CYP2C9 GENE POLYMORPHISM IN THE PHARMACOLOGICAL TREATMENT OF LONG QT PATIENTS

SŁAWOMIR SMOLIK*, EWA MORIC-JANISZEWSKA and LUDMIŁA WĘGLARZ

Department of Biochemistry, Medical University of Silesia,
Jedności 8, 41-200, Sosnowiec, Poland

The family of cytochrome CYP450 enzymes consists of more than 30 enzymes which are the major mediators of phase I metabolism of drugs and endogenous compounds. Genetic polymorphism of CYP450 enzymes can lead to adverse drug reaction or inadequate response to commonly prescribed doses of the therapeutic agents (1). CYP2C9 represents the most abundant form among human CYP2C isoforms and comprises approximately 20% of the total hepatic CYP450 content. It participates in the metabolism of number of drugs including non-steroidal anti-inflammatory agents, S-warfarin, phenytoin, tolbutamide and losartan (2). Polymorphism in the CYP2C9 gene seriously affects the toxicity of drugs, with S-warfarin in particular, and may lead to severe and life-threatening bleeding episode (3). Three variants of the CYP2C9 allele denominated as CYP2C9*1, CYP2C9*2 and CYP2C9*3 have been found in Caucasian population,. The mutant alleles CYP2C9*2 and CYP2C9*3 differ from the wild-type CYP2C9*1 by a point mutation, i.e., CYP2C9*2 is characterized by a C416T exchange in the exon 3 resulting in Arg144Cys substitution, whereas CYP2C9*3 has an exchange of A1061C in exon 7 causing Ile359Leu substitution. Both allelic variants are associated with reduced catalytic activity compared to the wild type; they are reported to show approximately 12% (CYP2C9*2) and less than 5% (CYP2C9*3) of the wild-type enzyme activity (4, 5).

Long QT syndrome is a rare inherited disorder of ion channels that may cause dangerous heart rhythms in response to exercise or stress. Inherited long QT syndrome affects about 1 among 7000 peo-ple. To date, mutations in 11 genes have been discovered and described as being relevant for this disease. The LQT1 type is caused by mutation in the KCNQ1 gene and is found in approximately 50–60% of cases. The LQT2 type can be caused by mutations in HERG gene and it is found in 30–35% of clinical cases (6, 7). The treatment of long QT syndrome is intended to prevent abnormal heartbeats, decrease hypertension and improve lipid profile and function of platelets. The treatment options include antiarrhythmic and antihypertensive agents (i.e., losartan), cholesterol lowering drugs, supplementation of potassium and anticoagulants (i.e., S-warfarin) (8).

The metabolism of the anticoagulant drug S-warfarin was found to be markedly lowered in patients having mutant CYP2C9 alleles. Moreover, a strong association was demonstrated between CYP2C9 variant alleles and low S-warfarin dose requirement, as well as a higher risk of bleeding complications, so it was suggested that dose adjustment of S-warfarin in patients carrying 2C9 allelic variants might be worthwhile in order to lower the risk of drug intoxication and bleeding (9). CYP2C9 genotype contributes to inter-individual differences in losartan activation and its hypotensive effect (10). Evaluation of losartan in the elderly showed that losartan reduced the QT dispersion in elderly patients with heart failure, so it may reduce mortality of long QT patients (11). Polymorphism of CYP2C9 has strong influence on long QT patients drug therapy and its allelic variants may cause severe adverse reaction. The purpose of the present study was to investigate the prevalence of the

* Corresponding author: e-mail: epimer@wp.p

CYP2C9*2 and CYP2C9*3 allelic variants in the group of 104 patients with long QT and discuss their clinical implication in the modern pharmacotherapy of long QT syndrome.

EXPERIMENTAL

Clinical study

A group of 104 patients sufferngi from long QT syndrome (58 males and 46 females, mean age 49 ± 12 years) were recruited for genotyping study after approval by the local ethics committee. The diagnosis of syndrome was based on clinical examination of patients, which was performed according to guidelines issued by Polish Cardiology Society. All patients had QTc interval > 440 ms for men and 450 ms for women. The exclusion criteria were QTc at baseline and history of additional risk factor (hypokalemia, heart failure, left ventricular hypertrophy).

