The meaning of the CYP2D6 gene polymorphism

At the end of the 70’s, it was shown that hydroxylation of debrisoquine and sparteine – anti-hypertensive and antiarrhythmic drugs, is catalyzed by polymorphic cytochrome P450 isoenzyme CYP2D6 (1–6). Several dozen mutations of CYP2D6 gene have been identified, but only a few have a significant effect on CYP2D6 protein structure, expression, or activity in Caucasian population (7). CYP2D6 alleles marked: *3A, *3B, *4A, *4B, *4C, *4D, *4E, *4F, *4G, *4H, *4I, *4K, *4L, *4M, *4N, *4X2 and *5 are totally inactive. Alleles *3 are the effect of a frame-shift mutations, alleles *4 – large splicing error, and *5 allele is a result of large deletion (1, 8). In Caucasian populations they occur with frequencies of 2, 22, and 4 percent, respectively. Their presence in a homozygous configuration means a lack of CYP2D6 activity and occurrence of the PM (poor metabolizer) phenotype. At the other end of scale, there is the UM (ultra-rapid metabolizer) phenotype arising from duplication or even multiplication of the number of CYP2D6 copies. The effect of copy number on nortriptyline metabolism was determined in the way of simulation of pharmacokinetic parameters basing on a single dose. It was found that with the dose of 3 × 25 mg per day, after 12 days, only the patients having no functional copy of CYP2D6 or just one copy were showing levels of the drug fitting the therapeutical window (that is 200–600 nM in blood plasma). Patients having 2 or more functional copies did not reach the lower level of the therapeutical window. With the usually ordered dose of nortriptyline 3 × 50 mg per day, patients with no or one functional copy of the gene...
showed their serum levels of the drug above the upper threshold before the 4th day of the simulated therapy. Patients with 2 functional copies (about half of the Caucasian population) were, after 12 days, in the middle of the therapeutic window. Patients with more copies may require doses of 3 x 75 mg per day, while patient with 13 copies of the CYP2D6 gene was showing the concentration of nortriptyline below the lowest therapeutical level even after 12 days of the simulated therapy (1).

The effect of the genetic polymorphism of drug metabolizing enzymes known as poor metabolizer may manifest itself as an accumulation of the drug or its metabolites caused by disturbed metabolism or elimination process. Therefore, the drug dose should be appropriately adjusted in order to obtain the assumed pharmacokinetic parameters of the drug and its metabolites (8). Rodents (mice) have multiple copies of CYP2D6, which remain in relation to their feeding habits as they consume significant amount of foods containing plant toxins (alkaloids), for which CYP2D6 shows a strong affinity. It can be assumed that the frequent appearance of multiple CYP2D6 copies in some human populations (e.g., north-eastern part of Africa), was in connection to the rapid increment in population which took place 20 to 10 years ago, and as a consequence, people were impelled to make a more extensive use of plants as a food source. Therefore, this evolutionary process ensured a safer use of such foods (9).

Clinical effects of the CYP2D6 genetic polymorphism

It is estimated that CYP2D6 metabolizes from 20 to 25 per cent of all clinically applicable drugs (6, 9). Patients with multiple copies of the CYP2D6 gene are ultra-rapid metabolizers (UMs), therefore, they don’t reach the therapeutic levels with the normal dosing regime. On the other hand, the patients devoid of a functional CYP2D6 are poor metabolizers (PMs), a group at high risk of drug side effects. Determination of the CYP2D6 genotype makes it possible to predict the clearance of antidepressants, such as desipramine, fluvoxamine, mexiteline, mianserine, nortriptyline and paroxetine, or neuroleptics, such as perphenazine and zuclopenthixol, as well as the muscarine receptor antagonists like tolterodine (9, 10).

It was found, after examination of 100 psychiatric patients, that the medication cost of the UM and PM genotype patients exceeds by 4 to 6 thousand dollars the expenses spent on the other patients (11). Time of treatment was the longest in the PM patients group. On the other hand, the group of patients not responding to treatment contains a significant number of the UM genotype patients. It is estimated that in Europe 40 to 60 million of people may have multiple copies of CYP2D6. This could explain frequently found lack of response to the antidepressive treatment in European Caucasians (9). Participation of CYP2D6 isoenzyme in biotransformation of some anti-anginal agents has also been noted. Monohydroxylation of perhexiline is mainly catalyzed by this CYP isoenzyme and is almost 100 times less efficient in PM than in EM subjects. The excessive plasma perhexiline concentrations are hepatotoxic and may cause peripheral neuropathies. It was found that symptoms of those perhexiline side effects in CYP2D6 PMs was 5 times more frequent, than in the control group (EMs) (12, 13).

Genetic mechanisms for CYP2D6 gene duplication

Differentiation of CYP2D6 at the genetic level is related to evolutionary process, in which phenomena like gene conversions, multiplications or other kind of mutations took place. Gene duplication may
occur as an effect of unequal crossing-over, chromosomal retroposition or genomic duplication. Unequal crossing-over causes tandem duplications situated in one chromosome. Depending on position of the crossing-over, duplication may involve whole gene or its part. It also may include several genes with their introns (14). Duplication of \textit{CYP2D6} is most likely caused by unequal crossing-over, during which a non-reciprocal exchange of genetic material takes place, with the result that a certain region is deleted from one chromosome and the other chromosome gets this region duplicated (Fig. 1). The mechanism of this phenomenon is still not fully elucidated (15, 16).

\textit{CYP2D6} gene is located on a longer arm of chromosome 22 (locus 22q13.1) (7). This locus is not conservative showing considerable degree of instability leading to rearrangements, although in most cases it contains one \textit{CYP2D6} gene and two pseudo genes: \textit{CYP2D7P} and \textit{CYP2D8P}. This region contains hot spots promoting unequal crossing-over, which in turn results in the presence of tandem repeats of multiple \textit{CYP2D6} copies (17, 18).

