CHAPTER 2.5

Pharmacogenomics research and practice: Snapshots from Malaysia

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Introduction

History of pharmacogenetics

Motulsky (1957) was the first to suggest that certain adverse drug reactions are caused by genetically determined variations in the activities of drug-metabolizing enzymes in the liver [1]. Thereafter, the relationship between genetic variance and drug therapy was investigated. Pharmacogenetics is the study of variability in drug response due to the inherent genetic differences in patients [2]. The term was first coined by Vogel (1959), and over the last decade, the subject has been extensively studied. Many examples of exaggerated responses to drugs attributable to inherited traits have been observed after a "normal" dosage [3]. To date, many of the pharmacogenetic differences that have been characterized on a molecular basis represent variability in metabolism and differences in relation to receptor affinity, transporters, or protein binding. Host genetic variation that cause changes in the the amino acid sequences affect the enzymatic functions and ligand-binding affinities of the transporters and receptors which further contribute to variable drug responses. Variabilities caused by pharmacogenetic differences in metabolism are generally more striking—greater than 10,000-fold—than pharmacogenetic differences in drug affinity to receptors which are generally less than 20-fold [2, 4, 5]. Individual differences in metabolic rates alter the expected relationship between the dose of a drug and its concentration in the blood, and the length of time it stays in the blood (t-half). Therefore, a polymorphism can lead to an excessive or prolonged therapeutic effect or drug-related toxicity after administration of a "typical" dose. The former is caused by a failure to clear the drug from the blood and the latter, by a change in the pattern of metabolism that produces toxic metabolites. Polymorphisms can confer a genetic predisposition to the adverse effects of medications, especially for drugs with a narrow therapeutic index (e.g., anticancer medications). Polymorphisms are defined as locations where the genetic variant has a frequency of at least 1% in the population, and a single-nucleotide polymorphism (SNP) is simply a single nucleotide change at any point in the DNA sequence (http://www.ncbi.nlm.gov/SNP/). Although not all SNPs alter gene function,
some can act as genetic markers to predict disease susceptibility or a patient’s response to drug therapy.

Pharmacogenetics defines the influence of genetic factors on the safety and efficacy of drugs, whereas pharmacogenomics combines large-scale genomic information, variations, and technologies with drug discovery and response [6]. Variations in the human genome, which include SNPs in genes encoding drug-metabolizing enzymes, transporters, and receptors, together with differences in gene expression, influence pharmacokinetic and pharmacodynamic factors. Therefore, knowledge of pharmacogenetics and pharmacogenomics is important in the implementation of personalized medicine. Some valid biomarkers are shown in Table 1, and the search for more such biomarkers continues. Information about pharmacogenomic biomarkers is published by the United States Food and Drug Administration (US FDA).

