gene, a c.94C>A mutation is significantly associated with an adverse thiopurine reaction. The aim of this study was to develop a quick and highly sensitive method for determining major *TPMT* and *ITPA* alleles. Here we present the molecular test for genotyping c.238G>C, c.460G>A, c.719A>G and c.94C>A changes based on multiplex high resolution melting analysis (HRMA). We analyzed DNA samples from 100 clinically diagnosed IBD patients treated with thiopurine drugs, and a known genotype in the positions 238, 460 and 719 of the *TPMT* gene as well as in position 94 of the *ITPA* gene. Our results obtained with multiplex HRMA indicated 100 % accuracy in comparison with data from restriction fragments length polymorphism (RFLP) and standard DNA sequencing. We conclude, that multiplex HRMA can be used as a quick, sensitive and efficient alternative diagnostic method compared to conventional techniques for the determination of *TPMT*2, TPMT*3A* and *TPMT*3C* alleles and c.94C>A change in the *ITPA* gene.

Key Points

The methodology described allows the simultaneous analysis of c.460G>A, c.719A>G and c.238G>C changes of the *TPMT* as well as c.94C>A of the *ITPA* gene by quick and economic multiplex HRMA.

The multiplex HRMA can be used as a sensitive and efficient alternative diagnostic method for the determination of *TPMT*2, TPMT*3 and ITPA* c.94C>A alleles.

1 Introduction

Thiopurine drugs, which include immunosuppressant azathioprine (AZA), anticancer agents 6-mercaptopurine (6MP) and 6-thioguanine (6TG), are currently widely used in the treatment of chronic inflammatory disorders as

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inflammatory bowel diseases (IBD), in hematological malignancies and in transplantation [1].

Peak plasma concentrations are reached after 1–2 h in most patients following oral intake. The thiopurine concentration then rapidly decline with half-lives of less than 1 h [2]. Particularly essential in the AZA biotransformation is thiopurine methyltransferase (TPMT, EC2.1.1.67). This enzyme catalyzes the S-methylation of thiopurines. An increased risk of adverse reactions from AZA and 6MP depends on accumulation of thioguanine nucleotide metabolites (6TGN). The concentration of 6TGN is inversely proportional to the activity of the TPMT enzyme and conditioned by the sequence variations in the thiopurine S-methyltransferase gene (*TPMT*, MIM 187680). 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 thiopurine methyltransferase activity [3]. Currently, 37 alleles responsible for TPMT deficiency (*TPMT*2-38*) are known [4]. However, three alleles: *TPMT*2* (c.238G>C, p.Ala80Pro, rs1800462 in the exon 4), *TPMT*3A* (a combination of c.G460A, p.Ala154Thr, rs1800460 in the exon 6 with c.719A>G, p.Tyr240Cys, rs1142345 in the exon 9) and *TPMT*3C* (c.719A>G) are responsible for 80–95 % of inherited TPMT deficiency in different populations all over the world [5]. According to the Single Nucleotide Polymorphism Database (dbSNP, 1000 Genomes) the variants c.460G>A, c.719A>G and c.283G>C of the *TPMT* gene are reported with the global minor allele frequency of 1.28, 3.91 and 0.22 %, respectively. In pharmacogenetic testing, these mutations are mainly analyzed according to the guidelines developed by the Clinical Pharmacogenetics Implementation Consortium, which provides dosing recommendations (updates at http://www.pharmgkb.org) for AZA, mercaptopurine (MP) and thioguanine [6].

A second significant protein, involved in the biotransformation of thiopurine drugs is inosine triphosphatase (ITPA; EC3.6.1.19). This enzyme catalyzes the pyrophosphohydrolysis of inosine triphosphate (ITP) to inosine monophosphate (IMP) preventing the accumulation of potentially toxic ITPs, which can be incorporated into nucleic acids and lead to cell apoptosis [7]. The ITPase is encoded by the inosine triphosphatase gene (*ITPA*, MIM 147520). So far, two mutations resulting in reduced activity of the ITPase have been identified: p.P32T (c.94C>A, rs1127354) and IVS2 + 21A>C (rs7270101). However, a stronger effect is caused by the exonic mutation (c.94C>A). The homozygosity of the *ITPA* c.94A allele leads to a deficiency in the ITPase activity in erythrocytes and lymphocytes, this occurs in approximately 1 in 1000 Caucasians. Heterozygotes constitute about 6.0 % of Caucasian populations, and have an average red cell ITPase activity of about 22 % of the control mean value. This allele is more common in Asian populations, with a frequency of 14–19 % [8]. Furthermore, it was observed that the ITPA c.94C/A genotype makes a contribution to the concentration of 6-methylmercaptopurine (6MMP) in red blood cells and the occurrence of hepatotoxicity [9] as well as the survival rate in pediatric patients with acute lymphoblastic leukemia (ALL) [9, 10].

