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Review article
Pregledni članak

INDIVIDUALIZED THERAPY: ROLE OF THIOPURINE S-METHYLTRANSFERASE PROTEIN AND GENETIC VARIANTS

INDIVIDUALIZOVANA TERAPIJA: ULOGA PROTEINSKIH I GENETSKIH VARIJANTI TIOPURIN S-METILTRANSFERAZE

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Summary: Thiopurine S-methyltransferase (TPMT: EC 2.1.1.67) is an enzyme that metabolizes immunosuppressive thiopurine medications, used in the treatment of autoimmune diseases, cancer and in transplantation medicine. In some individuals, TPMT enzyme activity is significantly increased or decreased compared to the normal TPMT activity level. Structural and biochemical analyses of the TPMT protein revealed the existence of certain protein variants with altered activity. It has been shown that certain TPMT gene polymorphisms exist, that define different TPMT allozymes. Decreased TPMT enzyme activity can also be a consequence of lower protein synthesis, which depends on the promoter transcription activity. Promoter polymorphisms, such as variable number of tandem repeats (VNTR), can modulate the transcription. Administering thiopurine drugs in patients with certain genetic TPMT variants leads to severe hematologic toxicity. To avoid toxicity, therapy is being modified according to the TPMT genotype (pharmacogenetics). We investigated the polymorphisms in exons and regulatory elements (promoter) of the TPMT gene which affect TPMT enzyme activity in the Serbian population. We used PCR-based methodology and sequencing in the detection of genetic variants on TPMT gene. We showed that genetic variants in exons account for 7.5% of all TPMT variants with decreased enzyme activity. The therapy for patients with these pharmacogenetic markers was modified, which contributed to the efficiency of treatment. Functional assays *in vitro* showed that the TPMT promoter activity and, therefore, the quantity of TPMT protein synthesized, depended on the architecture of VNTRs (i.e. number and type) in the promoter. Promoter of the TPMT gene specifically responds to mercaptopurine treatment of K562 cells in a VNTR-dependent manner. Study of DNA-protein interactions

Kratak sadržaj: Tiopurin S-metiltransferaza (TPMT: EC 2.1.1.67) jeste enzim koji metaboliše imunosupresivne tiopurinske lekove, koji se koriste za lečenje autoimunih bolesti, malignih oboljenja i u transplantacionoj medicini. Aktivnost enzima TPMT kod pojedinih ljudi je izrazito smanjena ili povećana u odnosu na normalni nivo aktivnosti. Istraživanja strukture i biohemijskih karakteristika proteina TPMT su ukazala na postojanje određenih proteinskih varijanti koje imaju izmenjenu aktivnost. Otkriveni su polimorfizmi u genu za TPMT koji daju različite TPMT alozime. Smanjenoj aktivnosti enzima može doprineti i manja količina sintetisanog proteina, što zavisi i od transkripcione aktivnosti promotora gena za TPMT. Polimorfizmi u samom promotoru, kao što je promenjiv broj tandemskih ponovaka (VNTR), mogu da modulišu transkripciju. Primena tiopurinskih lekova kod pacijenata sa određenim genetskim varijantama TPMT izaziva tešku hematološku toksičnost. Da bi se toksičnost izbegla, terapija se modifikuje u skladu sa genotipom TPMT (farmakogenetika). Mi smo izučavali polimorfizme u egzonima i regulatornim elementima (promotor) gena za TPMT koji dovode do promene aktivnosti enzima TPMT u srpskoj populaciji. Koristili smo metodologiju baziranu na PCR i DNK sekvenciranje za detekciju genetskih varijanti TPMT. Pokazali smo da su u našoj populaciji prisutne genetske varijante u egzonima koje ukupno daju 7,5% varijantnih alozima TPMT koji imaju smanjenu enzimsku aktivnost. Terapija za pacijente koji imaju ove farmakogenetičke markere je modifikovana, što je doprinelo uspešnijem lečenju. Funkcionalnim esejima *in vitro* smo pokazali da aktivnost promotora gena za TPMT, a samim tim i količina sintetisanog enzima TPMT, zavisi od arhitekture (broja i tipa) VNTR u promotoru. Promotor gena za TPMT specifično

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revealed that Sp1 and Sp3 transcription factors interact with VNTRs. Our research pointed out that the VNTR promoter region of the TPMT gene could become a new pharmacogenetic marker, clinically significant for the individualization of thiopurine therapy.