Ethnic differences have been reported, with different major alleles present in different populations. The CYP2D6*10 allele, which encodes the enzyme cytochrome P450 2D6 (CYP2D6), is rare in Caucasians and is thought to be derived from CYP2D6*4A, the most common inactive allele in Caucasians. In Chinese and the Malay populations, CYP2D6*10 accounts for 51–70% of all the CYP2D6 alleles, and results in a right shift in the median of the metabolic ratio (MR) among the Chinese and the Malays extensive metabolizers (EMs) [7-9]. This results in lower enzyme activity of CYP2D6 in Asians than in Caucasians. Among Africans, Masimirembwa et al. (1996) found a right shift in the MR in Zimbabweans. This was identified as being due to the CYP2D6*17 allele and its frequency was found to be 34% [10]. In contrast to the consistency in the percentage of poor metabolizers (PMs), the percentage of duplicated/multi-duplicated genes varies in different populations. In Swedish Caucasians, the frequency of subjects having duplicated/multi-duplicated genes is about 1–2% [11]. The frequency increases to 7–10% in Spain [12], and is as high as 29% in Ethiopians [13]. Studies in Asian countries have revealed that 1–2% of Japanese, Chinese, Malays, and Indians have duplicated genes [8, 14-17]. HLA-B*1502 is another allele that is clinically important in carbamazepine (CBZ)-induced hypersensitivity in Asians, but is irrelevant among Caucasians and Japanese [18]. Among Caucasians and Japanese, HLA-A*3101 is associated with a higher risk of cutaneous adverse drug reactions (cADRs) [19].
Table 1. Genes with single-nucleotide polymorphisms (SNPs) that have a functional impact on drug therapy and act as valid biomarkers.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene abbreviation</th>
<th>Selected substrates, ligands, or drugs for which the polymorphism may be relevant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>G6PDH</td>
<td>Involves many drugs generating electrophilic reactive metabolites in human cells</td>
</tr>
<tr>
<td>Butyrylcholine esterase</td>
<td>BCHE</td>
<td>Mivacurium, procaine, succinylcholine</td>
</tr>
<tr>
<td>N-acetyltransferase type 2</td>
<td>NAT2</td>
<td>Isoniazid, aromatic amines (occupational medicine and toxicology)</td>
</tr>
<tr>
<td>Cytochrome P450 2D6</td>
<td>CYP2D6</td>
<td>Amitriptyline, clomipramine, desipramine, doxepin, duloxetine, imipramine, nortriptyline, trimipramine, paroxetine, venlafaxine; haloperidol, perphenazine, chlorpromazine, perazine, promethazine, thioridazine, zuclopenthixol, aripiprazole, olanzapine, amphetamine, atomoxetine, carvedilol, metoprolol, nebivolol, propranolol, timolol, perhexilene, encainide, flecainide, mexiletine, ondansetron, tropiseton, codeine, tramadol, tamoxifen</td>
</tr>
<tr>
<td>Cytochrome P450 2C19</td>
<td>CYP2C19</td>
<td>Omeprazole, esomeprazole, lansoprazole, pantoprazole, rabeprazole, voriconazole, diazepam, alprazolam, amitriptyline, imipramine, doxepin; moclobemide, citalopram, S-mephentoin, phenytoin, primidone, clopidogrel, proguanil, cyclophosphamide, teniposide</td>
</tr>
<tr>
<td>Cytochrome P450 2C9</td>
<td>CYP2C9</td>
<td>S-Warfarin, acenocoumarol, phenprocoumon; glempiride, tolbutamide, glyburide, nateglinide; losartan, candesartan, irbesartan; celecoxib, diclofenac, ibuprofen, flurbiprofen, suprofen, naproxen, meloxicam, tenoxicam, piroxicam, lornoxicam, phenytoin; flavastatin; torsemide</td>
</tr>
<tr>
<td>Thiopurine S-methyltransferase</td>
<td>TPMT</td>
<td>6-Mercaptopurine, 6-thioguanine, azathioprine</td>
</tr>
<tr>
<td>Dihydropyrimidine dehydrogenase</td>
<td>DPD</td>
<td>5-Fluourouracil, capecitabine</td>
</tr>
<tr>
<td>Uridine diphosphate-glucuronic acid transferase type 1A1</td>
<td>UGT1A1</td>
<td>Bilirubin, irinotecan</td>
</tr>
<tr>
<td>Vitamin K epoxide reductase</td>
<td>VKORC1</td>
<td>Warfarin, acenocoumarol, phenprocoumon</td>
</tr>
<tr>
<td>Coagulation factor V</td>
<td>FV</td>
<td>Heparin, oral contraceptives, estrogens, selective estrogen receptor modulators (SERMs)</td>
</tr>
<tr>
<td>Organic anion-transporting polypeptide 1</td>
<td>OATP1B1</td>
<td>Almost all statins, methotrexate, repaglinide, rifampin, torsemide</td>
</tr>
<tr>
<td>Major histocompatibility locus</td>
<td>HLAB</td>
<td>HLA-B*5703 predicts Abacavir hypersensitivity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA-B*1502 predicts carbamazepine, phenytoin-induced Steven Johnson’s syndrome or Toxic Epidermal Necrolysis</td>
</tr>
</tbody>
</table>