Therefore, based on clinical and pharmacogenetic studies, it is crucial to generate an efficient diagnostic tool for the determination of *TPMT*2* and **3* alleles and the c.94C>A change in the *ITPA* gene. At the same time, due to the development of new, high-throughput molecular genetic techniques, the aim is to replace the previous standard methods for mutation detection (e.g. RFLP, SSCP, DHPLC, Sanger sequencing), which are time-consuming, laborious, and expensive. Also, in the literature reporting *TPMT* gene analysis, the evolution and search for new methods of detecting variants can be observed.

Recently, in a few studies, descriptions of modern methods for *TPMT* alleles determination using real-time polymerase chain reaction (PCR) machines have been presented. This confirms the high prevalence of this type of equipment in laboratories [11–13] and it highlights the need for improvements in *TPMT* genotyping tests. We demonstrate and encourage the use of a more cost-effective application than specific reactions with labeled dyes like TaqMan or hybridization probes. Here we describe a quick, sensitive and cost-effective genotyping methodology using multiplex high resolution melting (HRM) analysis for identifying *TPMT*2*, *TPMT*3A*, *TPMT*3C* and *ITPA* c.94C>A alleles.

2 Material and Methods

2.1 DNA Samples

DNA was collected in the Institute of Human Genetics Polish Academy of Sciences in Poznan from 100 IBD patients, treated in the Department of Gastroenterology, Human Nutrition and Internal Diseases, Poznan University of Medical Sciences. Furthermore, two control DNA samples (*TPMT*1/*2* and *TPMT*3A/*3A* alleles) from the Department of Experimental and Clinical Pharmacology at the Pomeranian Medical University in Szczecin (Poland) were used for validation of the multiplex HRMA. These patients had already been tested for *TPMT*2*, *TPMT*3A,* *TPMT*3C* and *ITPA* c.94C>A alleles by the standard restriction fragments length polymorphism (RFLP) analysis method and DNA sequencing. In this group, the distribution of alleles was as follows: *TPMT *1/*2,* one **3A/*3A*, seven **1/*3A* and the remaining 91 were wild type

**1/*1*. For the *ITPA* c.94C>A 12 individuals were heterozygotes, 2 homozygous for the variant allele and the remaining 86 were wild-type homozygous.

Informed consent of the subjects was given in written form. Ethical approval for this study was obtained from the Local Ethical Committee of the University of Medical Sciences in Poznan, Poland (Resolution No. 871/09). DNA was isolated from peripheral blood according to standard procedures using the method with guanidine isothiocyanate (GTC) and stored at 4 °C in an AE buffer containing 0.5 mM EDTA and 10 mM Tris-Cl.

2.2 High Resolution Melting Analysis

We designed primers to cover c.238G>C, c.460G>A and c.719A>G variants of the *TPMT* gene as well as c.94C>A of the *ITPA* gene using Primer3 software. These four primer pairs were combined into two multiplex PCR reactions: fragment c.460G>A with c.719A>G and fragment c.238G>A with c.94C>A. Primer sequences, the concentration used for multiplex PCR and amplicon size are shown in Table 1. The sequence of primers for exon 6 and 9 amplification of the *TMPT* gene had been published previously [14].

Products were amplified using the Type-it HRM PCR Kit [Qiagen] on a Rotor-Gene Q instrument [Qiagen]. The PCR was performed according to the manufacturer's instructions in a volume of 16 µL containing 8 µL of 2× HRM PCR Master Mix, 0.55–2.2 µL of each primer (5 pmol/µL) and 50 ng of DNA. PCR amplification conditions in the four loci were as follows: 95 °C of pre-incubation for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing for 30 s at 55 °C and extension at 72 °C for 10 s. The HRM analysis was carried out from 70 to 90 °C, raising the temperature by 0.1 °C degree at each step. Data collection and analysis was performed using Rotor-Gene Software, version 2.0.2. For allele recognition, HRM analyses were done and characteristic curves were visualized in difference graph. The melt normalization ranges for the first amplicon set were as follows: 74.4–74.6 °C and 76.5–76.7 °C for the fragment covering the position c.719, 77.0–77.3 °C and 79.4–80.2 °C for fragment corresponding to the position c.460 of the *TMPT* gene. For the second amplicon set, the melt normalization ranges constituted: 75.8–76.3 °C and 78.4–78.6 °C for the fragment with locus c.238 as well as 78.9–79.1 °C and 80.1–80.3 °C for c.94. The genotypes were identified with at least 99 % confidence.