Keywords: pharmacogenetics, TPMT gene polymorphisms, TPMT allozyme, VNTR in TPMT gene promoter

odgovara na tretman ćelija K562 tiopurinom zavisno od tipa VNTR. Izučavanje interakcija DNK i proteina je otkrilo da transkripcioni faktori Sp1 i Sp3 interaguju sa VNTR. Naša istraživanja ukazuju na to da bi region VNTR u promotoru gena za TPMT mogao postati novi farmakogenetički marker od kliničkog značaja za individualizaciju tiopurinske terapije.

Ključne reči: farmakogenetika, polimorfizmi u genu za TPMT, alozimi TPMT, VNTR u promotoru gena za TPMT

TPMT allozymes

Thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) represents one of the true examples of how pharmacogenetics can be applied to individualize drug therapy. TPMT is a cytosolic enzyme that catalyzes S-methylation, and consequent partial inactivation of immunosuppressive thiopurine medications, such as 6-mercaptopurine (6-MP), azathioprine and 6-thioguanine (1, 2). 6-MP is widely used in the treatment of many diseases, including acute leukemia, different types of inflammatory and autoimmune diseases and in transplantation medicine (3, 4). Some patients do not tolerate standard doses of thiopurine drugs and develop myelotoxicity (5–7), as a consequence of inherited TPMT deficiency. Structural and biochemical analyses of TPMT protein revealed the existence of certain protein variants with altered activity. Approximately 90% of individuals inherit two functional *TPMT* alleles resulting in high TPMT activity. These individuals are referred to as high methylators. Intermediate TPMT activity is seen in carriers of one nonfunctional *TPMT* allele. These intermediate methylators represent approximately 10% of the population. Low or undetectable TPMT activity is reported in 0.3% individuals who inherit two nonfunctional *TPMT* alleles (8). In addition, ultra-high methylators exist, who experience thiopurine treatment resistance and hepatotoxicity as a result of treatment with elevated 6-MP concentrations (9, 10). Patients with low TPMT activity are at high risk of severe, eventually fatal, hematologic toxicity owing to the accumulation of high levels of 6-thioguanine nucleotides (7). Consequently, thiopurine drug dose reduction is necessary to avoid toxicity (8). Patients with intermediate TPMT activity also require dose reduction (8, 11). Therefore, knowledge of the level of TPMT enzyme activity is essential for the balance of therapeutic and toxic effects of thiopurine drug dose.

TPMT polymorhisms

It has been shown that certain polymorphisms in TPMT gene exist that define different *TPMT* allozymes with different enzyme activity. *TPMT* gene exhibits significant genetic heterogeneity. At present, the *TPMT* allele nomenclature comprises at least 27 *TPMT* alleles (12), with wild-type allele designated as *TPMT**1. Among these, the most common polymorphisms are: c.238G>C, c.460G>A and c.719A>G. There are several TPMT variant alleles comprising one or more SNPs. On the basis of population studies, three alleles account for more than 95% of the clinically relevant TPMT variants: *TPMT**3A, *TPMT**3C and *TPMT**2, with the last of them contributing to a lesser extent (13). The *TPMT**2 allele contains a single c.238G>C polymorphism, the *TPMT**3A allele has two polymorphisms c.460G>A and c.719A>G, while *TPMT**3C has only the c.719A>G polymorphism. It is important to emphasize that the distribution of clinically relevant alleles is population specific (14–19). The *TPMT**3A allele is the most common variant allele in Caucasians (frequency approximately 5%), while *TPMT**3C is predominant in subjects with Asian or African ancestry (frequencies of 0.3–3% and 5.5–7.6% respectively). Majority of SNPs represent sequence variations that alter the encoded amino acid (20).

