Genetic Polymorphism of Cytochrome P450 and Methods for its Determination

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Abstract: The majority of human P450 dependent drug metabolism is carried out by polymorphic enzymes which can alter plasma concentration of the pharmacological active substance followed by an enhanced or suppressed pharmacological effect. The response of individual patients to drugs can be affected by variations in DNA sequence mainly by single nucleotide polymorphisms (SNPs). Knowledge of functionally important SNPs prior to the drug administrations may assist in the development of individualized pharmacotherapy avoiding unexpected drug responses, such as harmful adverse drug reactions or treatment failures. This review discusses both the basic characteristics of the major polymorphic cytochrome P450 enzymes and examines the pharmacogenetic methods employed to estimate metabolic status. We will focus mainly on the basic principles of genotyping assays involving molecular biology tools.

Cytochrome P450 represent an important family of enzymes involved in the metabolism of xenobiotics and drugs. Substantial inter-individual variability in the activity of CYP and other drug-metabolizing enzymes (DMEs) is frequently caused by polymorphism of genes coding individual proteins. Genetically determined rates of drug metabolism and thus elimination can, for many drugs, lead to variable and unpredictable responses, presenting clinically as insufficient efficacy or the manifestation of toxic adverse drug reactions in predisposed individuals [1]. Genetic polymorphisms are therefore nowadays extensively studied with respect to their clinical relevance.

In the early 1980s, the first P450 enzyme was purified to homogeneity, and, since this time, the recognition of the role in metabolism, genetic variability, its clinical relevance and other features of CYPs have rapidly developed. Over the years, diverse CYP terminology was used, but currently a standardised nomenclature is applied for all human CYPs (http:/drnelson.utmem.edu/CytochromeP450) and original trivial terms are used particularly for bacterial CYPs. The nomenclature of human CYPs is based on similarities in amino acids sequence. The CYP gene superfamily is divided into families designated by Arabic numerals following the abbreviation CYP (e.g. CYP2). There is at least 40% identity in amino acid sequence among the individual proteins within a family. Subfamilies are marked by a subsequent letter dividing groups of proteins with at least 55% similarity in primary structure (e.g. CYP2D). Individual enzymes are marked by a final Arabic numeral to complete the designation (e.g. CYP2D6). Sequence variation between two enzymes should be at least 3%.

Biochemically, CYPs belong to monooxygenase enzymes with systematic number E.C. 1.14.14.1. The principle of CYP mediated reactions is the enzyme ability to bind and to activate molecular oxygen to the heme iron in the centre of its structure. Then, a single atom of oxygen forms a molecule of water and the other one is activated and later introduced into the molecule of the substrate. The

complete catalytic cycle of CYP enzymes involves a multicomponent electron transfer chain, which is markedly more complicated.

CYP enzymes belong to the Phase I of metabolism, in which xenobiotics are subject to oxidation, reduction, or hydrolysis. This can be followed by other transformations, the Phase II reactions, which lead to more hydrophilic metabolites such as glucuronides as one of the many examples with increased solubility and facilitated elimination.

Cytochrome P450 is a major system of DMEs that contributes to biotransformation of endogenous as well as exogenous compounds. Generally it is possible to distinguish two groups of the CYP enzymes. The one covers families CYP 5–51 which are of great importance in biotransformation of endogenous substrates e.g. formation of steroid hormones and other low-molecular weight regulators of biological functions [2, 3]. The next groups, families CYP 1–3, play a role in the metabolism of such xenobiotics as drugs, food constituents, carcinogens, and pro-carcinogens, which can be either deactivated or activated by CYP metabolism. CYP4 family occupies an intermediate position in this classification, as determined by its substrate specificity.

Generally, the CYPs have broad substrate specificity. Frequently, two or more enzymes can catalyze the same type of reaction, indicating redundant and broad substrate specificity that allows alternative metabolic pathways in case where an enzyme is lacking due to genetic polymorphism, or is inhibited and has a decreased enzyme activity. This substrate overlap is considered to be an advantage in the metabolism of drugs, especially for poor metabolizers. However, the alternative metabolic pathway may not fully compensate the original pathway.