Adapted from Brockmöller and Tzvetkov, 2008 [20]
The cytochrome P450 proteins constitute the major family of drug metabolism enzymes. They are found mainly in the liver but also at other sites, and metabolize both endo- and exogenous substances. Studies at both the genetic (genotyping) and functional (phenotyping) levels of the cytochrome P450 system have revealed that much of the inter-individual variability in drug disposition can be attributed to genetic polymorphisms. Genetic polymorphisms can have varied consequences in drug metabolism. Some polymorphisms are silent, but some cause diseases or modify drug response, sometimes with dire consequences. Of the six human cytochrome P450 enzymes of major importance in the metabolism of clinically used drugs, CYP2A6, CYP2C9, CYP2C19, CYP3A4/5, and CYP2D6 are polymorphic. These enzymes account for more than 60% of human Phase I metabolism [21].

*In vivo* phenotyping with probe substrates to assess the activity of a specific drug-metabolizing enzyme is used to define the genetic polymorphism of that enzyme. For the more important cytochrome P450 enzymes, this can be done using a cocktail of non-interacting substrates. Thus, individuals can be “fingerprinted” with respect to metabolic capability. Based on the phenotyping studies that have been carried out, the polymorphic expression of P450 enzymes has been classified into four groups, none of which has a frequency of less than 1%. PMs express dysfunctional or inactive enzymes and possess a homozygous autosomal recessive allele (usually a mutant allele). EMs produce enzymes with normal activity and have heterozygous or homozygous dominant alleles with normal activities, while intermediate metabolizers have one mutant allele that causes reduced activity. At the other extreme, “ultra-rapid metabolizers” (UMs) have increased enzyme activity due to a duplicated gene. These individuals carry replicate functional copies of the relevant genes. The rates at which the drugs are metabolized may vary by 10- to 100-fold between PMs and EMs [4]. This can be illustrated using CYP2D6 as an example. A genetic polymorphism of CYP2D6 results in four phenotypes with significant clinical implications for PMs and UM (Figure 1). PMs metabolize drugs to a lesser extent and therefore require lower dosages to avoid toxic effects. Adverse effects due to elevated drug plasma levels occur more frequently in PMs in cases where drug clearance is dependent on CYP2D6 [22]. However, PMs can experience diminished effects with drugs that need to be metabolized to active compounds by CYP2D6. A popular example is codeine, which is a prodrug and requires conversion to morphine in patients. Inadequate transformation in PMs leads to diminished pain relief or even tolerance/addiction. At the other extreme are UM who have more than two functional alleles owing to gene duplication or multiplication. UM require higher doses than normal because drug metabolism and clearance are enhanced. These patients may be resistant to treatment, and more time may be required to adjust the dosage before therapy is achieved. In theory, identifying a CYP2D6 UM up front reduces the time needed to upwardly adjust a dosage, helping to achieve therapeutic success more quickly.
**POTENTIAL APPLICATION OF PHARMACOGENOMICS IN PERSONALIZED MEDICINE**

It is unfortunate that the problems associated with the genetic polymorphism of drug-metabolizing enzymes that result in varied drug responses have been underestimated. This has resulted in the slow adoption of pharmacogenomics-based approaches in clinical practice. Although the CYP2D6 polymorphism was discovered 50 years ago, there have been few controlled studies to evaluate its clinical significance and pharmacoeconomic impact. Nevertheless, genetic analysis is being increasingly applied in the pharmaceutical industry, in drug development, and in the design and assessment of clinical trials. In the current millennium, traditional therapeutic drug monitoring may even be complemented or supplemented with phenotyping or genotyping screens for human drug-metabolizing enzymes. When applied to drug selection and dosing, knowledge about the pharmacogenetics of drug metabolism could help physicians prevent adverse effects and therapeutic failures, and thus enhance therapeutic efficiency and reduce costs.

“One size does not fit all” reflects the importance of pharmacogenomics in solving problems in clinical settings, especially drug-induced adverse reactions (ADRs). A treatment that is effective in one patient might be ineffective in others. In the worst-case scenario, some patients may suffer ADRs due to a particular drug.

The following sections are reports of some of the information we have collected through our studies. We believe that our findings are clinically relevant and therefore may be appropriate for implementation in clinical settings.

