MOLECULAR AND EXPERIMENTAL BIOLOGY IN MEDICINE

THE VARIATION OF *CYP2C19* GENE IN THE ROMA POPULATION FROM CROATIA

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Abstract:
The *CYP2C19* gene is a member of cytochrome P450 family that codes for enzyme involved in the biotransformation of 10% of commonly prescribed drugs. Inter-individual variability in CYP2C19-mediated drug metabolism is mostly due to *CYP2C19* variations. This study analyses the pharmacogenetic profile of *CYP2C19* in three groups of Croatian Roma (440 individuals).
Ten *CYP2C19* loci were genotyped, seven of which were monomorphic (rs28399504, rs4986893, rs55640102, rs56337013, rs72552267, rs72558186 and rs41291556). Minor allele frequency (MAF) of rs3758581 (defining wild-type allele, *CYP2C19*1*) was 56.6% in the Roma, MAF of rs4244285 (*CYP2C19*2*) was 15.1% and of rs12248560 (*CYP2C19*17*) 28.3%. Frequencies of the same MAFs in the surrounding majority Croatian population were 61.5%, 14.8% and 23.7%, respectively (Ganoci et al 2017).
The more rapid metabolism (vs. wild type) of CYP2C19-targeted drugs is expected in 40% of the Roma (and in as much as 53.1% in the Baranja group). This is a consequence of a high prevalence (33%) of the "rapid metabolizers" (corresponding to the **1/*17* genotype) and 7% of "ultrarapid metabolizers" *(*17/*17*) in the Croatian Roma.
The results indicate that the genetic profile of CYP2C19 should be taken into account in modulating pharmacotherapy in the Roma population.

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INTRODUCTION

One of the frequently investigated genes involved in Adsorption, Distribution, Metabolism and Excretion (ADME) of drugs is the *CYP2C19* gene, a member of cytochrome P450 family. *CYP2C19*, mapped on chromosome 10q24.1-q24.3, contains nine exons and codes for the CYP2C19 protein (490 amino acids). CYP2C19 is found primarily in liver cells and plays a role in the processing or metabolizing of approximately 10% of commonly prescribed drugs.[1] These drugs are used for treating health conditions such as peptic ulcers (omeprazole), convulsions ((S)-mephenytoin), anxiety (diazepam), depression (citalopram, imipramine), for reducing the risk of heart attack and stroke due to platelets clotting (clopidogrel), etc.[2]
The CYP2C19 metabolic capacity is categorized in three phenotypes: (1) extensive metabolizers (EM), (2) intermediate metabolizers (IM), and (3) poor metabolizers (PM) of drugs.[2] Approximately 3% of Caucasian and 15 to 20% of Asian populations are "poor metabolizers", meaning that they have reduced or absent CYP2C19 activity.[3] This large interindividual variability observed in CYP2C19 drug metabolism *in vivo* is due to inherited variations in the *CYP2C19* gene.[4] Among many identified *CYP2C19* variants[5], three were recognized as major factors for pharmacokinetics and response to CYP2C19 substrates; rs4244285, rs4986893, and rs12248560. Unlike other genes, allelic variations and haplotypes in ADME are commonly described using the "star" (*) system. In most cases, **1* describes the default reference allele (wild type) of a haplotype (fully functioning enzyme), while other designations (**2, *2A, *2B, *3*, etc.) refer to haplotypes carrying one or more variants.[6] According to this "star" nomenclature,

loci rs4244285, rs4986893, and rs12248560 respond to *CYP2C19*2*, *CYP2C19*3* and *CYP2C19*17* alleles.

*CPY2C19*2* and *CYP2C19*3* are the two most frequent CYP2C19 loss-of-function polymorphic alleles; **2* (rs4244285) with single-base substitution (681 G>A), resulting in a splicing defect, and **3* (rs4986893) with a point mutation (636 G>A), producing a premature stop codon. Both variant alleles have insufficient activity, which can cause null function of the enzyme.[7] On the other hand, *CYP2C19*17*, which is a single-base substitution in the transcriptional regulatory region of the gene (806 C>T), results in the enhancement of the transcriptional activity of the CYP2C19 enzyme and rapid or ultrarapid metabolism of drugs.[8]

