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Distribution of the most common polymorphisms in *TYMS* gene in Slavic population of central Europe

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Thymidylate synthetase (TS) plays a critical role in the *de novo* synthesis of dTMP inside the cell. Therefore, TS is a suitable target for cytotoxic drugs such as fluoropyrimidines. Drug efficacy and toxicity depend on the intracellular level of TS, which is significantly influenced by the polymorphisms in the 5'UTR (TSER – rs45445694, TSER*3G>C – rs2853542) and 3'UTR (1494del TTAAAG – rs151264360) of *TYMS* gene. Polymorphic variants of *TYMS* gene affect TS activity via gene expression and transcript stability. Patients who undergo fluoropyrimidine therapy may benefit from genetic testing prior to the administration of chemotherapy. At the 5' terminus of *TYMS,* there is a polymorphic region represented by a variable number of 28bp long tandem repeats (2-9 tandems) with the G or C nucleotide variant (SNP G>C). The 3'end of *TYMS* gene may decrease the stability of mRNA in the case of 6 base deletion (1494del6, D). In our study, we have focused on testing of *TYMS* gene polymorphisms, determination of *TYMS* variant frequencies in Western Slavic population and comparison of Slovak population with other populations.

We performed identification of 5'UTR (rs45445694 – TSER*2 or TSER*3; rs2853542 – TSER*3G>C; TSER*3+*ins6*) and 3'UTR (rs151264360/1494del6/D) polymorphic regions of *TYMS* gene among 96 volunteers by PCR-RFLP and fragment analysis. Slovak frequencies of selected polymorphisms were established as follows: the frequency of TSER*2, TSER*3, TSER*3G>C, 1494del6/D and I to be 41%, 59%, 34%, 37.5% and 62.5% respectively. The high resolution of the capillary electrophoresis technique allowed among TSER*3 group identification of a subgroup of four individuals with rare 6bp insertion in 3R allele, *id est* 2.1% TSER*3+*ins6* allele frequency. In our study, we have revealed individuals with rare G>C substitution in the first 28bp tandem repeat of TSER*2 promoter enhancer region (rs183205964) as well, the overall frequency of this polymorphic allele in Slovak population was 2.1%.

Our results proved that Slovak population is in Hardy-Weinberg equilibrium and proportion of *TYMS* polymorphisms is in accordance with other published data.

Key words: fluoropyrimidines, thymidylate synthetase, pharmacogenetics, polymorphism, Slovak

Fluoropyrimidine drugs such as 5-fluorouracil (5-FU) and the prodrugs represented by capecitabine and tegafur are extensively used in cancer therapy. The fluoropyrimidine treatment efficiency depends on the intracellular level of the enzyme thymidylate synthetase (TS). If TS is inhibited by fluoropyrimidines, the production of dTMP in the cell is blocked, which leads to the misincorporation of 5-FU metabolites into RNA and DNA, and finally to cell-cycle arrest and apoptosis [1]. Thymidylate synthetase protein is coded by *TYMS* gene consisting of 7 exons located on chromosome 18 (18p11.32). The therapy outcome and also toxicity are influenced by TS cellular content which depends on the interpatient genetic variability [2].

Expression and stability of thymidylate synthetase vary according to the polymorphisms in the variable number of 28-bp tandem repeats and SNP (single nucleotide polymorphism) in the 5'-untranslated region (UTR) and also depend on 6-bp insertion/deletion polymorphism in the 3'UTR of *TYMS* gene (Fig. 1). *TYMS* promoter enhancer region (TSER) contains the variable number of 28bp tandem repeats (CCGCGCCACTT**G**GCCTGCCTCCGTCCCG) which are repeated from 2 up to 9 times (rs45445694 for-

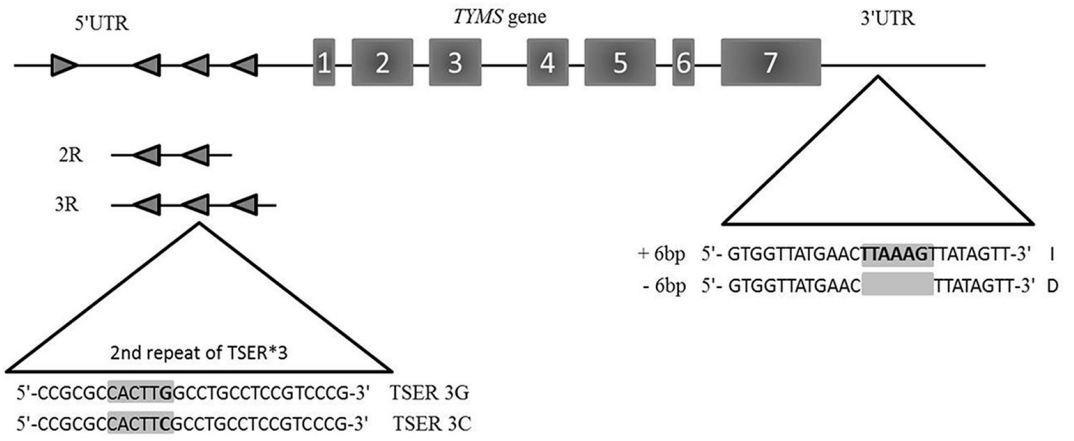

Figure 1. Structure of *TYMS* gene and 5'UTR and 3'UTR polymorphisms. TSER – Thymidylate Synthetase Enhancer Region, R – 28bp tandem repeat, I – I allele and D – D allele.

