UNRAVELING OF THE GENETIC POLYMORPHISM FOR ULTRA-RAPID METABOLISM OF PHENYTOIN

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LAPORAN AKHIR PENYELIDIKAN 'UNRAVELING OF THE GENETIC POLYMORPHISM FOR ULTRA-RAPID METABOLISM OF PHENYTOIN'

Merujuk kepada perkara di atas, bersama-sama ini di sertakan 3 (tiga) naskah laporan akhir penyelidikan bertajuk 'Unraveling of The Genetic Polymorphism for Ultra-Rapid Metabolism of Phenytoin'

Sekian, terima kasih.

Yang benar,

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Abstract

The frequency of functionally important mutations and alleles of genes coding for CYP2C9 metabolizing enzymes shows a wide ethnic variation. However, little is known of the frequency distribution of the minor allelic variants that could explain other metabolic capacity.

Methods: Using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) genotyping assays and allele specific PCR, the frequencies of three variants of the cytochrome P450 (CYP) 2C9 were determined in a sample of 93 CYP2C9 *1/*1 Swedish volunteers and 128 CYP2C9 *1/*1 Korean volunteer. The genotyping results were then compared to their Losartan metabolic ratio to find if there is a genotype-phenotype relationship.

Results: Three of the mutations were significantly lower in the Korean population. Mutation in intron 1 + 83 bp occurred in 0.78% (Korean) and 23.11% (Swedes) of alleles while the mutation at exon 2 +73 bp occurred in 0.78% (Korean) and 22.58% (Swedes) of alleles while mutation in intron 6 + 95 bp occurs at 0.78% (Korean) and 27.96% (Swedes) of alleles. The most homogeniously spread mutation ethnically, occurring in intron 8 - 109 bp occurs at 37.11% (Korean) and 31.72% (Swedes) of alleles.

Conclusion: The overview of different allele variation distribution of metabolizing enzymes among two different populations shows interesting results. The gathered information is inconclusive to explain the ultrarapid metabolizer, but it show that there is some ethnically differences that may lead to differences in metabolism capacity.

PENGHARGAAN

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A	Adenine
C	Cytosine
CI	Confidence Interval
CYP2C9	Cytochrome P450 2C9
dNTP	Doeoxynucleoside Triphosphates
G	Guanine
MT	Mutant
PCR	Polymerase Chain Reaction
SNP	Single Neucleotide Polymorphism
SSCP	Single-Stranded Conformational Polymorphism
T	Tyrosine
TBE	Tris Boric EDTA
TEAA	Triethylammoniumamine
TGGE	Temperature Gradient Gel Electrophoresis
Tm	Temperature
WHO	World Health Organization
WT	Wild Type

CHAPTER 1

INTRODUCTION

1.1 Phenytoin

Phenytoin is an anticonvulsant drug used for the treatments of epilepsy. The primary site of action is on the motor cortex to prevent and stop convulsion. Phenytoin (i) promotes sodium efflux from neurons; (ii) stabilises the threshold against hyperexcitability of membrane caused by stimulation or environmental factors; (iii) reduces post-tetanicpotentiation at synapses; and (iv) reduces the maximal activity of brain stem centeres responsible for the tonic phase of tonic-clonic (grand mal) seizures.

The plasma half-life in man after oral administration of phenytoin averages 22 hours, with a range of 7 to 42 hours. Steady state therapeutic levels are achieved at least 7 to 10 days (5 to 7 half-lives) after initiation of therapy with recommended doses of 300 mg/day.

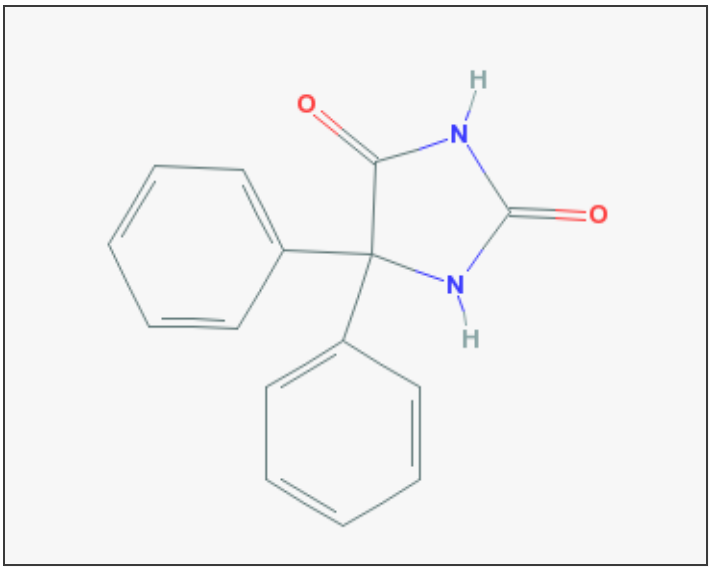

Figure 1: Chemical Structure of Phenytoin. Dilantin (phenytoin) is related to the barbiturates in chemical structure, but has a five-membered ring. The chemical name is 5,5-diphenyl-2,4 imidazolidinedione.