The Roma (Gypsy) are a large transnational minority of Indian origin numbering 15 million people worldwide, most of whom live in Europe. Their exodus from India occurred between the 5th and the 10th century, and in the 11th century they reached Europe. Upon arrival, a large part of the initial migrant population settled in the Balkans, where their descendants still live today. These Balkan Roma are represented by numerous groups, who differ between themselves genetically and linguistically. Another part of the Roma migrants continued their journey to western and northern Europe, and some of them crossed the Danube and settled in Wallachia (present-day Romania), where they were enslaved. After the abolition of slavery in the 19th century, these Vlax Roma, in Croatia called Bayash, migrated to Serbia, Hungary, Croatia and other Balkan states, but also to other parts of Europe and to the United States. Both Balkan Roma and Bayashs are the examples of population isolates with persistent, centuries-long socio-cultural and reproductive isolation.[9, 10]

The aim of this study was to determine the variation of the *CYP2C19* gene (allele frequency and haplotype distribution) among the Croatian Roma by genotyping ten SNP loci within the *CYP2C19* gene. These data might be taken into account for drug safety and dosage calculation of CYP2C19 substrates in the Croatian Roma population.

MATERIAL AND METHODS

Biological samples were collected during the on-going multidisciplinary anthropological, molecular-genetic and epidemiological investigation of Roma populations in Croatia. Study participants were volunteers and were informed about goals, methods and expectations of the study with the help of linguistically and culturally competent and trained Roma volunteers. They belonged to three socio-culturally different Roma groups: two groups of Vlax (Bayash) Roma, the Baranja group and the Međimurje group, and the Balkan Roma group.

CYP2C19 gene variation was investigated in 440 DNA samples by genotyping ten single nucleotide polymorphism (SNP) loci listed and described in more detail in Table 1. Genotyping was carried out using the KASP method.[11] This technology, until recently widely used only on plant species, has been successfully applied to human samples too.[12, 13]

Allele and genotype frequencies were calculated by the direct counting method. Hardy-Weinberg equilibrium and exact test of population differentiation were performed using Arlequin 3.5.2.2. Haplotypes were inferred using Phase ver. 2.1. Haplotypes were translated to the star nomenclature according to the guidance of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee.*5* Linkage disequilibrium (LD) and haplotype block analyses were accomplished with Haploview 4.1.

The study protocol was approved by the Scientific Board and Ethical Committee of the Institute for Anthropological Research in Zagreb, Croatia.

RESULTS

The genotyping results of ten SNP loci within the *CYP2C19* gene in DNA samples obtained from 440 Croatian Roma are given in Table 2. Seven of these loci were monomorphic and three were polymorphic; rs12248560, rs4244285, and rs3758581. The latter was

Table 1. Single nucleotide polymorphisms (SNPs) of *CYP2C19* gene analyzed in this study. Loci are sorted according to the ascending chromosome position. Only successfully genotyped SNPs were counted in column N. The star-allele *CYP2C19* variant nomenclature is assigned according to Pharmacogene Variation Consortium[5]

SNP	Chromosome position	Loci	1000 Genomes Major Allele	1000 Genomes Minor Allele	1000 Genomes Major Allele Frequency	Roma Major Allele	Roma Minor Allele	N	Roma Major Allele Frequency	Star-allele
rs12248560	94761900	polymorphic	C	T	85%	C	T	434	71.7%	*17
rs72552267	94762706	monomorphic	G	A	100%	G	A	435	100%	*6
rs4244285	94775416	polymorphic	G	A	78%	G	A	430	84.5%	*2
rs4986893	94775453	monomorphic	G	A	99%	G	A	418	100%	*3
rs56337013	94780653	monomorphic	C	T	100%	C	T	429	100%	*5
rs28399504	94781859	monomorphic	A	G	100%	A	G	436	100%	*4A, *4B
rs41291556	94781999	monomorphic	T	C	100%	T	C	436	100%	*8
rs3758581	94842866	polymorphic	G	A	95%	G	A	434	81.7%	*1
rs72558186	94852738	monomorphic	T	C	-	T	C	437	100%	*7
rs55640102	94852914	monomorphic	A	C	100%	A	C	420	100%	*12

Table 2. *CYP2C19* genotype frequencies (%) in the Croatian Roma for three subpopulations (Baranja, Međimurje and Balkan). Genotyped *CYP2C19* loci are listed according to their genomic positions in Chromosome 10. The differences in frequencies of genotypes and alleles in polymorphic loci between the three subpopulations were tested using Chi-square test (df=2).