merly described as rs34743033), more prevalent as duplet (2R, TSER*2) or triplet (3R, TSER*3) [3]. Increased number of repeats raises *TYMS* RNA concentration and protein synthesis [3-6]. Every repeat contains E-box (enhancer box) binding site for upstream stimulatory factors (USF-1/USF-2) functional only when guanine (G) at the last position of CACTTG sequence is present. Both 2R and 3R wild-type alleles contain a cytosine in the last repeat which abolished the USF binding site in the repeat [7]. A clinical association has been found between reduced toxicity and drug efficacy in case of the 3R/3R genotype, contrary patients with the 2R/2R genotype showed an increased risk of toxicity, an increased response to fluoropyrimidine chemotherapy, and an increased survival time compared to individuals with the 2R/3R or 3R/3R genotype. The frequency of TSER*3 (3R) in the world is in the range from 50 to 60% except for Asian populations where the prevalence of TSER*3 is significantly higher (over 80%) [3, 8, 9].

The SNP G>C substitution in the 12th nucleotide in the second repeat of TSER*3 (rs2853542) alters *TYMS* expression by abolishing a transcription factor binding site [7, 10]. Patients with TSER*3 G>C (TSER*3C, 3C) polymorphism have a greater risk of toxicity due to the reduction in *TYMS* expression [10]. The TSER*3 patient might be stratified into high and low *TYMS* expression groups according to the presence of the TSER*3 G>C polymorphism.

Decreased mRNA stability *in vitro* and lower gene expression *in vivo* was revealed by Mandola et al. [11] in association with the 3'UTR 6bp deletion allele located 447bp downstream of the *TYMS* transcription stop codon (rs151264360 formerly described as rs34489327 or rs16430; 1494del6, D) (Fig. 1). Frequency of variant with 6bp deletion in Caucasians is 26-29%, 50% in Africans, and up to 76% in Asians [11, 12].

Other genetic and clinical factors may be also taken into account that may affect patient's risk for toxicity, survival time and therapy response.

The aim of this study was to analyse *TYMS* variants in Slovak population for the first time, to determine the frequency of polymorphisms in *TYMS* gene and to compare our findings with other populations. This study provides valuable information about the genetic variability of *TYMS* gene in Western Slavic population, hitherto missing.

Materials and methods

Samples of healthy unrelated volunteers were analysed by PCR, RFLP and fragment analysis (capillary electrophoresis) to estimate polymorphism frequencies in Slovak population. The analysed group consisted of 96 generally healthy individuals of Caucasian origin, randomly selected from the database of volunteers available at the Institute of Medical Biology, Genetics and Clinical Genetics, participants signed a written informed consent before participation in this study. Acquired data were checked for Hardy-Weinberg equilibrium and genetic linkage between variations.

DNA isolation. Blood samples for DNA extraction were collected in 3 ml tubes containing potassium EDTA. Whole blood DNA was extracted from 200 ml uncoagulated blood using Purification DNA Kit (DNA NucleoSpin Blood Kit; Macherey-Nagel, Düren, Germany).

Allele analysis. The 5'UTR VNTR (variable number of tandem repeats) region and 3'UTR region were amplified by PCR, the primers and PCR conditions used were previously described by Kawakami et al. [5] and by Ulrich et al. [12] were optimized for our laboratory settings. Primers for 5'UTR region (polymorphisms 2R/TSER*2, 3R/TSER*3 – rs45445694; TSER*3+ins6) amplification primers and conditions as follow: 5'-[6FAM] GCGGAAGGGGTCCTGCCA-3' and 5'-TCCGAGCCGGCCACAGGCAT-3', PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), PCR conditions: 5 min at 95 °C; 30 cycles: 15 sec at 95 C, 30 sec at 68 °C, 30 sec at 72°C; 5 min at 72 °C.

For 3'UTR region (1494del6/*TYMS*del/D,I – rs151264360) amplification primers and condition as follow: 5'-[HEX] CAAATCTGAGGGAGCTGAGT-3'and 5'-CAGATAAGTGGCAGTACAGA-3, PCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA), PCR conditions: 5 min at 95 °C; 30 cycles: 15 sec at 95 °C, 30 sec at 60°C, 30 sec at 72 °C; 5 min at 72 °C.