ASA-PCR genotyping

Genomic DNA was extracted from the 5 mL whole blood samples with the use of Genomic Prep Plus method (A&A Biotechnology, Gdynia, Poland). Allele-specific amplification (ASA-PCR) was developed for genotyping CYP2C9*2 and CYP2C9*3 alleles. Each reaction mix included an external pair of primers (named forward and reverse) and two allele-specific primers, carrying either the wild type nucleotide or the mutant nucleotide in the 3' position. PCR would amplify the region between the wild type primer and the reverse external primer only if the wild type allele was present. Inversely, amplification of the region between the internal mutation specific primer and the forward external primer would occur only if the mutant allele was present. Wild type and mutant amplicons can be distinguished by a fragment size. A third fragment, which represents the region between the two external primers, was also generated and served as an internal control. The following reaction mixture was prepared for tetra-primer PCR assay to genotype CYP2C9*2: 100 ng of genomic DNA, 0.5 U of Taq DNA polymerase (Novazym, Poznań, Poland), a final concentration $1 \times$ PCR buffer, 1.5 mM of $MgCl_2$, 100 µM of each dNTP, and primers as follows: CYP2C9E3-F (0.24 µM), CYP2C9E3-R (0.16 µM), CYP2C9E3-T-F (0.16 µM), CYP2C9E3-C-R (0.24 µM) (Genomed, Warszawa, Poland). The mixture volume was adjusted to 25 µL with water. Primer sequences are listed in Table 1. The following thermal conditions were used: 95°C for 2 min for initial denaturation, and then 10 cycles at 95°C for 20 s, 66°C for 45 s and 72°C for 45 s, 20 cycles at 95°C for 20 s, 63°C for 45 s and 72°C for 45 s, and a final extension at 72°C for 5 min. In order to genotype the CYP2C9*3 polymorphism four primers were combined in one tetra-primer PCR assay. These were: CYP2C9E7-F (0.2 µM), CYP2C9E7-R (0.2 µM), CYP2C9E7A-F (0.2 µM) and CYP2C9E7C-R (0.2 µM) (Genomed, Warszawa, Poland. The other reaction mixture ingredients were identical to the ones applied for CYP2C9*2. The following thermal conditions were used: 95°C for 2 min for initial denaturation, 10 cycles at 95°C for 20 s, 59°C for 45 s and 72°C for 45 s, 20 cycles at 95°C for 20 s, 57°C for 45 s and 72°C for 45 s, and a final extension at 72°C for 5 min (12, 13). Primer sequences are listed in Table 1. The PCR products were separated by polyacrylamide gel electrophoresis and visualized by silver staining. The length of the ASA-PCR products were established based on the comparison with marker DNA with the use of BAS SYS 1D program (Biotec Fisher, Perth, Australia).

Table 1. Primers used for ASA-PCR.

Target	Primer	Sequence 5'→ 3'
CYP2C9*2	CYP2C9E3-F	AATAGTAACTTCGTTTGCTGTTATCTC
	CYP2C9E3-R	CAGTAGAGAAGATAGTAGTCCAGTAAGGT
	CYP2C9E3T-F	GGAAGAGGAGCATTGAGGACT
	CYP2C9E3C-R	GGGCTTCCTCTTGAACACG
CYP2C9*3	CYP2C9E7-F	GCCATTTTTCTCCTTTTCCAT
	CYP2C9E7-R	GGAGAACACACACTGCCAGA
	CYP2C9E7A-F	GCACGAGGTCCAGAGATACA
	CYP2C9E7C-R	TGGTGGGGAGAAGGTCAAG

All data were analyzed using the Statistica version 10 software. Differences in the distribution of all analyzed CYP genotypes were performed with χ^2 test. Differences were considered statistically significant at p value ≤ 0.05.

RESULTS

Amplification of CYP2C9*2 with the use of the external forward and external reverse primers yielded a 581 bp PCR product, amplification with CYP2C9E3T-F and external reverse primers indicating the presence of a T allele yielded a 127 bp product, amplification with CYP2C9E3C-R and external forward primers, indicating the presence of a C allele, yielded a 493 bp product. Amplification of CYP2C9*3 with the use of external forward and external reverse primers yielded a 434 bp product, amplification with CYP2C9E7A-F and external reverse primers, presenting the A allele, yielded a 295 bp product, amplification with CY2C9E7C-R and external forward primers presenting the C allele, yielded a 177 bp product.