SNP	Genotype	Genotype frequency						P
		N	Baranja	N	Međimurje	N	Balkan	
rs12248560	CC	131	37.4	127	53.5	176	58	<0.01
	CT		52.7		43.3		34.1	
	TT		9.9		3.2		7.9	
	T allele		36.3		24.8		25	<0.01
rs72552267	GG	131	100	129	100	175	100	-
	GA		0		0		0	
	AA		0		0		0	
rs4244285	GG	129	72.1	129	71.3	172	69.2	ns
	GA		27.1		27.9		27.9	
	AA		0.8		0.8		2.9	
	A allele		14.3		14.7		16.9	ns
rs4986893	GG	128	100	125	100	165	100	-
	GA		0		0		0	
	AA		0		0		0	
rs56337013	CC	128	100	130	100	171	100	-
	CT		0		0		0	
	TT		0		0		0	
rs28399504	AA	132	100	129	100	175	100	-
	AG		0		0		0	
	GG		0		0		0	
rs41291556	TT	132	100	128	100	176	100	-
	TC		0		0		0	
	CC		0		0		0	
rs3758581	GG	131	74.8	128	70.3	175	69.7	ns
	GA		17.6		22.7		21.1	
	AA		7.6		7		9.2	
	A allele		16.4		18.4		19.7	ns
rs72558186	TT	132	100	129	100	176	100	-
	TC		0		0		0	
	CC		0		0		0	
rs55640102	AA	123	100	130	100	167	100	-
	AC		0		0		0	
	CC		0		0		0	

the only locus that was not in Hardy-Weinberg equilibrium in any of the Roma subpopulations. Of the three polymorphic loci, rs12248560 showed significant differences between Roma subpopulations in both genotype and allele frequencies distribution, with its T allele being more frequent in the Balkan Roma than in the Baranja or the Međimurje Roma ($p<0.01$). The exact test showed significant differences between the Balkan and the Baranja Roma ($p<0.05$).

High-density SNP genotyping defined five distinct haplotypes for the loci ordered as follows: rs12248560, rs72552267, rs4244285, rs4986893, rs56337013, rs28399504, rs41291556, rs3758581, rs72558186, and rs55640102 (Table 3). All five haplotypes were present in all three subpopulations, but with different frequencies. The haplotype TGGGCATGTA, which was the most frequent in the Baranja sample (36.4%), was the second most frequent in the Roma from Međimurje and in the Balkan Roma. On the other hand, the haplotype CGGGCATGTA, which was the most frequent haplotype in the Međimurje and in the Balkan Roma (45.8% and 42.1%, respectively), was the second

Table 3. The frequency (%) and number (N) of *CYP2C19* haplotypes in the total Roma population and separately for the three Roma subpopulations (Baranja, Međimurje, and Balkan). Mutated loci are noted with underlined bold letters. The differences in haplotype frequencies between subpopulations were tested using Chi-square test ($\chi 2=13.25$; df=14; $p=0.1035$). The same test was used for testing the subpopulational differences in star allele structure ($\chi 2=12.77$; df=8; $p<0.05$).

No.	Haplotypes*	Star allele	Total %	Baranja %	Međimurje %	Balkan %	Total N	Baranja N	Međimurje N	Balkan N
1	CGGGCAT**G**TA	*1	41.4	36.0	45.8	42.1	364	95	120	149
2	CGGGCAT**A**TA	*1	15.2	13.6	15.3	16.4	134	36	40	58
3	CG**A**GCATGTA	*2	12.3	11.4	11.8	13.3	108	30	31	47
4	CG**A**GCATATA	*2	2.8	2.7	2.7	3.1	25	7	7	11
5	**T**GGGCATGTA	*17	28.3	36.4	24.4	25.1	249	96	64	89
		Total	100	100.1	100	100	880	264	262	354

Legend: *loci order: rs12248560, rs72552267, rs4244285, rs4986893, rs56337013, rs28399504, rs41291556, rs3758581, rs72558186, and rs55640102

most frequent in the Baranja sample. Chi-square test results showed no significant difference in haplotype frequencies between the Roma subpopulations ($\chi2$=13.25; df=14; p=0.1035).

Translation of haplotypes into star nomenclature resulted in three "star" (*) haplotypes, defined by polymorphic loci placed at the first, third and eighth position in haplotypes (Table 3). In all three Roma populations, the wild haplotype **1* was the most frequent (56.6%), followed by haplotypes **17* (28.3%) and **2* (15.1%). Subpopulational differences in star allele structure were statistically significant ($\chi2$=12.77; df=8; p<0.05).