PCR products of 5'UTR and 3'UTR region were analysed with the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) (Figure 2).

SNP analysis (PCR-RFLP). SNP of G or C in 2R and 3R allele (G>C substitution) [13]: VNTR PCR products were digested 16 hours at 37°C with *HaeIII* (New England Biolabs, Massachusetts, USA). Detection of the SNP was performed after electrophoresis (Origins 2100U, Elchrom Scientific, Switzerland) in Spreadex® EL 300 Wide Mini S-2x13 or Spreadex® EL 300 Wide Mini S-2x25 and staining with GelRed Nucleic Acid Gel Stain (Biotium, California, USA). Briefly, SNP detection: for 3G (3R-GGC) – fragments: 31bp – 28bp – 66bp – 10bp; 3C (3R-GCC): 31bp – 94bp – 10bp; 2GC (wt 2R allele): 31bp – 66bp – 10bp; 2GG: 31bp – 28bp – 38bp – 10bp; 2CG (2R-CG): 59bp – 38bp – 10bp; 2CC (2R-CC): 97bp – 10bp (Figure 3).

PCR-RFLP was used to confirm results of 3´UTR region fragment analysis using PCR product digestion with *DraI* (New England Biolabs, Massachusetts, USA), D allele -142bp fragment, I allele – 60bp and 88bp fragment (Figure 3) [12].

Statistic methods. Chi-square test was used to confirm or rule out whether the Slovak population is in accordance with Hardy-Weinberg equilibrium (Table 1, 2).

Fisher's exact test of contingency tables was applied to detect significant differences in frequency of alleles in Slovak and other population. If the P-value of appropriate test is $P < 0.05$; the corresponding distributions are significantly different at the 5% significance level. In the tables, significantly different P-values are marked bold (Table 3).

Sample power test was used to analyse the strength of Fisher´s exact test for significantly different results of allele distribution (Table 3).