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**Figure 1.** Graphical presentation of drug levels achieved in the predicted phenotype of CYP2D6 from a different allele of CYP2D6 (Adapted from Teh and Bertilsson, 2012) [23]

Legend: Parent drug: an active drug per se
Prodrug: An inactive drug, converted into an active metabolite
Pharmacogenetics of HLAB*1502

The US FDA recommends that patients with Asian ancestors should be genotyped before they are prescribed CBZ and phenytoin. Individuals who are carriers of HLAB*15:02 are advised to have their treatment designed according to their genetic background.

CBZ is a commonly used drug in Malaysia. It is indicated for various neurological and psychiatric disorders such as epilepsy, neuropathic pain, migraine, depression, and bipolar disorder. The pathogenesis of CBZ-induced Stevens Johnson syndrome and toxic epidermal necrolysis (SJS–TEN) is consistent with the delayed type of immune-mediated reaction, and is highly associated with the genetic variation of HLAB*15:02. The HLAB*15:02 allele is strongly correlated with CBZ-induced SJS–TEN in Han Chinese populations [24]. This association has subsequently been confirmed in Hong Kong, Malaysia, Thailand, India, and in descendants of immigrants from Southeast Asia—regions in which the HLAB*15:02 allele is prevalent [18]. To study the impact of pharmacogenetic testing in a local clinical setting, a HLAB*15:02 pharmacogenotyping test was implemented in a cohort of patients in a public hospital in Malaysia.

We recruited 86 patients to participate in a clinical trial under the Ministry of Health (MOH) Task Force. Of those 86 patients, 40 were newly registered patients who were not receiving CBZ. Genotyping was conducted before starting CBZ administration. Seven of the 40 patients in this group were positive for HLAB*15:02. Another 46 patients were recruited to a retrospective study. Out of this group, 15 patients were carriers of HLAB*15:02. CBZ was withdrawn from 14 patients owing to CBZ-cADRs (10 with SJS/TEN; 4 with rashes). One HLAB*15:02-positive patient did not develop SJS/TEN and was therefore tolerant of CBZ. The calculated odds ratio for SJS/TEN in subjects with the variant allele relative to the control patients was 609.0 (95% confidence interval (95%CI): 23–15873; \( P = 0.001 \)) [25].

Our findings suggest that screening patients for the HLAB*15:02 allele before the initiation of CBZ treatment, and withholding CBZ from HLAB*15:02-positive patients, can reduce the incidence of CBZ-induced SJS-TEN.

Patients who carry the HLAB*15:02 allele are prescribed other anti-epileptic drugs (AEDs). However, HLAB*15:02-positive patients may experience similar cADRs with alternative AEDs. Several reports on cADRs for HLAB*15:02-positive patients have been received from local Adverse Drug Reaction Surveillance Units. We successfully developed computational molecular models to predict the CBZ binding mode in both the HLAB*15:02/endogenous peptide-loaded complex and the HLA-A*3101/endogenous peptide-loaded complex to determine the nature of their molecular interaction with CBZ. A model was used to study the docking of 15 AEDs to determine their potential as cADR inducers via their binding to the complexes. Of the 15 AEDs, we found that oxcarbazepine, clonazepam, lamotrigine, phenytoin, and phenobarbital are potential cADR inducers for both alleles, whereas levetiracetam is a potential cADR inducer for HLA-A*3101 only. This information will provide
a warning to practitioners to be cautious when prescribing AEDs to prevent cADRs, especially with HLAB*15:02-positive patients.

**Pharmacogenetics of CYP2C9-VKORC1 and warfarin**

Warfarin has a very narrow therapeutic window but large individual variability (Lindh, 2009) [26-28]. Failure of patients to attain therapeutic international normalized ratio (INR) levels results in unwanted effects such as bleeding or the recurrence of thrombosis [29]. Many studies have proven that polymorphisms in genes involved in the pharmacokinetics (Figure 2) and pharmacodynamics (Figure 3) of warfarin are the significant causes of dose variability. Polymorphisms of the enzymes CYP2C9 and vitamin K epoxide reductase complex subunit 1 (VKORC1) are two important causes of the variation in the pharmacokinetics and pharmacodynamics of warfarin [30-32]. It has been reported that VKORC1 haplotypes are responsible for 20–30% of the inter-individual variability of warfarin, and CYP2C9 genotype variation accounts for another 6–10% [33, 34].