Linkage disequilibrium (LD) evaluated using polymorphic loci showed a complete disequilibrium (D=1.0) between loci rs12248560 and rs4244285 in all three Roma groups (r2 was 0.093, 0.071, and 0.056 in Baranja, Balkan, and Međimurje, respectively). In addition, another complete disequilibrium (D=1.0) was detected in the Međimurje Roma between rs12248560 and rs3758581 (r^2=0.076) (Figure 1).

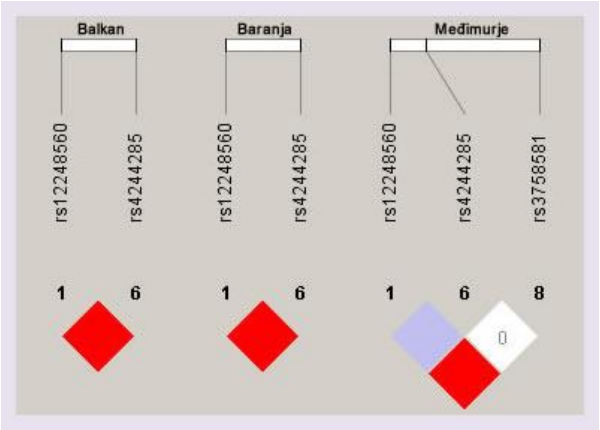

Figure 1. Linkage disequilibrium (LD) blocks within *CYP2C19* gene in the three Croatian Roma subpopulations.

The prevalence of *CYP2C19 *1/*1*, **1/*2*, **1/*17*, **2/*2*, **2/*17* and **17/*17* genotypes in the Croatian Roma is given in Table 4. Among the Baranja Roma sample, the majority of people (43.2%) had the **1/*17* genotype, meaning that they were rapid metabolizers, while it was the second most frequent genotype in the Međimurje Roma and in the Balkan Roma, where normal metabolism-defining **1/*1* genotypes were the most frequent. Actually, in the Međimurje Roma, the prevalence of **1/*1* and **1/*17* genotypes was quite similar, 35.1% vs. 33.6%. This Roma group, in comparison with the Baranja and the Balkan Roma, had the least ultrarapid metabolizers with the genotype **17/*17* (3% in the Međimurje Roma vs. 9.9% in the Baranja and 7.9% in the Balkan Roma). In the overall sample, the most frequent was the rapid metabolizing phenotype, detected in 33% of the Roma.

The *CYP2C19* star allele frequencies in different Roma populations are given in Table 5. When compared to other Roma from Europe, the Croatian Roma had the lowest prevalence of the **2* allele. It is interesting to note that one of the most important alleles in defining pharmacokinetics of the CYP2C19 enzyme, the **3* allele, is not present in any member of the Roma groups analyzed so far.

DISCUSSION

The Roma are the biggest and the most widespread ethnic minority in Europe. Uniparentally inherited markers, such as mitochondrial DNA and Y chromosome markers, confirm their ancestral Indian origin[14, 15], as do the genome-wide data.[16, 17] The genetic distinctiveness of the Roma has also been detected in various autosomal common[18-20] and rare disease loci.[21, 22] Similar genetic patterns were determined in the Roma in Croatia too.[23-27]

Recently, various ADME CYP genes in the Roma came to the attention of researchers, proving again the distinctive position of the Roma in the world's population variability.[13, 28, 29] Research of pharmaco-genes might be useful for modulation of pharmacotherapy in the Roma, a population with frequent and various health problems.[30-33] *CYP2C19* variants investigated in this research confirmed that loci found to be polymorphic in the 1000 Genomes populations were polymorphic in the Croatian Roma as well, but also that MAFs of rs3758581 (**1*) and rs12248560 (**17*) were higher in the Croatian Roma than in the 1000 Genomes populations (Table 1). In comparison with the surrounding majority Croatian population, **2* allele frequency in the Roma was

Table 4. Prevalence of *CYP2C19* star allele genotypes in the Croatian Roma subpopulations. The differences in frequency of each star allele genotype in the total population and in three subpopulations (Baranja, Međimurje and Balkan) were tested using the Chi-square test ($\chi2$=23.616; df=10; p<0.01).