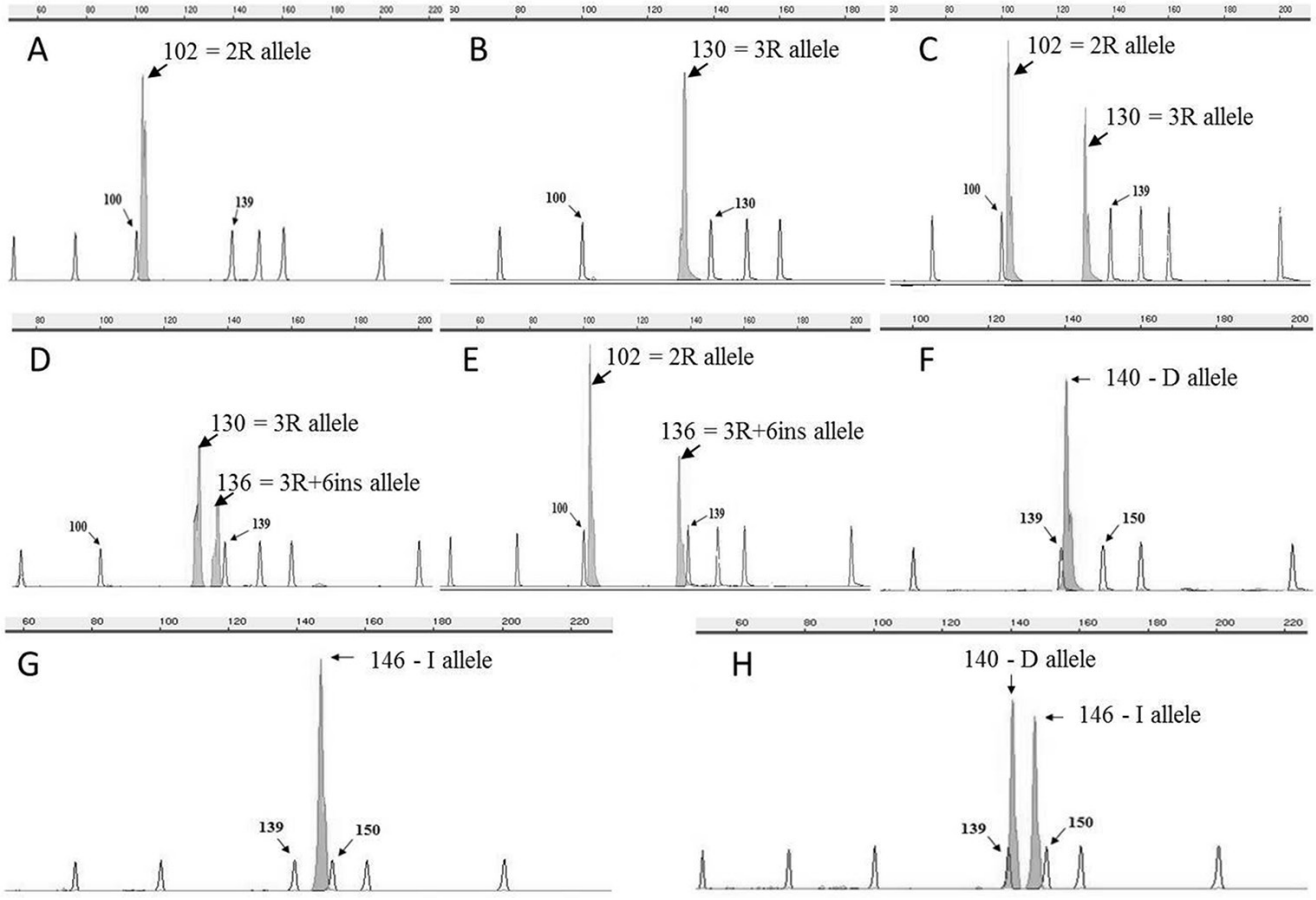

Figure 2. Fragment analysis of 5'UTR TSER region (2R, 3R, 3R+ins6) and 3'UTR region (D and I alleles) of *TYMS* gene. A) 2R/2R homozygote represented by fragment 102bp, B) 3R/3R homozygote – 130bp fragment, C) 2R/3R heterozygote – 102bp/130bp, D) 3R/3R+ins6 heterozygote – 130bp/136bp, E) 2R/3R+ins6 heterozygote – 102bp/136bp. The nearest size standard peaks 100 and 139bp. F) D/D homozygote represented by fragment 140bp, G) I/I homozygote – 146bp fragment, H) D/I heterozygote – 140bp/146bp. The nearest size standard peaks 139 and 150bp.

Results

We have genotyped 96 Slovak DNA samples of healthy volunteers and determined frequencies of *TYMS* polymorphisms in Slovak population for the first time. Using fragment analysis of the 5'UTR VNTR *TYMS* region, we have identified five different genotypes and the following frequencies: TSER*2/TSER*2 homozygotes represented by 20.8%, TSER*3/TSER*3 homozygotes with 35.4% portion, TSER*2/TSER*3 heterozygotes with 39.6% ratio, TSER*3/TSER*3+ins6 with 3.1% and TSER*2/TSER*3+ins6 with 1.1% (Table 1). The overall frequency of TSER*3 (with TSER*3+ins6*)* prevalent allele was 59%, the TSER*2 portion was 41% (Table 3). Within the TSER*3 allele, we have distinguished TSER*3 allele with 6 bp insertion (TSER*3+ins6) with 2.1% portion (Table 1). Based on the Chi-square test the distribution of the allelic variants

Table 1. Genotypes and allele frequencies of 5'UTR TSER region polymorphism (2R, 3R, 3R+ins6) and 3'UTR region polymorphism (D and I) in Slovak population.

Genotype	Number	Frequency (%)	Allele	Frequency (%)
2R/3R	38	39.6	3R	56.8
3R/3R	34	35.4	2R	41.1
2R/2R	20	20.8	3R+ins6	2.1
3R/3R+ins6	3	3.1		
2R/3R+ins6	1	1.1		
D/I	50	52.1	I	62.5
I/I	35	36.5	D	37.5
D/D	11	11.4		

Chi-square test confirmed the Hardy–Weinberg equilibrium in Slovak population (5'UTR TSER P=0.740, 3'UTR polymorphisms P=0.553).