For the validation of the established assays, analysis of each DNA sample was repeated in three separate runs. During the experiments, for the purpose of validation the DNA samples were blinded.

2.3 Sequencing of PCR Products

All polymorphisms detected in HRM were confirmed by direct PCR product sequencing. Selected samples were separately amplified, treated with the QIAquick™PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced bidirectionally on a MegaBACE™1000 DNA Analysis System using the DYEnamic™ ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences, Buckinghamshire, UK).

3 Results

The multiplex HRM analysis of the first primer set for the c.460G>A and c.719A>G changes in the *TPMT* gene showed three different melting profiles of exon 9 and five different melting profiles of exon 6 within the entire group studied (Fig. 1). Sequencing the samples representing all three melting profiles revealed the presence of three genotypes A/A, A/G and G/G in position c.719 (Fig. 1b) as well as three genotypes A/A, A/G and G/G in locus c.460 (Fig. 1c). Two additional profiles were caused by the

Table 1 Primers details and amplicon sizes used in multiplex HRMA

Set no.	Primer name	Gene	Sequence (5′→3′)	Amplicon size (bp)	Amount of primers per reaction (pmol)
1	Exon6_F	*TPMT*	TGTTGAAGTACCAGCATGCAC	173	2.75
	Exon6_R		CTTACCATTTGCGATCACCTG		2.75
	Exon9_F		GAATCCCTGATGTCATTCTTCA	213	11.0
	Exon9_R		CCTCAAAAACATGTCAGTGTGA		11.0
2	Exon4_F		CCCTCTATTTAGTCATTTGAAAACA	241	11.0
	Exon4_R		AAAACTTTTGTGGGGATATGGA		11.0
	Exon2_F	*ITPA*	TAGGAGATGGGCAGCAGAGT	163	6.75
	Exon2_R		TCAATTTTCTGTGCCACCAA		6.75

HRMA high resolution melting analysis, *F* forward, *R* reverse

polymorphism c.474C>T (rs2842934, p.Ile158Ile), which is found next to the c.460G>A. The frequency of T allele is 24.7 % (dbSNP, 1000 Genomes). It was not possible to design a PCR product for position c.460 analysis skipping position c.474 of the *TMPT* gene. Nine combinations of these two variants are possible. We did not observe changed homozygous in both loci, the possibility of their occurrence is low and may be observed approximately in 1 case per 10,000. From our experiment we can assume that the occurrence of the polymorphism does not alter the possibility of the correct identification of genotype in the locus.

Figure 2 presents the results of the melting analysis of PCR products of the second primer set encompassing a c.238G>C change locus of the *TPMT* gene and c.94C>A of the *ITPA* gene. For exon 4 of the *TPMT* gene, a different melting profile was observed once within the group investigated (Fig. 2b), and sequencing of this DNA fragment indicated a heterozygous substitution c.238G>C. On the basis of the HRMA of exon 2 of the *ITPA* gene, three repetitive melting profiles were observed within the entire group (Fig. 2c), and sequencing revealed three corresponding genotypes T/T, T/C, and C/C in position c.94.

In terms of the validation all three repeats of the HRM analyses for each sample showed consistent results. Moreover, the genotypes identified with multiplex HRMA for *TPMT* gene loci c.238G>C, c.460G>A and c.719A>G as well as ITPA gene locus c.94C>A indicated 100 % accuracy for the whole group compared to the data from RFLP analysis and DNA sequencing. The dropout rate from the established multiplex HRMA was 4.17 % (3 samples per 72), thus staying above our general research mutation screening success rate target of 95 %. The optimization was designed to obtain equal peak height of both products in melt profile. Therefore, the PCR containing primer ratios 1:1, 2:1, 3:1 and 4:1 were prepared and analyzed by HRMA. In the next step, primer concentrations, based on analysis of melt profiles, were corrected.