![Figure 2. The metabolism of warfarin [35]](image-url)
VKORC1 haplotypes have been used to stratify patients into low-, intermediate-, and high-dose warfarin groups. Ten common non-coding SNPs have been used to assign nine haplotypes. Genealogic analysis has revealed two highly divergent haplotype groups, namely groups A and B, associated with different warfarin dose requirements [36, 37]. Haplotypes H1 and H2 have been assigned to group A with a lower warfarin dose requirement, whereas haplotypes H7, H8, and H9 have been assigned to group B with a higher warfarin dose requirement [37-39]. VKORC1 haplotype groups A and B correspond to approximately 25% of the variance in dose. The warfarin maintenance dose differed significantly among the three haplotype groups at 2.7 ± 0.2 mg per day for A/A, 4.9 ± 0.2 mg per day for A/B, and 6.2 ± 0.3 mg per day for B/B [37, 40].

Inter-ethnic variations in VKORC1 haplotype frequencies provide a good explanation for the different warfarin requirements of different ethnic groups [34]. The frequency of VKORC1 haplotypes varies among different ethnic groups, as shown in Table 2. Asian Americans have a higher proportion of group A haplotypes and African Americans have a higher proportion of
group B haplotypes [37]. Other studies have shown that Japanese and Chinese require lower doses than Caucasian patients [41].

Pharmacogenetic studies have proven a strong correlation between warfarin dose and genetic polymorphisms in CYP2C9 and VKORC1. Malaysia is a multiracial country with people from different ethnic groups and genetic backgrounds. Traditional warfarin doses might place patients at risk because patients from different ethnic groups might require different titrations of warfarin dose. Genotyping patients prior to initiation of therapy may identify at-risk patients and those not likely to benefit from the therapy, and this will help us move away from a trial-and-error approach to more individualized and effective warfarin therapy. Furthermore, warfarin dosing algorithms based on a patient’s genotype, age, and height have been. Developing a nomogram for warfarin will facilitate individualization of the warfarin dose, during both the initial and maintenance stages of therapy.

### Table 2. Percentage frequencies of VKORC1 haplotypes according to ethnic groups.

<table>
<thead>
<tr>
<th>Population</th>
<th>H1</th>
<th>H2</th>
<th>H7</th>
<th>H8</th>
<th>H9</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>European</td>
<td>12%</td>
<td>26%</td>
<td>21%</td>
<td>14%</td>
<td>24%</td>
<td>[37]</td>
</tr>
<tr>
<td>African</td>
<td>7%</td>
<td>6%</td>
<td>42%</td>
<td>1%</td>
<td>6%</td>
<td>[37]</td>
</tr>
<tr>
<td>Asian</td>
<td>89%</td>
<td>0</td>
<td>10%</td>
<td>0</td>
<td>0</td>
<td>[37]</td>
</tr>
<tr>
<td>Chinese</td>
<td>86.4%</td>
<td>12.5%</td>
<td>0</td>
<td>1.1%</td>
<td></td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td>87%</td>
<td></td>
<td></td>
<td></td>
<td>*9%</td>
<td>[39]</td>
</tr>
<tr>
<td>Indians</td>
<td>7.1%</td>
<td>85.7%</td>
<td>0</td>
<td>7.2%</td>
<td></td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td>12%</td>
<td></td>
<td></td>
<td></td>
<td>*82%</td>
<td>[39]</td>
</tr>
<tr>
<td>Malays</td>
<td>80.0%</td>
<td>20.0%</td>
<td>0</td>
<td>0</td>
<td></td>
<td>[42]</td>
</tr>
</tbody>
</table>