Individualization of thiopurine therapy

Administration of thiopurine drugs in patients with certain genetic TPMT variants leads to severe hematologic toxicity. To avoid toxicity, therapy is being modified according to the *TPMT* genotype of patients. For heterozygous carriers of *TPMT* polymorphisms thiopurine drug dose has been reduced (7, 21, 20). In 1–2% of patients with ultra-high TPMT enzyme activity doses of administered thiopurine drugs are 50% higher than standard. Even then, they often do not respond to therapy (14, 17). Since an inherited decrease of TPMT activity results in potentially life-threatening clinical consequences for patients treated with thiopurine drugs, a need for measurement of TPMT enzyme activity emerged. *TPMT* genotyping is a highly sensitive and specific alternative to expensive TPMT enzyme activity determination. More than 95% concordance exists between the *TPMT* genotype and phenotype (23, 24). Therefore, *TPMT* genotyping is a reliable method for guiding thiopurine therapy. It is of great importance that *TPMT* genotyping becomes a part of standard diagnostic protocols for diseases treated with thiopurine drugs (25).

TPMT gene polymorphisms is Serbia

We investigated polymorphisms in the exons of the *TPMT* gene which affect TPMT enzyme activity in the Serbian population in 100 unrelated blood donors and 100 children with acute lymphoblastic leukemia. We used PCR-based methodology for the three most common exon polymorphisms in the detection of genetic variants on the *TPMT* gene (11).

We showed that genetic *TPMT* variants in exons account for 7.5% of all TPMT variants with decreased enzyme activity (11). Similar to the other Caucasian subjects, *TPMT**3A is the most prevalent variant allele in Serbia (3.2%). The second most frequent allele in European populations, *TPMT**2, was found in a frequency of 0.2%. The *TPMT**3C allele was not detected among the 400 studied alleles, indicating that it must be very rare, if present at all, in the population of Serbia. Interestingly, the rare *TPMT**3B allele, detected in less than 10 individuals so far (26), was found in two independent families in Serbia. Patients with detected *TPMT* polymorphisms were all heterozygous carriers, and for the *TPMT**3A allele that was confirmed using family studies. The therapy for patients with these pharmacogenetic markers was modified, which contributed to the efficiency of treatment. For children carriers of *TPMT* polymorphisms thiopurine drug dose has been reduced by 25–50% (11). With this modified treatment all children, carriers of polymorphic *TPMT* alleles, did not miss the therapy (because of myelotoxicity). Time of therapy for those children was the same as for children without *TPMT* polymorphisms. Thiopurine therapy was gradually increased till full dose in those children after a while, because of good tolerance to the drug (11, 27). Our study showed that the application of pharmacogenetics principles in clinical practice is needed and indispensable even for the heterozygous carriers of *TPMT* polymorphisms.

One step ahead: quantitative *TPMT* polymorphisms

TPMT protein degradation has been the most frequent cause of decrease in the quantity of the TPMT protein (28, 29). A chaperone protein-dependent, proteasome-mediated pathway including aggresome formation and protein misfolding (30, 31) and autophagy (32), have been shown to contribute to degradation of TPMT variant allozymes (12). An alteration at the active site of the enzyme also accounts for the loss-of-function of the TPMT enzyme (33). Altered protein quantity, as a consequence of TPMT protein degradation, is an important mechanism responsible for the functional effects of an inherited variation in the amino acid sequence. Decreased TPMT enzyme activity, as a result of decreased protein quantity, can also be a consequence of lower protein synthesis, which depends on the transcription level. The most important transcription regulatory element is a promoter which is located upstream from the gene coding region.

TPMT gene promoter

Promoter of the *TPMT* gene is a eukaryotic promoter, with neither TATA nor CAAT boxes (32). It is highly polymorphic, containing a variable number of tandem repeats (VNTR) (9), from 3 to 9 (VNTR*3 to VNTR*9). There are three types of repeats: A, B and C type, differing in the length of the unit core (B and C repeats are 17 bp long, and A repeat is 18 bp long), with the first 14 nucleotides common to all three repeats. Since all alleles share one invariant C repeat, the differences between them are due to variations in the number of A and B repeats. The architecture of repeats (AnBmC) is maintained with no intervening sequences between them (35). Each repeat is GC-rich, and has putative binding sites for transcription factors from Sp and Krüppel-like factor (KLF) families (34, 36). The most common number of VNTR repeats in Caucasians is four or five (9, 28). Promoter polymorphisms, such as VNTRs, by influencing the level of *TPMT* gene transcription, could be responsible for altered TPMT protein quantity and altered TPMT enzyme activity.