Genetically determined variability in CYP activity is caused mainly by single nucleotide polymorphisms. Generally, genetic polymorphism is defined as a deviation in DNA sequence with an allele frequency of at least 1%. Therefore, heterozygotes for such alleles occur with a frequency of at least 2%. Basically, there are three different types of mutation involving SNPs – substitution (exchange), deletion (loss) and insertion (addition). These small deviations of the genotype can occur in introns as well as in exons. If the polymorphism appears in introns it can change the extent of gene expression or conceivably a multiplication of alleles. When the polymorphism is observed in exons, this can alter the function of the protein product.

A substitution may alter a codon so that a wrong amino acid is present at this site. It either has no effect on the reading frame (missense mutation) or the variant nucleotide base pair sequence is translated into a stop codon.

Deletion or insertion causes a shift of the reading frame (frameshift mutation). Thus the sequence that follows no longer codes for a functional gene product (nonsense mutation).

Although single nucleotide polymorphisms occur frequently throughout the genome, only part of them manifests functional significance.

In general, according to the genotype, four major CYP phenotypes could be expected. With respect to the metabolic capacity of the enzyme, two of the four groups are found at the fringes of the population. Poor metabolizers (PM) pronounced decrease enzyme activity, being homozygous for either functionally variant alleles or having a complete deletion of the gene. Ultrarapid metabolisers (UM) are the opposite extreme of the possessing gene duplications or multiplications. Metabolic dissimilarities between intermediate metabololizers (IM), who are heterozygous for specific variant alleles, and extensive metabolisers (EM) who have two functionally competent alleles, are of clinical relevance only with respect to the treatment by specific and poorly tolerated drugs.

Genotyping or phenotyping can be used to determine the polymorphisms of CYP activity. Genotyping is based on relatively simple molecular biology methods that have several practical benefits in comparison to phenotyping.

Polymerase chain reaction (PCR) based analysis is most commonly used to detect genotypes. Usually, restriction fragment length polymorphism (RFLP), a single strand conformation polymorphism (SSCP) or allele specific and sequence specific PCR analysis methods are applied subsequently. The PCR allows massive exponential amplification of the target sequence in DNA followed in RFLP by a digestion using a specific restriction enzyme that distinguishes between the wild type and variant sequence differing by only one nucleotide. The restriction fragments are analyzed by gel electrophoresis on either agarose or polyacrylamide gels, depending on the size differences among the fragments. Agarose gels can be used to separate fragments with difference greater than 40bp [4].

The principle of SSCP method is based on molecular size- and shape-dependent electrophoretic mobility of nucleic acids in a non-denaturing gel. Unlike double-strand DNA, single-strand DNA is flexible and adopts a conformation determined by intramolecular interactions that uniquely depend on the sequence composition. Conformational changes induced by even one single base substitution, can be detected as a variation in electrophoretic mobility of the single-strand DNA in non-denaturising polyacrylamide gels.

Many of the probe-based systems, as another specific detection method used for allelic discrimination, rely on the principle of fluorescence resonance energy transfer (FRET) for signal generation. Variably designed probes are able to detect SNPs within an amplified PCR product using the variability in level of energy needed to dissociate bonds between base pairs in double stranded DNA fragment of the amplified template. If there is no mismatch – a probe is completely complementary to the wild-type DNA strand. Then the probe melts off at characteristic temperature that is higher than in presence of a single nucleotide polymorphism. A single base mismatch under the probe decreases the Tm by as little as 3°C for G–T and as much as 10°C for A–C substitutions [5] as a specific example for the TagMan probe. Melting temperatures are monitored in real time by decline of fluorescent signal at the time of loosing the probes.

Mini/sequencing can be another approach to analyse point mutation (SNP). Minisequencing is based on annealing of 3' end primer with one marked nucleotide that is annealed only in case of complementary to template oligonucleotides.

DNA chips and pyrosequencing are modified methods of the minisequncing.