**EXPERIMENTAL AND RESULTS**

**Long-PCR method for detection of \textit{CYP2D6} gene duplication**

Long-PCR is a specialized PCR variant for amplification of DNA regions longer than 5 kb. It finds application for amplification of bigger genes and gene fragments. It differs from the traditional PCR having a modified temperature and time profile of the reaction, such as extended duration of denaturation and elongation procedures. Long-PCR uses slightly higher Mg\(^{2+}\), dNTP and primer concentrations. General guidelines for successful long-PCR include application of two polymerase mixture, one of which has a 3'-5' proofreading exonuclease activity and the other one is exclusively a 5'-3' synthesizing (non-proofreading) enzyme. Both should have a "Hot Start" function and pH optimum of 8.7. Primers should be 20–23 nucleotides long (19).

For detection of \textit{CYP2D6} duplication, the following primers were used: forward 5'-TCACCC-CACTGACCCAACTCT-3' and reverse 5'-CAGTGTCAGGGCACCTAGAT-3' (Oligo.pl, Poland). They allow to obtain a 5.2 kb product as a positive internal control in every sample, and 3.6 kb product in case of patient with \textit{CYP2D6} duplication. The first of the products (5.2 kb) is derived from an intergenic region between \textit{CYP2D6} gene and \textit{CYP2D7P} pseudogene, and the second (3.6 kb) from the region between the duplicated \textit{CYP2D6} genes (17).

**Optimization of long-PCR program**

Long-PCR method was optimized in a series of experiments with varying: time spans of initial denaturation, annealing time and temperature, number of cycles, Mg\(^{2+}\) concentrations, the amount of polymerases and DNA. DNA was isolated from venous blood using Puregene Kit (Gentra Systems, USA). Long-PCR reagents and its optimized concentrations are given in Table 1. Amplifications was carried out in a gradient thermocycler (Eppendorf, Germany).

Optimal long-PCR program for detection of \textit{CYP2D6} gene duplication was described in Table 2. The long-PCR products were analyzed by 2.0% agarose (Qiagen, Germany) gel electrophoresis (BioRad, USA). Gel inspection and image analysis

---

**Figure 2.** Long-PCR products: 5.2 kb – positive internal control; 3.6 kb – \textit{CYP2D6} duplication
was carried out using digital gel imaging and documentation system (Kodak, USA). Figure 3 shows an exemplary electropherogram.

**CONCLUSION**

The contemporary medicine aspires to a state where the treatment could be fitted to the patient’s individual metabolic capacity. The possibility to evaluate the pharmacogenetic profile of patients together with the increasing knowledge about the mechanisms of inhibition, repression and also induction of enzymes participating in biotransformation of xenobiotics and endogenous compounds creates increasing possibilities of elaborating optimal individualized pharmaetherapeutic strategies.

---

**Table 1. Optimized concentrations of mixture components.**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Tube 50 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Polymerase 2.5 U/µl (Perpetual OptiTaq DNA Polymerase, EURx, Poland)</td>
<td>1 U</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>AmpliBuffer C 10x (Roboklon, Germany/EURx, Poland)</td>
<td>–</td>
<td>5.0 µL</td>
</tr>
<tr>
<td>dNTP 100 mM (EURx, Poland)</td>
<td>2 mM</td>
<td>1.0 µL</td>
</tr>
<tr>
<td>Primer f 100 µM (Oligo.pl, Poland)</td>
<td>0.3 µM</td>
<td>0.15 µL</td>
</tr>
<tr>
<td>Primer r 100 µM (Oligo.pl, Poland)</td>
<td>0.3 µM</td>
<td>0.15 µL</td>
</tr>
<tr>
<td>DNA</td>
<td>260 ng</td>
<td>5 μL</td>
</tr>
<tr>
<td>Mg²⁺ 50 mM (EURx, Poland)</td>
<td>3.0 mM</td>
<td>1.25 µL</td>
</tr>
<tr>
<td>H₂O</td>
<td>–</td>
<td>37.05 µL</td>
</tr>
</tbody>
</table>

| Table 2. Optimal long-PCR program for detection of CYP2D6 gene duplication. |
|-----------------------------|-----------------|------------|
| Steps                      | Temperature (°C) | Time (min) |
| Initial denaturation       | 94              | 10         |
| Denaturation               | 94              | 1          |
| Annealing                  | 68              | 0.5        |
| Elongation                 | 72              | 6          |
| Final elongation           | 72              | 10         |

---

Figure 3. Exemplary electropherogram: bands obtained for the subject with duplicated CYP2D6 gene (1) and the subject without duplication (2)
using the knowledge about individual specificities in the scope of reaction to the drug and about interactions occurring between the administered drugs, their metabolites, food components and environmental toxins, under conditions of natural variation of the physiological and pathological state of the organism (7). It is suggested that pharmacogenetic tests would be useful, especially in patients treated with drugs with narrow therapeutic index and strong side effects.

Patients with duplication or multiplication of CYP2D6 are ultra-rapid metabolizers and require the use of a higher than standard doses of many drugs. In this work, we have tried to optimize the PCR method useful for screening of the patients with duplicated CYP2D6 gene, finding the procedure capable of detecting the duplications. On the other hand, we have found that procedure is cumbersome and time-consuming, which makes it hardly applicable in large-scale pharmacogenetic studies and diagnostic programs. Therefore, although the research undertaken seems to be justified, a new generation methods, like RT-PCR, promise significantly quicker procedures.

REFERENCES


Received: 6. 08. 2009