Phenytoin exhibits a non linear pharmacokinetics with narrow therapeutic window (10-20 µg/mL) (Winker and Luer, 1998). Unusually high levels maybe a result from liver disease, congenital enzyme deficiency or concomitant medication interactions which result in metabolic interference. The patient with large variations in phenytoin plasma levels, despite standard doses, presents a difficult clinical problem. Large interindividual differences in the pharmacokinetics and requirement of doses with varying therapeutic effects have also been reported (Taguchi *et. al.*, 2005).

1.2 Drug Metabolism by Cytochrome P450

Foreign chemicals like phenytoin are detoxified in the body by a process called drug metabolism. Drug metabolism is a biochemical modification process of xenobiotics such as warfarin, antidepressants, antiepileptic drugs, and statins by the human body trough a specialized enzymic system. Phenytoin is extensively metabolized in the liver to *para* hydroxylated metabolites by the cytochrome (CYP) P450 enzymes 2C9 and 2C19, and is around 90% bound to serum albumin. The CYP450 enzymes are so named because they are bound to membranes within a cell (cyto) and contain a heme pigment (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide (Lynch & Price, 2007). Cytochrome P450 enzymes metabolise more than 30 different classes of drugs and many endogenous compounds. The CYP450 proteins are categorized into families and sub families by the similarities of their DNA sequences. Up to date, there are more that 2500 of the P450 sequences archived (Nelson, 2003). Humans have 18 families and 45 subfamilies of CYP450 (Nebert and Rusell, 2002; Nelson, 2003). Variations that occur in the genes that encode these enzymes were found to influence their ability to metabolise certain drugs. Many of these drugs share the same enzyme systems for metabolism and thus many drug interactions have resulted from inhibition or induction of these CYP450 enzymes (Table 1). Up to date findings have reported polymorphism of these enzyme with some having inhibited activity while others with multi-duplicated activities. Genetic polymorphisms have been found to be responsible for the varying enzyme

activities observed. Genetic mutations that result in inactive forms of CYP enzymes have been reported to cause toxicities among patients prescribed the specific substrate. This is due to the accumulation of the toxic substrate.

Table 1: Significant Cytochrome P450 Enzymes and Their Inhibitors, Inducers, and Substrates (Lynch & Price, 2007)

Enzyme	*Potent inhibitors*	*Potent inducers*	*Substrates*
CYP1A2	Amiodarone (Cordarone), cimetidine (Tagamet), ciprofloxacin (Cipro), fluvoxamine (Luvox)	Carbamazepine (Tegretol), phenobarbital, rifampin (Rifadin), tobacco	Caffeine, clozapine (Clozaril), theophylline
CYP2C9	Amiodarone, fluconazole (Diflucan), fluoxetine (Prozac), metronidazole (Flagyl), ritonavir (Norvir), trimethoprim/sulfamethoxazole (Bactrim, Septra)	Carbamazepine, phenobarbital, phenytoin (Dilantin), rifampin	Carvedilol (Coreg), celecoxib (Celebrex), glipizide (Glucotrol), ibuprofen (Motrin), irbesartan (Avapro), losartan (Cozaar)
CYP2C19	Fluvoxamine, isoniazid (INH), ritonavir	Carbamazepine, phenytoin, rifampin	Omeprazole (Prilosec), phenobarbital, Phenytoin
CYP2D6	Amiodarone, cimetidine, diphenhydramine (Benadryl), fluoxetine, paroxetine (Paxil), quinidine, ritonavir, terbinafine (Lamisil)	No significant inducers	Amitriptyline, carvedilol, codeine, donepezil (Aricept), haloperidol (Haldol), metoprolol (Lopressor), paroxetine, risperidone (Risperdal), tramadol (Ultram)
CYP3A4 and CYP3A5	Clarithromycin (Biaxin), diltiazem (Cardizem), erythromycin, grapefruit juice, itraconazole (Sporanox), ketoconazole (Nizoral), nefazodone (Serzone), ritonavir, telithromycin (Ketek), verapamil (Calan)	Carbamazepine, *Hypericum perforatum* (St. John's wort), phenobarbital, phenytoin, rifampin	Alprazolam (Xanax), amlodipine (Norvasc), atorvastatin (Lipitor), cyclosporine (Sandimmune), diazepam (Valium), estradiol (Estrace), simvastatin (Zocor), sildenafil (Viagra), verapamil, zolpidem (Ambien)