Pyrosequencing is [6] a method based on sequencing by synthesis, when the repeated incorporation of dNTP is accompanied by a release of proportional amount of pyrophosphate that immediately emits visible light from luciferase

Table 1 – Major drug substrates for CYP2C19, CYP2C19 and CYP2D6

CYP2C9	CYP2C19	CYP2D6	
Amitriptyline	Amitriptyline	Ajmaline	Hydrocodone
Antipyrine	Carisoprodole	Alprenolol	Imipramine
Candesartan	Carisoprodol	Amiflamine	Indoramin
Carbamazepine	Citalopram	Amitriptyline	Lidocaine
Celecoxib	Clomipramine	Amphetamine	Maprotilin
Diclofenac	Clozapine	Aprindine	Methoxyamphetamine
Dronabinol (THC)	Cyclophosphamide	Aripiprazole	Methoxyphenamine
Fluoxetine	Diazepam	Atomoxetine	Metiamide
Flurbiprofen	Fluoxamine	Bufuralol	Metoclopramide
Fluvastatin	Fluoxetin	Bupranolol	Mexiletine
Glibenclamide	Hexobarbital	Captopril	Mianserin
Glimepride	Imipramine	Carvediol	Minaprime
Glimpiride	Indometacin	Cinnarizine	Nortriptyline
Glipizide	Isoniazid	Citalopram	Ondasetron
Glipizide	Lansoprazole	Chlorpheniramine	Otycodone
Glyburide	S-mephenytoin	Chlorpromazine	Paroxetine
Ibuprofen	Mianserin	Clomipramine	Perhexiline
Indometacin	Moclobemide	Clozapine	Perphenazine
Irbesartan	Nelfinavir	Codeine	Phenacetin
Losartan	Nilutamide	Debrisoquine	Phenformin
Meloxicam	Omeprazole	Deprenyl	Propafenone
S-Naproxen	Pantoprazole	Desipramine	Propranolol
Nateglinide	Phenobarbital	Desmethylcitalopram	Quinidine
Phenytoin	Phenobarbitole	Dexfenfluramine	Risperidone
Piroxicam	Phenytoin	Dextromethorphan	S-metoprolol
Rosiglitazone	Primidone	Dihydrocodeine	Sparteine
Sulfonyureas	Progesterone	Encainide	Tamoxifen
Suprofen	Propranolol	Ethylmorphine	Thioridazine
Tamoxifen	Proquanil	Flecainide	Timolol
Tolbutamide	R- mephobarbital	Flunarizine	Tomoxetine
Tolbutamide	Rifampicin	Fluoxamine	Tramadol
Torsemide	Teniposide	Fluoxetine	Trifluperidol
S-Warfarin	Trimipramine	Fluperlapine	Trimepranol
	R-warfarin (8-OH)	Fluphenazine	Tropisetron
		Galanthamine	Venlafaxine
		Haloperidol	Zuclopenthixol

reaction. Nucleotide sequence is determined from the signal peak in the pyrogram in real-time and the divergences in sequence are immediately visible.

To facilitate and automate screening for clinically relevant CYP genes new technologies are emerging. These technologies are able to detect many SNPs during one reaction. With the exception of custom-designed gene chips, the commercial test is available and certified as a diagnostic chip for variations in two genes, CYP2D6 and CYP2C19. A DNA chip is a small piece of silicon glass ($\sim$ 1 cm^2) to which a large number of synthetic, single-stranded DNA oligonucleotides (oligos) have been chemically bonded. Oligos function as DNA probes: they anneal selectively only to those DNA molecules whose nucleotide sequences are exactly complementary. They can therefore be used to identify the presence of specific DNA sequences in a heterogeneous mixture of genes.

Another approach that was mentioned – the phenotyping – involves either methods with administration of probe drug that is metabolized via examined metabolic pathway with subsequent measurement of drug/metabolite concentration ratio in blood, urine or saliva or methods without administration of probe drugs e.g. not so widely used direct measurement of enzyme activity or possibly measurement of protein levels.

Phenotyping is a more accurate method to determine enzyme activity but the risk issue of adverse drug reaction is a limiting step in general analyzes of phenotype for the patients although low risk probe drugs are used. Reluctance in compliance accompanies these assays, and the results are modified by non-identified factors such as intraindividual variations extending from enzyme inhibition to induction, diet or concurrent disease, and variant levels of endogenous factors. Finally, by phenotyping we determine actual enzyme activity, by genotyping we describe genetic predisposition.

Genetic predisposition allows to assign patients into one of the phenotyping groups (PM, UM, IM, EM). Despite the methodological progress in genotyping it is necessary to be aware of forecasting the real enzyme activity based on gene structure without any direct information about the real phenotype. Existence of unknown functional SNPs could lead to a misclassification of the subject with regard to enzyme activity. Therefore indirect determination and expense of analysis are the major disadvantages of genotyping. However, indisputable advantages of genotyping exist. It is not so time-consuming analysis is not altered by co-administration of another drug, environmental or constitutional factors; intraindividual variability is not an issue and whether the phenotype is closely linked to genotype, it is used preferentially and successfully in the most of the pharmacogenetics assays.