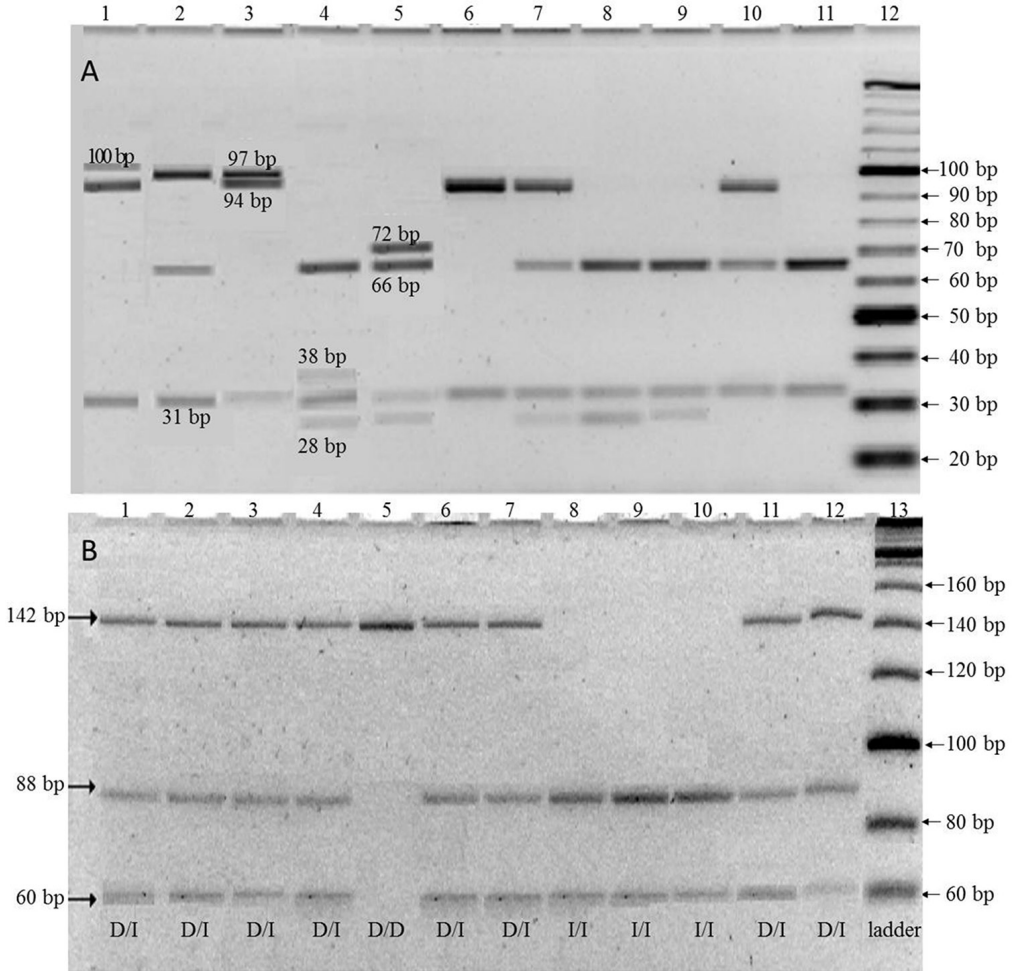

Figure 3. PCR-RFLP A) of 5'UTR region fragment analysis using *HaeIII* digestion of PCR products (sample, genotype, fragments). 1 – 3C/3C+*ins6* – 100bp and 94bp fragments; 2 – 2CC/2GC – 97bp and 66bp, 3 – 2CC/3C – 97bp and 94bp, 4 – 2GG/2GC – 38bp, 66bp and 28bp, 5 – 2GC/3G+*ins6* – 72bp, 66bp and 28bp, 6 – 3C/3C – 94bp; 7 – 3C/3G – 94bp, 66bp and 28bp; 8 – 3G/3G – 66bp and 28bp; 9 – 2GC/3G – 66bp and 28bp; 10 – 2GC/3C – 94bp and 66bp; 11 – 2GC/2GC – 66bp, 12 – DNA ladder. B) of 3'UTR region fragment analysis using *DraI* digestion of PCR product. D allele –142bp fragment, I allele – 60bp and 88bp fragment. PCR-RFLP confirmed results of fragment analysis of all 96 samples. Sample genotypes: 1, 2, 3, 4, 6, 7, 11, 12 – D/I heterozygote; 5 – D/D homozygote; 8, 9, 10 – I/I homozygote; 13 – DNA ladder.

Table 2. Distribution of SNP (G>C substitution) genotypes and alleles of *TYMS* in Slovak population.

Genotype	Number	Frequency (%)	Allele	Frequency (%)
2GC/3C	23	24	2GC	38.5
3C/3G	21	21.9	3C	32.8
2GC/2GC	17	17.7	3G	24.0
2GC/3G	13	13.5	2CC	2.1
3C/3C	7	7.3	3C+ins6	1.6
3G/3G	6	6.3	2GG	0.5
3C/3C+ins6	3	3.1	3G+ins6	0.5
2CC/2GC	2	2.1		
2CC/3C	2	2.1		
2GC/2GG	1	1		
2GC/3G+ins6	1	1		

Chi-square test confirmed the Hardy–Weinberg equilibrium in Slovak population (P=0.999).

of *TYMS* in Slovak population (Table 1) is in Hardy–Weinberg equilibrium. The detection of the TSER*3+ins6 allele was probably not within the detection limit in the studies with which we compared our population. Therefore, when comparing the frequencies of different populations, we have merged the frequency of TSER*3+ins6 allele with TSER*3 allele frequency. The frequencies of investigated polymorphisms correlate with data previously reported for Caucasians. Based on the calculated P-value the significant difference was found in frequency of 2R and 3R polymorphic allele (rs45445694) between Chinese, Kenyan, Ghanaian populations comparing to Slovak population, while the proportion of TSER*2 and TSER*3 was similar in the rest of the analysed populations (Macedonia, African-Americans, American and British Caucasians, Southwest Asian, and Spain) (Table 3). The sample power for comparative analysis of Slovak and: the population of Kenya (0.95), the population of Ghana (0.93) and Chinese population (0.999) is strong.