1.3 Phenytoin Metabolism

Drugs interact with the CYP450 system in several ways because they may be metabolised by only one CYP450 enzyme (eg., metoprolol by CYP2D6) or by multiple enzymes (Daly & King, 2003). Studies associated the pharmacokinetic and dose variations of phenytoin with a variation that occurs in its multiple metabolising enzymes, namely CYP2C9 and CYP2C19 (Figure 2). These variations are known to be caused by polymorphisms of the CYP2C9 and CYP2C19 encoding genes (Lee *et. al.,* 2007). It has been reported that individuals carrying mutant CYP2C9 alleles are likely to develop higher serum concentrations than normal, on account of their impaired metabolism (Aynacioglu *et. al.,* 1999). Besides the inherent genetic differences of the patients that cause different therapeutic outcomes, inhibition of CYP2C9 or CYP2C19 enzymes by concurrent drug use can alter the metabolic clearance of phenytoin.

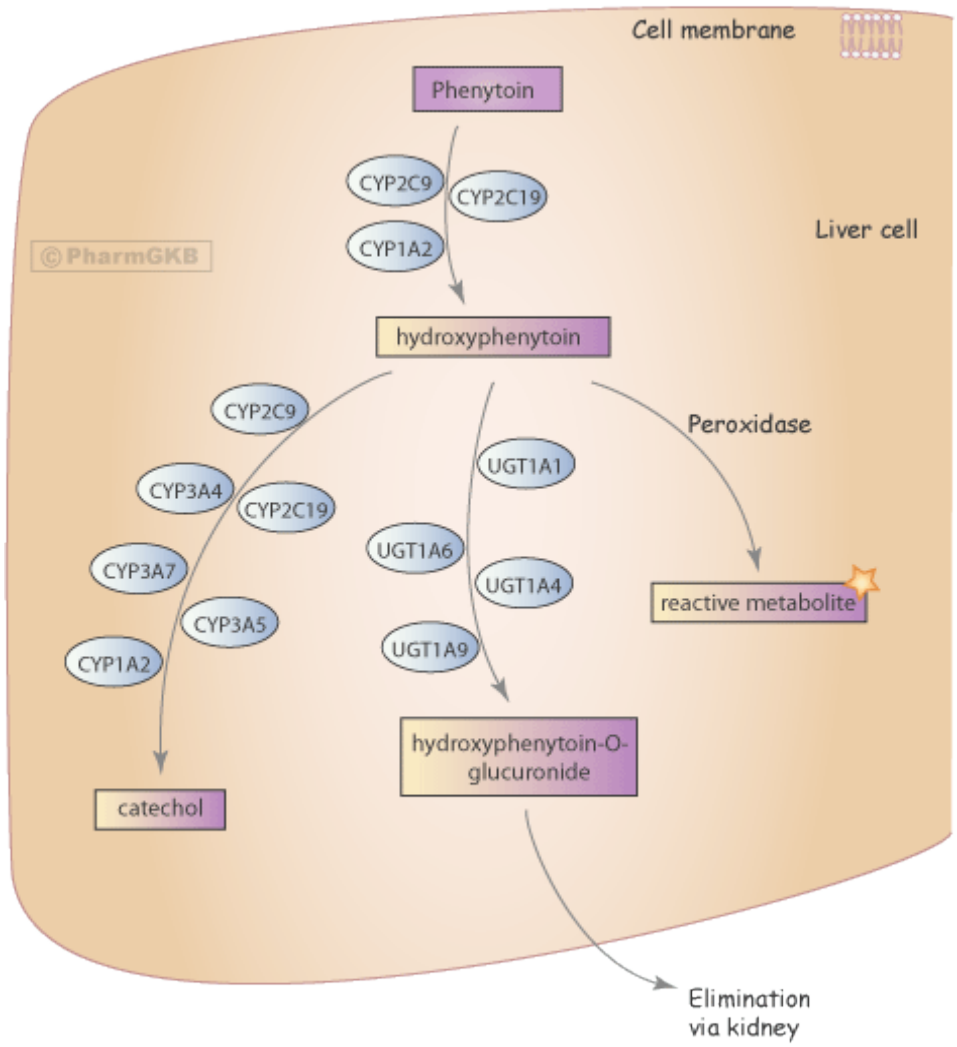

Figure 2: Phenytoin Metabolism Pathways. Phenytoin is metabolized by CYP2C9, CYP2C19 and CYP1A2 enzymes primarily to 5-(*p*-hydroxyphenyl), 5-phenylhydantoin (HPPH), which may be further metabolised to a catechol that spontaneously oxidizes to semiquinone and quinone species that covalently modify proteins. CYP2C9, CYP3A4, CYP2C19, CYP3A7, CYP3A5 and CYP1A2 catalyze HPPH metabolism to catechol, proposed to be the final enzymatic step in phenytoin bioactivation. Hydroxyphenytoin is glucuronidated by UGT1A1/A6/A4/A9 and is excreted as hydroxyphenytoin O-glucuronide. It has been proposed that this glucuronidaton prevents a peroxidase-mediated conversion of hydroxyphenytoin to a toxic reactive metabolite which can oxidize proteins, lipids and DNA (Klein *et. al*., 2001).

1.4 Pharmacogenetics of CYP2C9

Human cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) on chromosome 10 is one of two P450s known to have crystal structure (Williams *et. al*., 2003). The CYP2C9 enzyme catalyses the metabolism for a number of drugs, an example would be phenytoin, an anti-epileptic drug (van derWeide *et. al*., 2000). Genetic composition of *CYP2C9* among different ethnic in different part of the world was reported to be different (Nakai *et. al*., 2005; Tanira *et. al*., 2007). It is fairly important for clinician and pharmacies to recognise the importance of inter-individual differences in the genetic profile and thus pharmacogenetics and personalised drug therapy can be used as an aid tomaximise therapeutic outcomes.