Genotyping of CYP2C9*2 revealed that 2 persons were homozygous for the defective CYP2C19*2 allele, heterozygosity for CYP2C19*2 was found in 17 individuals. For the CYP2C9*3 polymorphism, 10 of 104 individuals carried the CYP2C9*3 allele (one of them was homozygous, 9 showing heterozygosity).

The allelic frequencies and final genotype distribution in investigated group of patients are shown in Table 2. Statistical analysis did not reveal any significant statistical differences between observed in European population and predicted genotype frequencies, however, the study group is too small to evaluate the frequency of CYP2C9 *2 and * 3 alleles in the Polish population. In statistical analysis, the British frequency of CYP2C9*2 and *3 alleles was used (14).

DISCUSSION AND CONCLUSION

CYP2C9 exhibits a genetic polymorphism and to date more than 35 allelic variants have been described. The two most common allelic variants are CYP2C9*2 and CYP2C9*3 which occur in approximately 85% of poor metabolizers and their frequencies are reported to range 10% to 14% and 6 to 7%, respectively, for Caucasian population, but are relatively rare in Asian and African populations (15). Both of these polymorphisms lead to reduced activity of CYP2C9 which can influence on the therapy with S-warfarin and losartan, the drugs commonly prescribed for long QT patients.

Warfarin is a natural product and given as racemic mixture of the R and S stereoisomers of the drug. S-warfarin is 3–5 times more potent an inhibitor of the vitamin K epoxide reductase complex, the target of action, than R-warfarin. The stereoisomers are metabolized by different phase 1 enzymes; the predominant metabolism of the S isomer is *via* CYP2C9 whereas metabolism of R-warfarin is mainly *via* CYP3A4 with involvement of CYP1A1, CYP1A2, CYP2C8, CYP2C9, CYP2C18 and CYP2C19 (16). Both CYP2C9*2 and CYP2C*3 cause a reduction in S-warfarin clearance with 10-fold variation observed from the genotype with the highest (CYP2C9*1/*1) to the one with the lowest (CYP2C9*3/*3) activity (*1/*1 > *1/*2 >*1/*3 > *2/*2 > *2/*3 > *3/*3). The effect of CYP2C9*3/*3 genotype is the most severe one with clearance of S-warfarin being 10% of the wild type genotype (17). According to the clinical studies, individuals with CYP2C9*1/*2 and *1/*3 genotypes require 10–20% and 20–50% lower average maintenance doses of warfarin, respectively, compared to wild type individuals. The CYP2C9*2 allele seems to have less influence on the dose of acenocoumarol or phenprocoumon compared to warfarin. It is important to identify the subjects having both polymor-

Table 2. Number and frequencies of variant alleles of CYP2C9*2 and CYP2C9*3 in a cohort of long QT patients.

Genotype	Number of individuals	Observed frequency (%)	Frequency in European population (%)
CYP2C9*1/*1	75	72	80.9
CYP2C9*1/*2	17	16	19.1
CYP2C9*2/*2	2	2	0.2
CYP2C9*1/*3	9	9	8.5
CYP2C9*3/*3	1	1	0.05

phisms as poor metabolizers because of them having the highest susceptibility to severe and life-threatening episodes when treated with anticoagulants. Numerous guidelines have been published on the use of pharmacogenomic test in dosing of S-warfarin, two algorithms are recommended that both provide reliable and very similar results (18).

Losartan is a selective angiotensin II receptor antagonist used in the treatment of hypertension in long QT patients. After oral administration, approximately 14% of the losartan dose is converted to E-3174. The E-3174 metabolite is at least 10-fold more potent than losartan. *In vitro* and *in vivo* studies have demonstrated that losartan is metabolized by CYP3A4 and CYP2C9 to E-3174 (19). Oxidation of losartan was significantly reduced in liver microsomes from individuals homozygous for the CYP2C9*3 allele or homozygous for the CYP2C9*2 allele compared with CYP2C9*1 (approximately 30- and 4-fold, respectively). Individuals with slow CYP2C9 metabolism showed an impaired therapeutic response to losartan, but still there is no algorithm of pharmacogenomic dosing of this drug (20, 21).