We have used PCR-RFLP analysis to confirm the distribution of genotypes and allele among Slovaks and to subdivide groups into detailed categories according to the content of G or C within the twelfth nucleotide of the 28bp repeats of TSER*2, TSER*3 or TSER*3+ins6 (Table 2). Frequency of wild-type TSER*2 allele (2GC) was 38.5%, 3C allele (without 3C+ins6) 32.8%, 3G allele (without 3G+ins6) 24%; we have also come across rare alleles, such as the 2CC allele with 2.1% frequency, 3C+ins6 allele with 1.6% portion, 2GG allele 0.5% and 3G+ins6 0.5%. Based on the Chi-square test, the distribution of TSER*2 (2GC, 2CC, 2GG), TSER*3G, TSER*3C allelic variants of *TYMS* in Slovak population is in Hardy–Weinberg equilibrium (Table 2). In order to compare our population with other studies, we merged Slovak 2GC, 2CC and 2GG frequencies to TSER*2 frequency (41%) as well as the 3C+ins6 frequency with 3C frequency (34%) and also the 3G+ins6 frequency with 3G (25%). In addition to this, the detailed investigation of SNP G or C variants in TSER*3 (rs2853542) revealed a significant difference between Slovaks and African-Americans, and between Slovak and Chinese populations as well (Table 3). The sample power for comparative analysis of Slovak and African Americans (0.96), as well as Chinese population (1), is strong.

In our work, the polymorphism rs151264360 in the 3'UTR region of *TYMS* (1494del6/*TYMS*del/D, I) was analysed by fragment analysis and the results were confirmed using PCR-RFLP with 62.5 % presence of I allele and 37.5% of D allele. The proportion of genotypes was as follows: more than half (52.1%) D/I heterozygotes, 36.5% I/I homozygotes, 11.4% D/D homozygotes (Table 1). Distribution of D and I alleles is in Hardy-Weinberg equilibrium in Slovak population. Statistically significant difference in frequency of distribution of D and I alleles was found comparing Slovak and Spanish populations (Table 3). The sample power of Fisher´s exact test comparing the population of Slovakia with the population of Spain is moderately strong (0.726).

In our study, we have analysed the distribution of combined genotypes of 5'UTR TSER region polymorphism (2R, 3R) and 3'UTR region polymorphism (D and I) in 37 volunteers (Table 4). We have found 8 genotype combinations from possible genotypes and following frequencies: most prevalent genotype 3R/3R – I/D with 24.4%; then 2R/3R – I/D 21.6%; 2R/2R – I/I, 2R/2R- I/D and 2R/3R – I/I equally 10.8%; 3R/3R – I/I and 3R/3R – D/D equally 8.1%; and 2R3R – D/D 5.4%. We did not identify individuals with the genotype 2R/2R – D/D.

Discussion

TYMS gene genotyping is a way that can potentially help predict patient response to fluoropyrimidines prior to the use of chemotherapy, thus leading to better-personalized treatment. We have completed the genotype study of *TYMS* gene in Slovak population that shows the distribution of *TYMS* variants among Slovaks. Our study is unique not only because of the analysis of VNTR of *TYMS* gene but also because of G and C determination within the twelfth nucleotide of the 28bp repeat in every tandem. According to our knowledge, such analysis has been not published for any Western Slavic population yet. The *TYMS* allele frequencies in Slovaks are comparable with other published Caucasian populations. The most prevalent allele in our study was TSER*3 (3R) with 59% frequency, less frequent allele was TSER*2 allele with 41% portion, and we have also detected the rare 6-bp insertion in the TSER*3 allele (TSER*3+ins6) in the frequency of 2.1%. The TSER*3+ins6 variant was firstly described by Thomas et al. with the frequency of 0.4% in Caucasians and 1.3% in African-Americans [13]. The functional effect of the 6-bp insertion in 5'UTR of the 3R allele remains to be determined. There were attempts to determine Western Slavic population, but the data are not detailed. In the study of Goricar and colleagues [14], the frequencies of two groups of genotypes for Slovenians are mentioned. The frequency for the first

Table 3. Differences in frequency of 2R and 3R alleles; 2R, 3G and 3C alleles; D and I alleles in Slovak and other populations analysed by Fisher's exact test of contingency tables.