Furthermore, genotyping assays are accompanied by acceptable compliance of the patient since only a small volume of whole blood is required for PCR-based assays.

Subsequently, when compared to phenotyping, there are no requirements to adhere to restricted regimens during the test or unload the organism of the probe

drug. Moreover, it is sufficient if it is performed once in the lifetime of the patient.

As a rule, genotyping is faster especially for large populations, and the techniques used are easily adaptable in any molecular biology laboratory.

Three of the P450 enzymes CYP2C9, CYP2C19 and CYP2D6 are consider being clinically most important polymorphic DMEs among I phase metabolism P450 enzymes since they mediated over 40% of all CYP-mediated drug metabolism and all display indispensable incidence of the PM in Caucasian populations. Concurrently about 59% of drugs that are frequently cited in adverse drug reaction studies in the USA are metabolized by at least one polymorphic enzyme of which 86% are P450 substrates [7]. Some non-preventable adverse drug events and therapeutic failures are today thought to depend on individual genetic background of many genes involved in drug disposition and efficacy. These ADRs represent a substantial financial cost in healthcare systems worldwide. For example, in the USA the cost of management of serious ADRs exceeds $100 billion per year.

CYP2C9

CYP2C9 is the principal CYP2C in human liver, where its relative content is about 10% [2]. It catalyzes oxidative metabolism of 10–20% [8] widely prescribed drugs including groups such as oral hypoglycemic agents – sulfonylureas mainly, angiotensin II blockers, some of NSAIDs and others (see more in Table 2). Most of these compounds are weak acids [9] weakly anionic and fairly lipophilic [2]. A tolbutamide oral hypoglycemic agent is used as a probe drug for phenotyping, the metabolic ratio is measured in urine, lornoxicam is tested as an alternative probe drug [10].

Table 2 – Relative allele frequencies of selected cytochrome enzymes

Cytochrome P450 enzymes		Allele frequencies (%)		
		Caucasians	Asians	African Americans
CYP2D6	2×N	2.3	0.9	4
	*3	2–3	0.2	0.2
	*4	12–21	1	2
	*5	2–7	6	4
	*6	1	0	0
	*10	1–2	51	6
	*17	0	0	20–35
	PM	6–10	1	5
CYP2C9	*2	8–14	0	4
	*3	4–16	2–3	2
	PM	14–37	0–1	0–8
CYP2C19	*2	13	23–32	13
	*3	<1	6–10	–
	PM	3–5	12–23	4–18

References: 8, 9, 15–18

More than 50 single nucleotide polymorphisms (SNPs) have been described in the regulatory and coding regions of the CYP2C9 gene, but only two coding variants CYP2C9*2 and CYP2C9*3 have functional consequence to be genotyped among the white population, where the allelic frequencies are detected in the range of 8–14% and 4–16%, respectively (to compare see Table 2). Some other variant alleles CYP2C9*5 (protein variant D360E) CYP2C9*6 (null allele) CYP2C9*8 (protein variantR150H) CYP2C9*11 (protein variant R335W) have been detected in population of African origin only [8]. Another variant CYP2C9*4 (protein variant I359T) has been exclusively identified in Japanese epileptic patients [11]. Major variant alleles of CYP2C9 and its molecular as well as functional consequences are described in Table 3. Clinical consequence of CYP2C9 PM is evident in decreased clearance of hypoglycaemics that may cause severe hypoglycaemia, for example S-warfarin, where bleeding is a known ADR of overdose. Hence homozygous persons require lower warfarin doses to achieve anticoagulation that is accompanied by more frequent inspection of INR (international normalization ratio). However, genetic polymorphism does not modulate the plasma concentration solely. Other factors, such as co-administration of other medication with induction or inhibition effects are also involved.