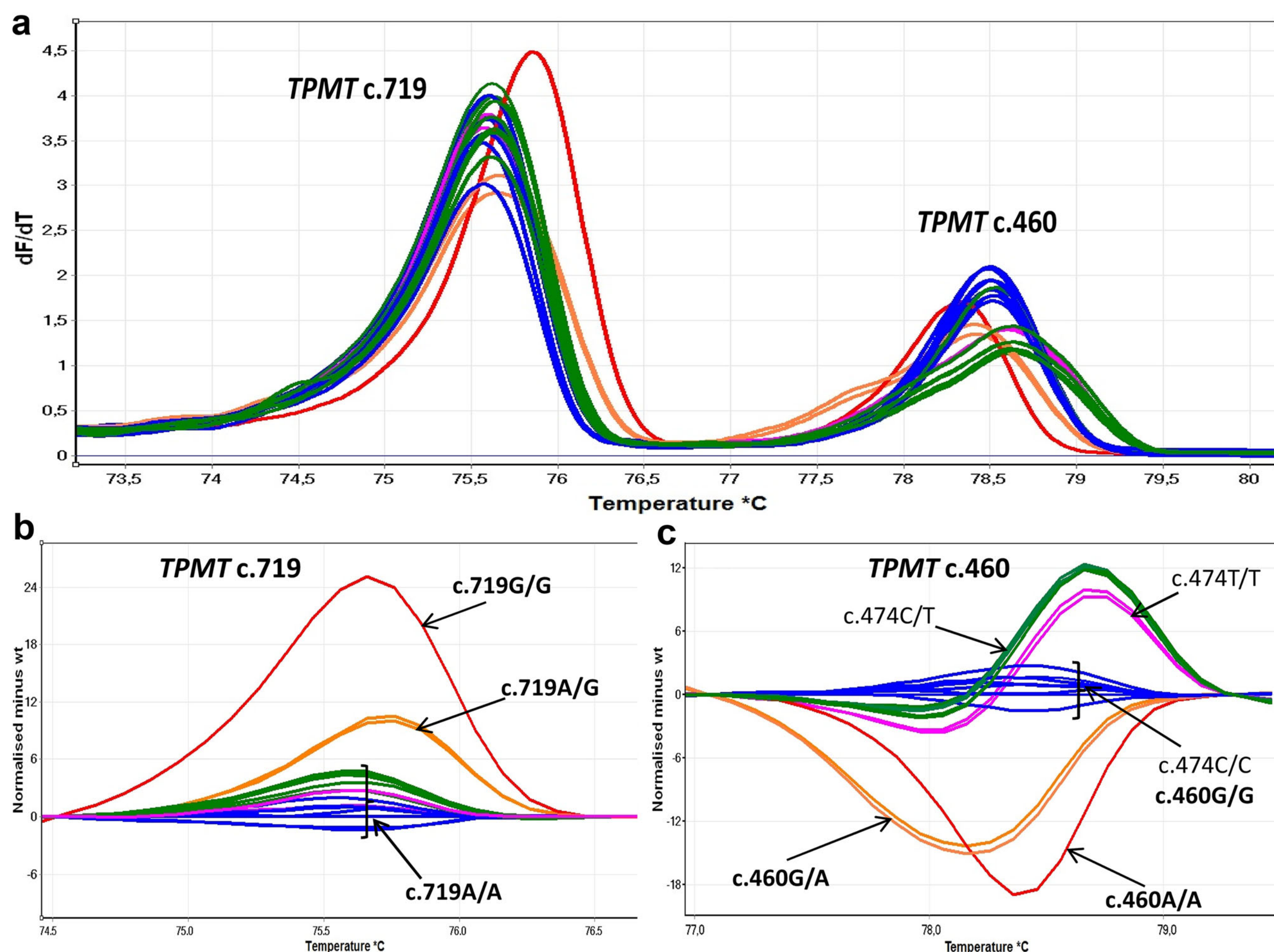

Fig. 1 Multiplex high resolution melting (HRM) analysis for exon 6 and 9 of the *TPMT* gene. **a** Melting profile of both polymerase chain reaction (PCR) products. **b** Normalized difference plots of amplicons corresponding to exon 9 (c.719). **c** Normalized difference plots of amplicons corresponding to exon 6 (c.460 and additionally locus c.474)