Star allele genotypes	Total Roma N (%)	Baranja N (%)	Međimurje N (%)	Balkan N (%)	Phenotype
****17/*17***	31 (7)	13 (9.9)	4 (3)	14 (7.9)	Ultrarapid metabolizer
****1/*17***	145 (33)	57 (43.2)	44 (33.6)	44 (24.9)	Rapid metabolizer
****1/*1***	138 (31.4)	26 (19.7)	46 (35.1)	66 (37.3)	Normal metabolizer
****1/*2***	77 (17.5)	22 (16.7)	24 (18.3)	31 (17.5)	Intermediate metabolizer
****2/*17***	42 (9.5)	13 (9.9)	12 (9.2)	17 (9.6)	Intermediate metabolizer
****2/*2***	7 (1.6)	1 (0.7)	1 (0.8)	5 (2.8)	Poor metabolizer

Table 5. Prevalence of *CYP2C19* star alleles in Roma populations in Europe. The two studies carried out in Portuguese and Spanish Roma reported data only on *CYP2C19*2* prevalence. The *17 star-allele prevalence among the Croatian Roma was significantly the highest in the Baranja Roma sample ($\chi 2=12.138$, df=1, p<0.01).

Ethnic groups	N	Star alleles (%)				Ref.
		*1	*2	*3	*17	
Roma - Croatia	440	56.6	15.1	0	28.3	this study
Baranja	132	49.6	14	0	36.4	
Međimurje	131	61.1	14.5	0	24.4	
Balkan	177	58.5	16.4	0	25.1	
Roma - Hungary	500	79.5	20.5	0	n.g.	Sipeky *et al.* 2013
Roma - Portugal	116		21.1			Teixeira *et al.* 2015
Roma - Spain	62		23.4			Pimenoff *et al.* 2012

Legend: n.g. – not genotyped

similar to Croatians[34, 35] (15.1% vs. 15%[34] and 14.8%[35]), **3* allele was not detected neither in the Roma nor in Croatians[34], while **17* allele was more frequent in the Roma sample than in Croatians[35] (28.3% vs. 23.7%). Still, prevalence of ultrarapid metabolizers *(*17/*17*) and rapid metabolizers (**1/*17*) in the Croatian sample roughly corresponded to the Roma sample: 5.4% of ultrarapid and 31.3% of rapid metabolizers in the Croatian population[35] vs. 7% of ultrarapid and 33% of rapid metabolizers in the Roma. Poor metabolizing **2/*2* genotype was found in 2.4% of Croatians[35] and in 1.6% of the Roma.

The **17* allele frequency in the Baranja Roma was significantly higher than in the other two Roma populations, and increased the overall **17* allele prevalence. The finding that more than a half of Baranja Roma were rapid and ultrarapid metabolizers has important implications for the selection of adequate drugs and drug dosages in these Roma, who self reported a rather high prevalence of diagnosed hypertension (13.6%), stomach pain (20.8%), frequent headaches (24.9%) and anxiety/insomnia (16.2%)[32] - health problems frequently treated with drugs metabolized or processed by the CYP2C19 protein.

Of all the *CYP2C19* loci we investigated, other Roma population studies reported mostly only on **2* allele frequency: it was found in 23.4% of the Spanish Roma[36], in 21.1% of the Portuguese Roma[37] and in 20.5% of the Hungarian Roma[38]. In the latter study, the **3* allele was also investigated, but no carrier was found in the Hungarian Roma. The **3* variant is present in low frequencies (0.6-2.7%) in the populations of India, a bit lower in the south of India (0.6-1.8%) than in the north of India (1.2-2.7%).[39] It is possible that strong bottleneck and founder effects, which occurred approximately 1.5 thousand years ago[16], led to the loss of the **3* allele in the Roma.

In a review paper on the *CYP2C19* worldwide variation, the authors joined the Hungarian Roma population with Indians and Sri Lankans into a group named "South Asians" and frequencies of **2*, **3* and **17* alleles in this joined population were 30.3%, 0.5% and 13.8%, respectively.[40] The data on **2* and **3* allele frequencies in Roma populations in this study and papers cited here question grouping populations only according to geographical origin: centuries-long reproductive isolation should have been taken into account because it clearly affected allele frequencies. What is also interesting is that the Roma are not the only European population isolate with high *CYP2C19*2* allele frequency: higher **2* allele frequencies than in the Roma were determined in Sami and Basques.[36]

The *CYP2C19*2* nonfunctional allele may have been positively selected in Europeans[36] and in people of African descent[41], while the other nonfunctional allele, *CYP2C19*3* may have been positively selected in Asian populations.[41] Fast metabolizing **17* allele also seems to be positively selected in Europeans.[41] The biological factors responsible for these selective pressures are currently still unknown.

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