Population	Allele frequency (%)				P-value	Sample power	Reference
	2R	3R	4R	9R			
Slovakia (n =192)	41	59	ND	ND			Current study
Macedonia (n=210)	38	62	ND	ND	0.476	NC	[16]
Kenya (n=196)	44	49	7	ND	**0.000**	0.950	[8]
Ghana (n=496)	40	56	3	1	**0.028**	0.930	[8]
African-Americans (n=184)	46	52	2	ND	0.062	NC	[8]
American Caucasians (n=208)	46	54	ND	ND	0.364	NC	[8]
British Caucasians (n=194)	45	54	1	ND	0.295	NC	[8]
China (n=192)	18	82	ND	ND	**0.000**	0.999	[17]
Southwest Asian (n=190)	38	62	ND	ND	0.532	NC	[17]
Spain (n=250)	47	53	ND	ND	0.248	NC	[34]
	2R	3G	3C	other			
Slovakia (n=192)	41	25	34	ND			Current study
Macedonia (n=210)	38	23	39	ND	0.570	NC	[16]
Spain (n=240)	47	23	30	ND	0.448	NC	[34]
White Ethnicity (n=198)	41	26	33	ND	0.909	NC	[10]
Hispanic (n=196)	42	30	26	2	0.062	NC	[10]
African Americans (n=118)	48	37	15	ND	**0.001**	0.960	[10]
China (n=160)	19	51	30	ND	**0.000**	1	[10]
	D		I				
Slovakia (n=192)	37		63				Current study
Caucasians (n=190)	29		71		0.131	NC	[12]
Northern Ireland (n=888)	32		68		0.150	NC	[35]
Spain (n=256)	26		74		**0.013**	0.726	[34]

Significantly different P-value is marked bold, n = number of alleles, ND = not detected, NC = not calculated.

group of TSER*2/TSER*2 homozygotes is 17.9% and for the other group, consisting of individuals with TSER*2/TSER*3 and TSER*3/TSER*3 genotypes, it is 82.1%. Jakubowska et al. [15] published an analysis of Polish population for the genotype groups: 22% of TSER*2/TSER*2 homozygotes, 30% of TSER*3/TSER*3 homozygotes and 48% of heterozygotes consisting of individuals with TSER*2/TSER*3 or TSER*2/TSER*4 genotypes. Macedonian population, one of the South Slavs, has been studied by Kapedanovska and colleagues [16]. The distribution of Macedonian genotypes is as described: 12% TSER*2/TSER*2 homozygotes, 30% TSER*2/TSER*3C, 20% TSER*2/TSER*3G, 19% TSER*3C/TSER*3C, 10% TSER*3C/TSER*3G.

In our group of volunteers, we have not found individuals with 4 or more 28bp tandem repeats. We have found differences in the distribution of TSER polymorphic alleles between Slovak population comparing the populations of Kenya, Ghana, and China. According to the opinion of Marsh and colleagues [17], polymorphism of TSER in humans may be a by-product of migration rather than allele evolution; environmental and/or other epigenetic factors can shift the allele frequencies in different regional dietary variations. In selective pressure of low intake of thymidine individuals with higher thymidylate synthase, due to the multiplication of TSER tandem repeat have selective advantage. Zhang and colleagues [18] demonstrated that besides humans, *TSER* is length polymorphic in many nonhuman primates while monomorphic in others. They suggested that the most recent common ancestor of hominoids and Old World monkeys probably possessed triple repeats. But now triple and double repeats, via deletion of one repeat, are two dominant types in hominoids and Old Word monkeys.

Table 4. Distribution of combined genotypes of 5'UTR TSER region polymorphism (2R, 3R) and 3'UTR region polymorphism (D and I) in Slovak population.

Genotype (N=37)	Number	Frequency (%)
2R/2R, I/I	4	10.8
2R/2R, I/D	4	10.8
2R/2R, D/D	0	0
2R/3R, I/I	4	10.8
2R/3R, I/D	8	21.6
2R/3R, D/D	2	5.4
3R/3R, I/I	3	8.1
3R/3R, I/D	9	24.4
3R/3R, D/D	3	8.1

N = number of genotypes

There have been numerous attempts to associate TSER polymorphisms with clinical outcomes in cancer patients receiving fluoropyrimidine therapy, conclusions have been inconsistent. Several studies have revealed links between TSER genotype and the response to chemotherapy, and mention that TSER*2/TSER*2 genotype or patients with at least one TSER*2 allele have a better response to fluorouracil as compared to patients with TSER*3/TSER*3 genotype [4, 19-22]. *In vitro* studies have shown up to four times more efficient translation from a construct with three repeats compared with two repeats [3, 23]. In the study of *in vivo* analysis of colorectal cancer tissues, no relation between genotype and transcription has been revealed, but TSER genotype was associated with TS protein expression. Specifically, cancer tissues with the 3R/3R genotype had a significantly higher TS protein expression level than did those with the 2R/3R genotype. Cancer tissue with 2R/2R genotype had the lowest TS protein expression [23].

De Bock and colleagues [7] have analysed thymidylate synthase activity *in vivo* in patients with colorectal cancer according to TSER polymorphism by measurement of 2´-deoxyuridine (dUrd) plasma level, a surrogate marker of TS inhibition. Plasma levels of dUrd were significantly different between genotypes, but in contrast to others, not to the absolute number of functional repeated elements (USF E-box) [7]. This study suggests that not the number of functional sequences, but their position within the promoter determines *TYMS* gene activity [7].