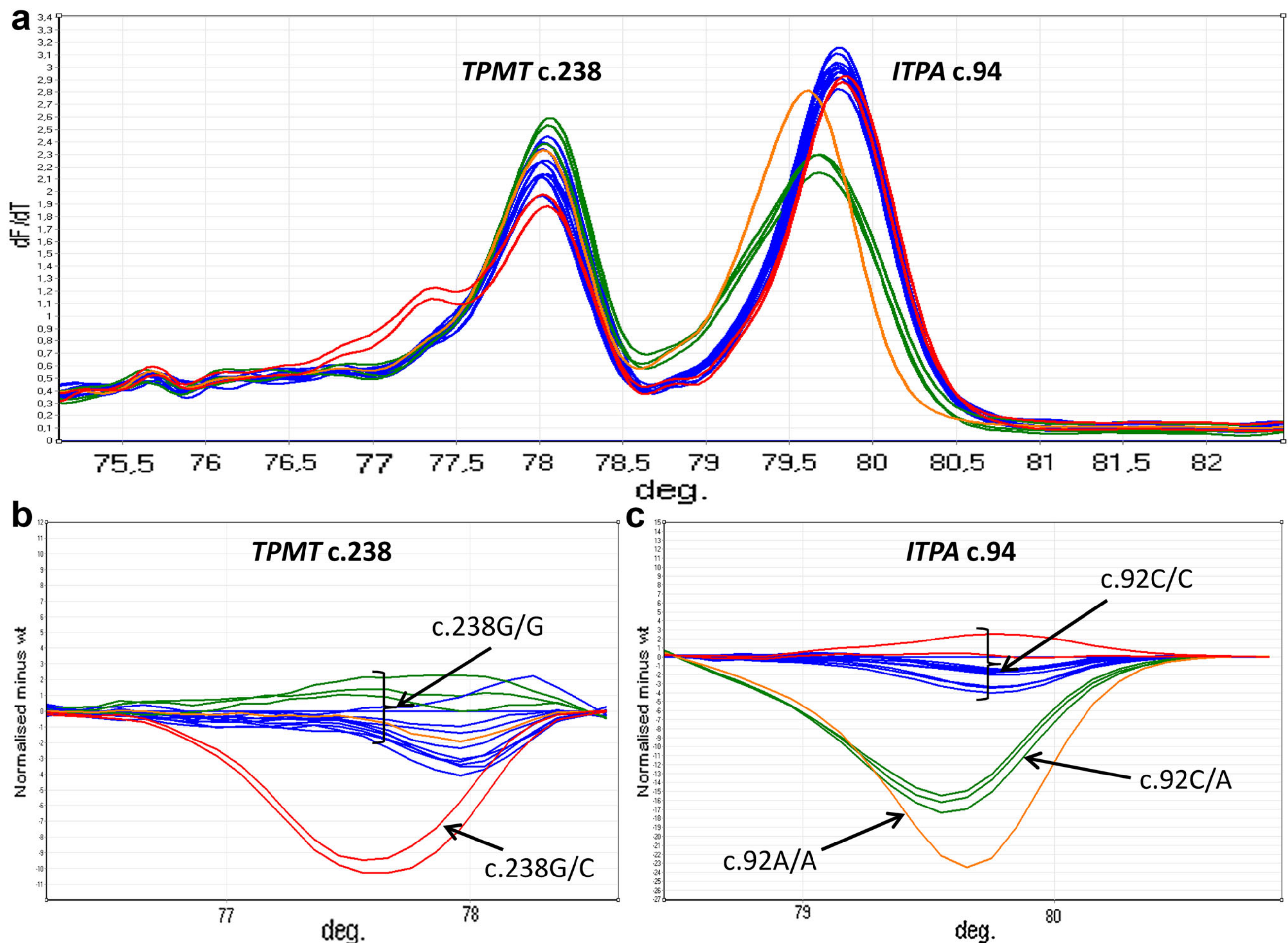

Fig. 2 Multiplex high resolution melting analysis (HRMA) for exon 4 of the *TPMT* gene and exon 2 of the *ITPA* gene. **a** Melting profile of both polymerase chain reaction (PCR) products. **b** Normalized difference plots of amplicons corresponding to exon 4 (c.238). **c** Normalized difference plots of amplicons corresponding to exon 2 (c.94)

The optimization of primer concentration in two runs takes approximately 5 h. For the first multiplex reaction, optimal results were obtained with the primers ratio 4:1 and for the second set, ratio 2:1.22 were accepted (Table 1).