VNTRs in the *TPMT* promoter: potential pharmacogenetic markers

TPMT gene polymorphisms are recognized pharmacogenetic markers which enable the individualization of thiopurine drug therapy (4, 11). Polymorphisms in the promoter region of the TPMT gene are still candidate pharmacogenetic markers. A modulatory effect of VNTR alleles *in vitro* (regarding the VNTR number) has been previously observed, but the repeat polymorphism did not display a significant role in TPMT gene regulation *in vivo* (9). It has also been shown that an inverse correlation exists between the red blood cell TPMT activity and the sum of VNTR repeats on both alleles (14). Several studies determined a significant association of the TPMT activity level and VNTR genotype (28, 37). The highest level of TPMT activity was associated with the VNTR*4/VNTR*5 genotype. Lower activity levels were associated with genotypes containing at least one allele with more than five repeats. Another study showed that the highest level of TPMT activity was associated with the VNTR*5/VNTR*5 genotype (38). All conclusions were mostly based on the study of the overall number of repeats on both chromosomes and their effect on *TPMT* gene expression. The role of the TPMT promoter in a clinical context is controversial, because it has not been clarified how the VNTR region within the TPMT gene promoter influences TPMT gene transcription, and whether promoter TPMT polymorphisms contribute to tolerance to 6-MP (39, 40).

Pharmacogenetic potential of VNTRs in the *TPMT* gene promoter

In our previous study on the TPMT gene polymorphisms in Serbia, it was noticed that almost half of the patients with no common *TPMT* polymorphisms experienced hematopoietic toxicity (11). Therefore, we investigated polymorphisms in the regulatory elements (promoter) of the *TPMT* gene which affect TPMT enzyme activity, using PCR-based methodology and sequencing. We have determined the distribution of VNTR alleles and genotypes in the Serbian population (41). We have detected 11 different VNTR alleles in the TPMT gene promoter in the Serbian population. VNTR alleles contained 4 to 8 repeats. The most frequent VNTR alleles were VNTR*4a (54.22%) and VNTR*5a (30.02%) (41). We have detected 17 different VNTR genotypes in the TPMT gene promoter in the Serbian population. The most frequent VNTR genotypes were VNTR*4a/VNTR*5a (33.3%), VNTR*4a/VNTR*4a (28.7%), VNTR*5a/VNTR*5a (9.15%), VNTR*4a/ VNTR6a (8.50%) and VNTR*5a/ VNTR6a (5.88%) (*Table I*). The remaining alleles and genotypes were less represented (41). Functional assays *in vitro* showed that the *TPMT* promoter activity and, therefore, the quantity of TPMT protein synthesized, depended on the architecture of VNTRs (i.e. number and type) in the promoter (*Figure 1*). Both the number and type of VNTR repeats determine the level of the *TPMT* gene transcription. Number of tandem repeats in the VNTR region of the *TPMT* gene promoter influences TPMT gene transcription. The TPMT promoter with four repeats (VNTR*4b allele) had the highest activity and the activity decreases in promoters with 5, 6 and 7 repeats. Activity of a promoter with 8 repeats is increased compared to promoters with 7 repeats. Different configuration, type of repeats inside the group with the same number of repeats, also influences *TPMT* transcription. The most prominent difference is detected in the VNTR*4 group. Activity of a promoter with the VNTR*4b allele is four times higher than the activity of a promoter with the VNTR*4a allele. The positive regulatory element located 5′ to the VNTR region (–180 to –130) is responsible for constitutive *TPMT* transcription, and the conformational architecture of VNTR region (number and type of repeats) influences the TPMT transcription fine tuning. VNTR*4b architecture (AB_2C) has the best conformational architecture for the most efficient *TPMT* transcription. Our functional assays demonstrate that the most frequent VNTR alleles in the Serbian population (comprising almost 90% of the general population) can be clustered in three distinct groups, namely low (A_2BC), medium (A_2B_3C) and high *TPMT* expressers (A_2B_2C). Our study has shown that the VNTR genotypes responsible for hypomethylation phenotype (A_2BC and A_2B_3C VNTR types) occur in one third of the Serbian population. For that reason, the VNTR genotype could be considered as a pharmacogenetic marker for our population. It remains to be seen whether the VNTR promoter region of *TPMT* gene can be employed as a pharmacogenetic marker of clinical importance for individualizing thiopurine therapy.