Up to date, there are no prescription and genotyping recommendations of *TYMS* gene prior therapy. Accurate information about the activity of thymidylate synthase depending on TSER genotype is missing as well. There are several studies trying to find out TSER genotype-specific guidelines for fluoropyrimidines dosing. Haller et al. [24] demonstrated regional differences in the tolerability profiles of fluoropyrimidines in the retrospective study. More treatment-related toxicity was reported in the US patients compared with the rest of the world for bolus fluorouracil/leucovorin and capecitabine in first-line metastatic colorectal cancer and adjuvant colon cancer. In the adjuvant setting, a range of fluoropyrimidine tolerability was observed, with East Asian patients having the lowest, and US patients the highest [24]. Soo and colleagues [25] sought to develop TSER specific guideline for capecitabine dosing. In their phase I study, they revealed a possibility to escalate the capecitabine dose of TSER 3R/3R advanced and/or metastatic cancer patients from lower the FDA-approved dose (from 1250mg/m^2 to 1500mg/m^2) [25].

In accordance with previous studies, the significant inverse association between the 5-FU toxicity and number of 28bp tandem repeats in 5'UTR region of *TYMS* gene was reported by some authors [4, 26, 27]. In the Saif's case report, they have described the first case of severe takotsubo cardiomyopathy related to DPD deficiency (heterozygous for the c.85T>C mutation) and homozygous polymorphism of *TYMS* (TSER*2/TSER*2, 2R/2R) in a patient with colon cancer following 5-FU containing regimen [28]. Despite controversy in the literature, overall TSER*2/TSER*2 finding predicts improved survival of patients receiving 5-FU chemotherapy but also increases the risk for 5-FU toxicity. Wang and colleagues have also observed pancytopenia and severe gastrointestinal toxicities in Caucasian TSER*2/TSER*2 homozygous patient with squamous cell rectal cancer after initiated 5-FU therapy in combination with mitomycin-C and radiation therapy followed after surgical excision [29]. The G>C substitution within the 12th nucleotide of the second repeat of TSER*3 (rs2853542) alters the *TYMS* expression due to abolished USF-1 binding site [10]. Morganti et al. demonstrated the reduction in *TYMS* expression in the case of G>C substitution in the second repeat of TSER*3 (TSER*3C) comparing to TSER*3G homozygotes and other genotypes in the colonic mucosa of 48 colorectal cancer patients [30]. The frequency of TSER*3C varies among world population from 15% in African Americans up to 33% in whites [10]. In Slovak population, TSER*3C allele frequency was 34% including TSER*3C+ins6 allele with frequency 1.6%. Marcuello and colleagues showed an improved overall response in metastatic colorectal cancer patients receiving 5-fluorouracil with the low expression genotypes (patients without any TSER*3G alleles) [31]. This SNP may further stratify TSER*3 individuals into high and low *TYMS* expression groups.

Another identified SNP with reduced effect on TS activity was analysed by Meulendijks and colleagues. They studied the G>C substitution in the first 28bp tandem repeat of 2R promoter-enhancer region of *TYMS* (rs183205964 known as the 2RC allele) among 1605 patients of this 28 patients (1.7%) carried the 2RC (our tag 2CC) allele. They observed significantly more frequent early severe toxicity and toxicity-related hospitalization in risk-associated genotype carriers (2RG/2RC, 3RC/2RC and 2RC/2RC). There was only one patient with the rare genotype 2RC/2RC in the study, who had to be hospitalized twice and had severe febrile neutropenia, diarrhoea, and hand-foot syndrome [32]. The G>C substitution in the first 28bp tandem repeat of promoter enhancer region of *TYMS* was firstly described by Lincz and colleagues in 2007 [33]. In our study, we have identified 2 individuals with genotype 2RC/2RG (our tag 2CC/2GC) and 2 with 2RC/ TSER*3C (our tag 2CC/3C), the overall frequency of the rs183205964 polymorphic allele in Slovak population was 2.1%.

On the other hand, Mandola et al. have measured TS mRNA amount in liver metastasis of 43 patients with advanced metastatic colorectal carcinoma and determined genotype – mRNA level correlation according to D and I allele presence. They found that patients with D/D genotype had decreased intratumoral TS mRNA to approximately 24% of TS mRNA amount of intratumoral TS mRNA in individuals with I/I genotype, while TS expression of D/I heterozygotes fell between two extremes with roughly 48% [11].

The knowledge of patient genotype prior fluoropyrimidine may help therapists to adequately set up treatment and prevent undesirable complications and life-threatening conditions in the future, despite the fact that clear predictive strategy has

not been developed for clinical use yet. Other comprehensive haplotype studies involving analysis of all aforementioned polymorphisms in a relationship with therapy effectiveness and the toxicity risks are necessary to achieve the prescription and genotyping recommendations of *TYMS* gene prior to therapy.

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