4 Discussion

For around a decade it has been well established, that the equilibrium between 6MMP and toxic 6TGN in patients undergoing thiopurine drug therapy, depends on the genetic polymorphism of the *TPMT* and *ITPA* gene. A strong genotype-phenotype correlation was supported by a number of clinical trials resulting in the development of dosing recommendations for thiopurine drugs based on *TPMT* genotype. Worldwide the most common inactive alleles are *TPMT*2* and **3*, and these alleles constitute the basis for pharmacogenetic testing during thiopurine drug treatment [11–13, 15, 16].

The participation of other genes, *ITPA* in particular, in adverse reactions and toxic side effects of thiopurines, had been controversial for a long time. In 2004, two mutations resulting in different levels of ITPase-reduced activity were identified: p.P32T (c.94C>A) and IVS2 + 21A>C [17]. Some studies confirmed the importance of only one, c.94C>A [18] or IVS2 + 21A>C [19], of these two *ITPA* gene changes in predisposition to AZA intolerance. Other investigations showed that none of the *ITPA* variants had a significant association with renal transplant recipients [20], while in contrast to the above, other researchers suggested the importance of both *ITPA* substitutions in response to thiopurine treatment and side effects [21]. Finally, in the last five years several independent studies on the large groups of ALL patients, the influence on 6MP metabolism and the toxic effect of multiple loci in the *TPMT* gene and the variation c.94C>A in the *ITPA* gene has been confirmed [9, 22]. Moreover, in research by Smid et al. [10], performed on a group of 408 ALL pediatric patients

undergoing maintenance therapy with 6MP, the association between one non-functional *ITPA* allele and the lower risk of suffering early ($p = 0.003$) and/or bone marrow relapse ($p = 0.017$) was observed [10]. The authors concluded that the *ITPA* genotype can be used as a genetic marker for the improvement of survival rate and therapy individualization for patients with ALL [10].

Therefore, we decided to evaluate a quick and cost-effective test for identification of *TPMT2** and *TPMT*3* alleles of TPMT as well as a c.94C>A variant of *ITPA* by multiplex HRMA. The multiplex real-time PCR for the detection of major *TPMT* and *ITPA* alleles is no longer used in the new approach [11–13, 16]. What is innovative about our test, compared to other real-time PCRs, is its use of HRMA as a more cost-effective and sensitive application, also additionally in a multiplex combination. The advantages of our assay, as a type of real-time PCR analysis, are its high sensitivity, efficiency and lack of an agarose electrophoresis stage compared to standard PCR-based techniques. Moreover, expensive allele-specific fluorescent probes are not necessary and they significantly reduce the costs of the analysis. An estimated cost of HRMA reagents per sample is approximately EUR1.00 and is over two times lower than allele-specific fluorescent probe assay. In the case of RLFP, the reagents have an equivalent cost, but this technique is much more time consuming. The final price per sample for DNA sequencing is about EUR5-6.

For the reliable interpretation of multiplex HRMA results it is crucial to design amplicons with discriminating melting points and a similar efficiency in terms of PCR reactions. We designed PCR products up to 300 base pairs to correct allele discrimination and the melting points of multiplex reactions products differ by at least 2 °C. However, HRM analysis as a screening method has its limitations, all samples with melting profiles different from the wild type should be directly sequenced (or analyzed with the use of a second method) to confirm genotyping results.

The similarity of the PCR products' yield was optimized within sets by proportions of primer pairs. For the first multiplex reaction, a four times greater amount of exon 9 primers relative to the exon 6 of the *TPMT* gene were used (Table 1). In the second set, optimal results were obtained with a 61.36 % increased amount of exon 4 primers of the *TPMT* gene relative to the primers of the *ITPA* gene fragment (Table 1).

5 Conclusion

The methodology we describe here allows the simultaneous analysis of c.460G>A, c.719A>G and c.238G>C of the *TPMT* gene variants, as well as the c.94C>A of the *ITPA* gene by rapid and economic multiplex HRMA. Our results indicated 100 % accuracy for the whole group studied when compared to the data from RFLP and sequencing. Therefore, we conclude that multiplex HRMA can be used as a sensitive and efficient alternative diagnostic method, compared to conventional techniques for genotyping of *TPMT*2*, *TPMT*3* and *ITPA* c.94C>A alleles.

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Compliance with Ethical Standards

Conflict of interest The authors MSZ, PB, ABK, OZB, MW, AD, MK, MW, DL, AP and RS declare no conflicts of interest.

Ethical approval and informed consent All patients declared informed consent and the studies were approved by the local Ethics Committee of the Poznan University of Medical Sciences (Approval No. 871/09).

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