Carriers of *CYP2C9*2* and **3* variant alleles were found to have lower enzyme activity compared with carriers of the wild type *CYP2C9*1* allele (Kirchheiner *et. al*., 2003). Another variant allele, *CYP2C9*4* was found to cause a complete deficient enzyme activity in Afican-American subjects (Kirchheiner *et. al*., 2003). The *CYP2C9*2* allele represents the amino acid exchange of Arg144Cys in exon 3 and *CYP2C9*3* allele represents the amino acid change of Ile359Leu in exon 7 (Scordo *et. al*., 2001; Yasar *et. al*., 2001; Kirchheiner *et. al*., 2004). *CYP2C9*4* represents the amino acid change of Ile359Thr and *CYP2C9**5represents the amino acid change of Asp360Glu (Dickmann *et. al*., 2001). Other variation reported is *CYP2C9*6* variant which is a new null polymorphism carrying a deletion of nucleotide adenine at position 818. This variant results in a premature stop codon and a truncated inactive protein (Lee *et. al*., 2002).

Previous studies showed that the frequencies of *CYP2C9*1* was the most common variant foundamong the Malays and Chinese in Malaysia, the occurrences of *CYP2C9*2* and *CYP2C9*3*were at 6-10% (Ku *et. al*., 2003; Ngow *et. al*., 2009). Some studies show that there is no solid evidence of the effects of *CYP2C9*2* on its catalytic activity, but the variant have been reported to occur mostly in Japanese population (Hanatani *et. al.,* 2001). On the contradict, other studies verified that the *CYP2C9*2* protein has moderately impaired catalytic activity *in vitro*perhaps due to impaired binding of the reductase (Capri and Miller, 1997; Goldstein, 2001).Meanwhile a few studies done by different groups found that individuals who were genotyped with *CYP2C9*3* have reduced clearance of S-warfarin, tolbutamide and phenytoin (Sullivan-Klose *et. al.,* 1996; Odani *et. al.,* 1997; Steward *et. al.,* 1997; Hanatani *et.al.,* 2001).

The implication of the genetic polymorphism of CYP2C9, the main enzyme responsible for the metabolism of phenytoin is however not clear. More works need to be carried out to unravel the genetic polymorphism of CYP2C9 which influence the pharmacokinetics, pharmacodynamics and dose requirement for the therapeutic effects of phenytoin. Understanding the impact of genotyping at the induction of therapy would thus be of great value in order to lower the risk of intoxication or non-effectiveness of therapeutic agents.

1.5 Objectives

The objectives of this study are:

1. To unravel the genetic mutation underlies the ultra-rapid metabolism of phenytoin;
2. To perform functionality study of genetic variants by cloning;
3. To develop of a PCR-based diagnostic test kit for determination of defective genetic variant.

CHAPTER 2

MATERIALS AND METHODS

2.1 Methodology

The overall study design is as depicted in Figure 2.1.