Table 3 – Molecular and functional consequences of selected cytochrome enzymes

Major variant alleles		Molecular consequence (nucleotide changes)	Protein variant	Functional consequence (enzyme activity)
CYP2C9	*1			Normal activity = wild type
	*2	430C>T	R144C	Decreased activity
	*3	1075A>C	I359L	Decreased activity
CYP2C19	*1			Normal activity = wild type
	*2	681G>A	Defective splicing	Inactive enzyme
	*3	636G>A	Stopcodon	Inactive enzyme
CYP2D6	*1			Normal activity = wild type
	*2X*N*	Gene multiplication	*N* active genes	Increased activity
	*3	2549A>del (2637)	Frameshift	Inactive enzyme
	*4	1846G>A	Defective splicing	Inactive enzyme
	*5	Gene deletion	No enzyme	Inactive enzyme
	*6	1707T>del (1795)	Frameshift	Inactive enzyme
	*10	100C>T (188)	P34S S486T	Unstable enzyme
	*17	1023C>T 2850C>T	T107I R296C S486T	Altered affinity for substrates

CYP2C19

In the 1980s mephenytoin polymorphisms were identified. Later on, additional drugs were added to the list of S-mephenytoin hydroxylase substrates. They should be neutral or weakly basic and moderately lipophilic. They are presented in the following drug classes: proton pump inhibitors, tricyclic antidepressants, barbiturates, beta-blockers, antimalarial drug (proguanil). (see details in Table 1) The CYP2C19 gene is localized on chromosome 10q24.1–3. The two alleles (CYP2C19*2 and CYP2C19*3) are associated with production of truncated proteins, when the absence of activity is a result of amino acids substitution on the position 681 and 636. These deficiencies that lead to poor metabolizers affect approximately 12–23% of Asians, 4–18% of Blacks and about 3–5% of Caucasians (caused mainly by CYP2C19*2) (Tables 2, 3). The hydroxylation of omeprazole is metabolised by CYP2C19. There is approximately a 10-fold difference in the oral clearance of omeprazole in PMs. Hence the cure rates for gastric and duodenal ulcers were significantly higher in PMs when compared to homozygous wild-type subjects [12].

CYP2D6

CYP2D6 is a polypeptide of 497 amino acids; its relative content in the uninduced human liver is about 4% of P450 enzymes [2]. Despite its small percentage accounts, the role of cytochrome P450 2D6 in the drug metabolism is extensively high. CYP2D6 catalyse the oxidative biotransformation of about 25% clinically important drugs. Most of the CYP2D6 substrates are known to be lipophilic bases with a protonable nitrogen atom.

Debrisoquine immediately followed by spartein was the first substrate on which the variable levels of enzyme activity was described and followed by identification of three phenotypes [13]: extensive metabolisers (EM), ultrarapid metabolisers (UM) and poor metabolisers (PM). Approximately 1% of Asians and 6–10% of Caucasians lack CYP2D6 activity (PM phenotype). On the other hand, there are 2% of Asians and 1–7% Caucasians with increased enzyme activity (UM phenotype). Their CYP2D6 gene locus is multiplied, they have one extra copy of the CYP2D6*1 or CYP2D6*2 allele resulting in faster than average rate of the metabolism (Tables 2, 3).

Cytochrome P4502D6 gene is localized on chromosome 22q13.1. It is a highly polymorphic gene locus with more than 80 variant alleles [11]. All variant alleles are presented at the home page of the human CYP allele nomenclature committee (http://www.imm.ki.se/cypalleles/cyp2d6.htm). The optimum amount of genotyping required to accurately predict the phenotype remains in debate. Gaedigk et al. affirm that a test for 2D6*3, 2D6*4, 2D6*5, and*6 identified 97,5% of poor metabolizers [14]. All the CYP2D6 pharmacogenotyping assays are difficult by the presence of two neighbouring pseudogenes CYP2D7, CYP2C8. CYP2D6 major drug substrates are antidepressants (tricyclic antidepressants or serotonin

reuptake inhibitors), neuroleptics, cardiological drugs (beta-blockers, antiarrhytmics), antiemetics and many others (see Table 1).

The knowledge of CYP2D6 polymorphism has several clinical implications. The issue that is often discussed is the recommended reduction in dosage of the antidepressants that may have decreased the incidence of adverse drug reaction prior to the first administration of a psychiatric drug to patients who are poor metabolizers. However, genotyping is still not routine in most healthcare centres.

It is known that the biological response of the individual patients to a specific drug varies considerably from the therapeutic effect through the ineffective drug treatment to adverse drug reaction. Thus the consequences of all these and many other polymorphisms are being examined clinically. To improve the drug treatment maximally we need to individualize a conception to which the pharmacogenetics can help.

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