We therefore investigated the contribution made by genetic polymorphisms of common alleles of CYP2C9 and VKORC1, as well as by other variables, and developed a new algorithm, which was validated in a small group of patients. The dose predicted using this algorithm was compared with the maintenance doses. Developing population-specific algorithms would be useful for achieving personalized medicine because algorithms developed by other studies may not be applicable to the local population. The covariates affecting the patient’s warfarin dose requirement could vary between different geographical and ethnic groups owing to diet and environmental factors. A linear regression model including the variables age, CYP2C9 and VKORC1 genotype, and height proved to be the optimal choice for estimating warfarin dose because it had the largest R² value (Table 3). This dosing algorithm was assessed in a second unrelated population of 28 patients on warfarin therapy and with a stable control of anticoagulation. Pearson correlation analysis of the data showed a very close and significant relationship between the calculated warfarin dose using the algorithm derived from the regression dose model and the actual dose (r = 0.598; P = 0.001). The dose predicted for the 28 patients who had achieved INR 2–4 was 3.30 mg (SD 0.84) compared with the currently
prescribed dose of 3.45 mg (SD 1.42). There was also significant correlation between the predicted dose and the dose at initiation \( (r = 0.609; \ p = 0.001) \) [43].

Table 3. Dose prediction of warfarin using VKORC1 and CYP2C9 genotypes.

<table>
<thead>
<tr>
<th>Dosing equation</th>
<th>R2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Dose} = 7.728 - 0.993(G-1639A) - 0.564(C1173T) - 0.811 \text{ (CYP2C9)} - 0.037 \text{ (Age)} - 0.008 \text{ (Weight)} + 2.180 \text{ (Height)} )</td>
<td>36.5%</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Legend: G-1639A = 1, 2, 3 for homozygous G-1639G, heterozygous G-1639A, homozygous A-1639A; C1173T = 1, 2, 3 for homozygous C1173C, heterozygous C1173T, homozygous T1173T; CYP2C9= 1, 2, 3 for homozygous CYP2C9*1/*1, heterozygous CYP2C9*1/*3

We further studied the relevance of VKORC1 haplotypes to the dose requirement of warfarin. In the multiple linear regression (MLR) analysis adjusted for clinically important covariates, only four haplotype pairs were independently associated with the warfarin dose (MLR; \( P < 0.05 \)). The multiple regression result indicated that VKORC1 haplotypes accounted for 25.5% variation in the warfarin dose. The mean maintenance doses for haplotype pairs H1-H1 and H1-H10, which were associated with a low warfarin dose requirement, were 2.92 mg (MLR; \( P < 0.05 \)) and 3.07 mg (MLR; \( P > 0.05 \)) per day, respectively, whereas haplotype pairs H1-H6 and H1-H7, which were significantly associated with a high dose requirement, had mean maintenance doses of 4.91 mg (MLR; \( P < 0.05 \)) and 4.33 mg (MLR; \( P < 0.05 \)) per day, respectively. Patients with haplotype pair H1-H1 required a significantly lower dose than those with haplotype pairs H1-H6 and H1-H7 (t-test; \( P < 0.05 \)), whereas the dose requirement for patients with pair H1-H10 was not significantly different from that for patients with pair H1-H1 (t-test; \( P > 0.05 \)). The result was in accordance with a previous study by Rieder et al. (2005). The average INR was approximately 2.21, and did not differ significantly among the patients classified according to VKORC1 haplotype pairs (MLR; \( P < 0.05 \)).

An understanding of the pharmacogenomics of warfarin is the next step towards personalized warfarin therapy. CYP2C9 and VKORC1 genotyping is useful for predicting a patient’s response to warfarin therapy, and minimizes ADRs. Warfarin genotyping reduces the risk of hospitalization for bleeding events or thromboembolism in patients initiated with warfarin treatment in outpatient settings. Clinicians should seriously consider genotyping patients who are beginning warfarin treatment to improve therapeutic outcomes. Genotyping provides potential benefits for patients on warfarin therapy as well as for patients using warfarin in combination with aspirin.
Pharmacogenetics of DPYD and 5-FU

In cancer therapy, the response to drugs varies greatly between patients. Understanding the factors that cause this variability helps improve personalized medicine in clinical practice and drug development. The proper implementation of pharmacogenetics/genomics to maximize therapeutic effects and reduce adverse effects is needed because chemotherapeutic treatment has a low success rate. The “trial and error” approach to therapy is inefficient and often causes severe toxicity. The advent of molecular analysis will reduce “trial and error” prescriptions and allow safer dosing options by reducing ADRs [44].