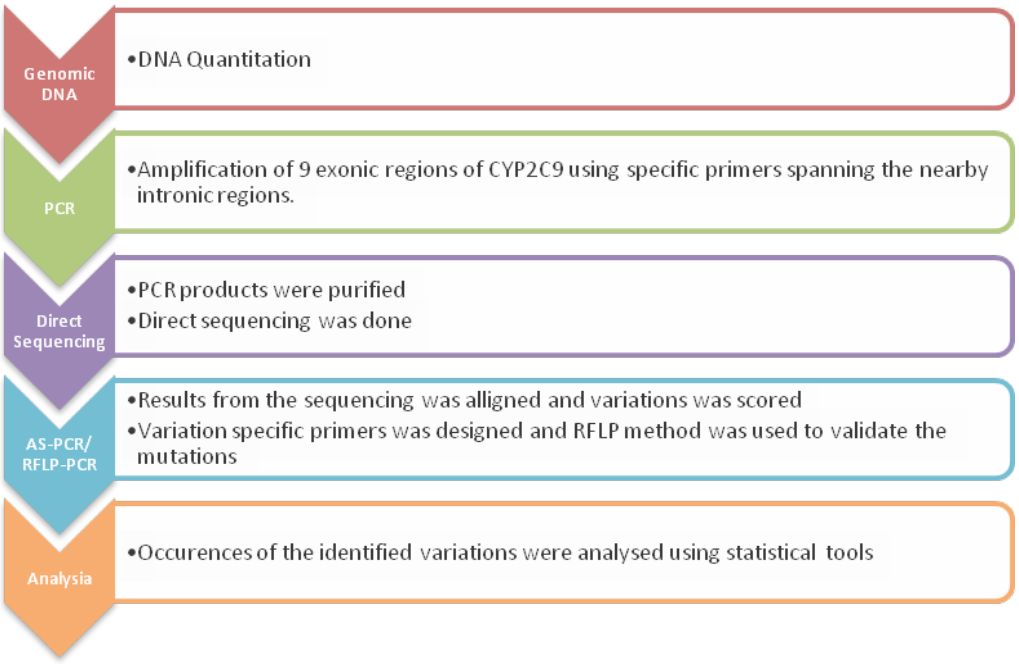

Figure 3: Flow Chart of the Research Methodology

2.2 Subjects

This study was approved by the local ethics committee at Huddinge University Hospital, KarolinskaInstitutet, Stockholm, Sweden and Inha University Hospital, Incheon, Korea and was performed in accordance to Helsinki Declaration.

The DNA sample from the subject who were identified as ultra-rapid metaboliser (MR = 0.13) by Helldén e*t. al.*, (2010) and three (3) other subjects with losartan metabolic ratio of 1 SD± 0.05, genotypes of CYP2C9*1/*1 and CYP2C19*1/*1 (Ramsjo *et. al.*, 2010) were selected as a control. The exonic- intronic and the 5'flanking regions of CYP2C9 were amplified using the primers listed in Table 1 before sequencing.

2.3 Chemicals, Reagents and Instruments

Chemicals, reagents and the instruments used in this study are listed in Table 2.

Table 2: Chemicals, Reagents and Instruments Used in this Study

	Chemicals and Reagents	Supplier
1.	Agarose LE, Analytical Grade	Promega, Promega Corporation, Madison USA.
2.	Biotools® DNA Polymerase (recombinant E.coli) , 1U/μL - storage Buffer: [10mMTris-HCl (pH 8.0), 50 mMKCl, 1 mM EDTA, 0.1% Triton X-100, 50% glycerol (v/v)] - Reaction Buffer: [10 X concentration : 75 mMTris-HCl (pH 9.0), 50 mMKCl, 20 mM $(NH_4)_2SO_4$] - 50 mM $MgCl_2$ (supplied separately)	
3.	Ethanol Denatured, Anhydrous (500ml) - biotechnology grade (94.9-96.0%)	Amresco® Solon Ind. Pkwy. Solon, Ohio USA..
4.	Ethidium Bromide (10 mg/ml) - molecular biology grade	Promega, Promega Corporation, Madison USA.
5.	GeneRuler™ 100 bps DNA Ladder® - PME-80 DNA completely digested.	Promega, Promega Corporation, Madison USA.
6.	PCR Primers Synthesis	InvitrogenCorperation, Custom Primers, Life Technologies, Faraday Avenue Carlsbad, California, USA.
7.	Sucrose (1kg) Ultra Pure Grade	Amresco® Solon Ind. Pkwy. Solon, Ohio USA.
8.	Water : distilled, deionised (Ω 18.2)	Mili-Q® Reagent Water System, Milipore MA, USA.
9.	Centrifuge 5410	Eppendorf AG, Hamburg, Germany.