The presented assay allow rapid genotyping of CYP2C9 alleles and decrease the chance of contamination intrinsic to the generally used two-step procedures. The method used in the study is very simple and provides results of genotyping theoretically within 3 h. To increase the efficiency and facilitate genotyping, a real-time PCR method with fluorometric melting point analysis of PCR product was developed. The real-time PCR method produced 100% reliable results as confirmed by sequencing, however the costs of genotyping with use the hybridization probe are higher than the costs for conventional PCR genotyping. Method presented in this paper is more time-consuming but less expensive than sequencing and real-time PCR assays (22).

Molecular genetic analysis constitutes a determinative tool for the definitive validation of the right dose of the drug to the right patient. Genotyping for CYP2C9 allelic variants can be carried out rapidly and at low cost by ASA-PCR based assays. When genotyping is to be performed in all patients before starting treatment with losartan or S-warfarin, drugs often used in some of long QT patients, it should be adjusted in the high-risk individuals at the initiation of treatment. This procedure, together with serum drug concentration monitoring, will facilitate and accelerate a proper drug dosing, with a lower risk of intoxications, which contributes to the individualization and optimalization of pharmacotherapy patients with long QT syndrome.

REFERENCES

1. Samer C., Lorenzini K., Rollason V., Daali Y., Desmeules J.: Mol. Diagn. Ther. 17, 165, (2013).
2. Yip V., Pirmohamed M.: Am. J. Cardiovasc. Drugs 151, 13 (2013).
3. Eby C.: Semin. Thromb. Hemost. 661, 38 (2012).
4. Flachsbart F., Ufer M., Kleindorp R., Nikolaus S., Schreiber .S, Nebel A.: J. Gerontol. A Biol. Sci. Med. Sci. 66, 1186 (2011).
5. Hiratsuka M.: Drug Metab. Pharmacokinet. 27, 68 (2012).
6. Priori S., Cerrone M.: Ital. Heart J. 6, 241 (2001).
7. Goldenberg I., Zaręba W., Moss A.: Curr. Probl. Cardiol. 33, 629 (2008).
8. Visscher H., Amstutz U., Sistonen J., Ross C., Hayden M., Carleton B.: J. Cardiovasc. Pharmacol. 58, 228 (2011).
9. Hamberg A., Wadelius M.: Pharmacogenomics 15, 361 (2014).
10. Bae J., Choi C., Lee H., Lee Y., Jang C., Lee S.: Int. J. Clin. Pharmacol. Ther. 50, 683 (2012).
11. Feichao X., Caiping M., Yali H., Can R., Zhice X., Lubo Z.: Curr. Med. Chem. 16, 3841 (2009).
12. Spohn G., Geisen C., Luxembourg B., Sittinger K., Seifried E., Bonig H.: Mol. Diagn. Ther. 13, 15 (2011).
13. Arvanitidis K., Ragia G., Iordanidou M., Kyriaki S., Xanthi A., Tavridou A., Manolopoulos V.: Fundam. Clin. Pharmacol. 21, 419 (2007).
14. Xie H., Prasad H., Kim R., Stein M.: Adv. Drug Deliv. Rev. 54, 1257 (2002).
15. Smires F., Habbal R., Moreau C., Assaidi A., Loriot M., Nadifi S.: Pathol. Biol. 61, 88 (2013).
16. Rettie A., Korzekwa K., Kunze K., Lawrence R., Eddy A., Aoyama T., Gelboin H. et al.: Chem. Res. Toxicol. 5, 1 (1992).
17. Daly A.K.: Arch. Toxicol. 87, 407 (2013).
18. Cavallari L., Kittles R., Perera M.: N. Eng. J. Med. 18, 1763 (2014).
19. Stearns R., Chakravarty P., Chen R., Chiu S.: Drug Metab. Dispos. 2, 207 (1995).
20. Yasar U., Forslund-Bergengren C., Tybring G., Dorado P., Llerena A., Sjöqvist F., Eliasson E., Dahl M.: Clin. Pharmacol. Ther. 71, 89 (2002).
21. Hatta F., Teh L., Helldén A., Hellgren K., Roh H., Salleh M., Aklillu E., Bertilsson L.: Eur. J. Clin. Pharmacol. 68, 1033 (2012).
22. Kim S., Lee H., Lee W., Um T., Cho C., Chun S., Min W.: Thromb. Res. 130, 104 (2012).