Several pharmacogenomic tests have proved useful in clinical settings. For example, molecular tests for the detection of human epidermal growth factor receptor 2 (HER2) are useful for identifying breast cancer patients who will benefit from an antibody drug called Herceptin [45].

The pharmacogenomics approach is also useful for predicting 5-fluorouracil (5-FU)-related toxicity. A previous study identified a splice mutation in the gene that encodes dihydropyrimidine dehydrogenase (DPYD), IVS14+1G>A (DPYD*2A), as the most common variant associated with 5-FU toxicity. According to Morel et al. (2007), 50–60% of patients who carry genetic variations of DPYD develop severe 5-FU toxicity [46]. A study by Kristensen et al. (2010) indicated that pre-screening for the DPYD variant prior to 5-FU administration could avoid 20% of 5-FU-related toxicity cases [47].

The association between the serum concentration of 5-FU and toxicity was demonstrated by Findlay et al. (1996) [48]. Subsequently, several other studies have reported that deficiency in DPYD is associated with 5-FU-related toxicity symptoms such as diarrhea, stomatitis, mucositis, neurotoxicity, and in some cases, death [49-51]. DPYD is a pyrimidine catabolic enzyme. It is the initial and rate-limiting factor in the catabolism pathway of toxic 5-FU metabolites. It is polymorphic and many DPYD variants are reported to be associated with 5-FU-induced toxicity [52-54]. For example, Kleibl et al. (2009) reported that patients with IVS14+1G>A or DPYD*6 (V732I) mutations have a higher risk of mucositis and leucopenia after 5-FU chemotherapy [55].

However, there is wide inter-ethnic and inter-geographical difference in the allele types and frequency of DPYD. It has been reported that 3% of the Caucasian population carry DPYD*2A in exon 14 of DPYD, whereas none of the Japanese examined was found to carry this variant [56]. Moreover, the prevalence of DPYD*5 (rs1801159) in exon 13 of DPYD was reported to be 11.5% in Egyptians, 14% in Caucasians, and 12% in Tunisians [57]. Higher frequencies of DPYD*5 (rs1801159) were observed in Japanese (35%), Taiwanese (21%), and African-Americans (22.7%). Hence, it is important to know the polymorphic nature of DPYD in different population as different types and frequencies of alleles of DPYD are present in different ethnic groups.
Understanding the effect of DPYD polymorphisms on the serum level of 5-FU and its associated toxicity is crucial to preventing adverse reactions and optimizing therapy outcomes. This information is essential in Malaysia with its multi-ethnic population, which has presented a great challenge for the clinical management of patients.

Convenient and accurate genetic analysis that is clinically useful for the prediction of DPYD enzyme activity and 5-FU efficacy is required. We therefore developed easy-to-use polymerase chain reaction (PCR) methods for the rapid screening of DPYD variants. The serum level of 5-FU was quantified using high-performance liquid chromatography (HPLC), and DPYD genotypes were correlated with the serum level of 5-FU and clinical outcomes.

The Pharmacogenetics Working Group Guidelines from the Royal Dutch Association for the Advancement of Pharmacy recommend the therapeutic doses shown in Table 4 (http://www.pharmgkb.org) for 5-FU, based on DPYD genotype. An alternative drug or a reduced dose is recommended for poor and intermediate metabolizers to avoid severe side effects due to 5-FU.

Table 4. Dose recommendation for 5-fluorouracil (5-FU) based on DPYD genotype.