Table I Frequency of VNTR genotypes in the TPMT gene promoter in Serbian population.

VNTR genotype	frequency (%)
VNTR*4a/VNTR*4a	28.70
VNTR*4a/VNTR*4b	1.31
VNTR*4a/VNTR*5a	33.31
VNTR*4a/VNTR*5b	1.96
VNTR*4a/VNTR*6a	8.50
VNTR*4a/VNTR*6b	0.65
VNTR*4a/VNTR*7a	3.27
VNTR*4a/VNTR*8a	0.65
VNTR*5a/VNTR*5a	9.15
VNTR*5a/VNTR*6a	5.88
VNTR*5a/VNTR*6b	0.65
VNTR*5a/VNTR*7a	1.96
VNTR*5a/VNTR*7b	0.65
VNTR*6a/VNTR*6b	0.65
VNTR*6a/VNTR*7a	1.31
VNTR*6b/VNTR*6b	0.65
VNTR*7a/VNTR*7a	0.65

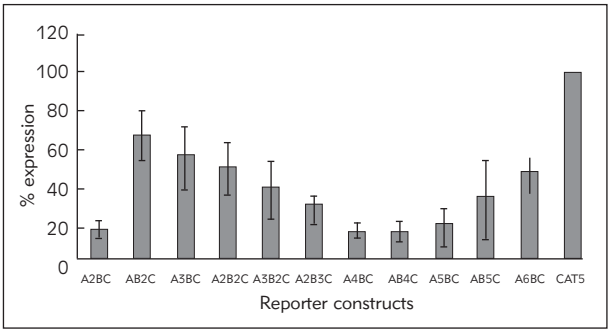

Figure 1 Functional analysis of 11 different VNTR type TPMT gene promoters detected in the Serbian population. K562 cells were transiently transfected with CAT constructs containing TPMT gene promoters with a different VNTR type. The pCH110 vector expressing β-gal was used to adjust for differencies in transfection activity. Vector pBLCAT5 (CAT5) (*tk* promoter driving *cat* gene) was used as positive control. The normalized CAT activities were evaluated as a percentage of pBLCAT5 vector activity, which was set as 100%, and are presented as the means±standard deviation of at least three independent experiments. Statistical significance was determined by Student's *t*-test, with two-tailed paired samples, and a p-value of less than 0.05 was considered significant.

Epigenetic control of TPMT gene transcription

Preliminary results have shown that the VNTR region has not been methylated in patients to whom thiopurine therapy was administered (41).

The *TPMT* gene promoter demonstrated a specific response to 6-MP treatment (10 μmol/L) in a VNTR--specific manner in K562 cells, namely a reduction of reporter gene expression of 40–50% (41). Study of the DNA-protein interactions (electrophoretic mobility shift assay) revealed that transcription factors Sp1 and Sp3 are involved in the *TPMT* transcription mediated by VNTR repeats. Electrophoretic mobility shift assay and south-western assay have shown that transcription factors involved in transcription mediated by the VNTR region under the influence of a 6-MP drug were different than when no drug was involved. However, the exact mechanism by which 6-MP influences *TPMT* gene transcription still remains unknown. 6-MP could influence DNA through DNA methylation (42).

Conclusion

TPMT pharmacogenetics has been studied extensively because of its clinical significance. Many patients got benefits from the knowledge of the *TPMT* genotype-phenotype correlation. However, further research is needed to elucidate the influence of the TPMT polymorphism on drug metabolism (pharmacokinetics) and drug targets (pharmacodynamics) (18).

The characterization of new *TPMT* polymorphisms and their effect on the level of enzyme activity will be the subject of future studies.

TPMT pharmacogenetics represents a promising example of how individualized therapy and personalized medicine will enter everyday medical practice in the near future.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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