<table>
<thead>
<tr>
<th>Phenotype (genotype)</th>
<th>Therapeutic dose recommendation</th>
<th>Types of allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor metabolizer</td>
<td>Alternative drug required</td>
<td>2 inactive alleles; or 2 decreased activity alleles; or 1 inactive and 1 decreased activity allele</td>
</tr>
<tr>
<td>Intermediate metabolizer</td>
<td>50% dose reduction or alternative drug required</td>
<td>1 active allele and 1 inactive; or decreased activity allele</td>
</tr>
</tbody>
</table>


Adapted from Ezzeldin and Diasio, 2004 [58]

Our study revealed that patients with the DPYD allele that causes deficient enzyme activity had higher median serum levels of 5-FU compared with the normal DPYD group (Mann–Whitney test, P-value = 0.010) (Figure 4). Regression analysis was carried out to determine the influence of DPYD*5 and 1896T>C on 5-FU levels. DPYD*5 (rs1801159) and 1896T>C (rs17376848) were accountable for 5-FU levels when tested as individual variables at 3.4% and 27.6%, respectively. DPYD*5 (rs1801159) and 1896T>C (rs17376848) were accountable for 36.6% of inter-individual variation of 5-FU serum levels achieved among patients. Significant correlation (P < 0.05) was observed between patients who were carriers of 1896T>C (rs17376848) and neutropenia (RR of 2.3; 95%CI: 1.01–5.09). The results obtained from the regression equation for the prediction of 5-FU levels and the risk of neutropenia were: 5-FU levels = -0.8628 + 3.605 (DPYD*5/rs1801159) + 6.844 (1896T>C/rs17376848) (P-value = 0.0041) and neutropenia = -0.612 + 0.349 (DPYD*5/rs1801159) + 0.411 (1896T>C/rs17376848) (P-value = 0.017) [59].
Figure 4. Association between *DPYD* genotypes and serum concentration of 5-fluorouracil (5-FU)

*DPYD*5 (rs1801159) and 1896T>C (rs17376848) are useful predictors of a patient’s response to 5-FU chemotherapy. The phenotype–genotype correlations demonstrated in our study prove the usefulness of genotyping as a tool to monitor 5-FU efficacy instead of using conventional drug level measurements. Genotyping of *DPYD* variants is therefore recommended to prevent severe toxicity, particularly neutropenia due to 5-FU. An alternative drug or a reduced dose of 5-FU is recommended for poor or intermediate metabolizers to avoid severe adverse effects due to 5-FU administration [59].

**UGT1A1 gene and irinotecan**

Irinotecan was first introduced in Japan as Campto®. During development, it was more commonly known as CPT-11. It is a derivative of camptothecin and is widely used in the treatment of metastatic colorectal cancer, either as mono-therapy or second-line therapy. It is used in the FOLFIRI regimen (FOL: folinic acid (leucovorin); F: 5-fluorouracil (5-FU); and IRI: irinotecan) [60].

Irinotecan is a potent inhibitor of topoisomerase I (Topo-I). It binds to the 3’-phosphoryl end of a nicked DNA strand to form a stable DNA–Topo-I complex during DNA replication [61]. Thus, it prevents the ligation of a nicked DNA strand, resulting in the collision of the replication fork with the stabilized DNA–Topo-I complex. Subsequently, the DNA double strand breaks and triggers apoptotic cascades resulting in cell death [61].
After intravenous (IV) administration, irinotecan is converted to its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), by a carboxylesterase. SN-38 is detoxified by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) to its inactive form SN-38 glucuronide (SN-38G), which is then excreted into the bile and urine [62].

Polymorphisms of UGT1A1 are responsible for the large inter-individual differences in the pharmacokinetics of irinotecan and the risk of severe toxicity [62, 63]. More than 30 genetic variants in the promoter region and exon 1 of UGT1A1 have been reported to decrease enzyme activity [64, 65]. Under-expression of the UGT1A1 enzyme impairs the metabolism of SN-38 to its inactive form (SN-38G) and causes an excessive accumulation of toxic SN-38. Ando et al. (2000) showed that 80% of the patients they examined who suffered from life-threatening toxicities had variants UGT1A1*6 (211G>A) and UGT1A1*27 (686C>A). Patients with non-small cell lung cancer and homozygous UGT1A1*6 had significantly lower tumor response rates, and shorter progression-free and overall survival rates when treated with irinotecan-based chemotherapy [66]. The study by Ando et al. (2000) also revealed that Japanese patients who were heterozygous for UGT1A1*27 experienced severe toxicities such as leucopenia and/or diarrhea [67].

In other cases, there was a positive correlation between UGT1A1 polymorphism and irinotecan-related toxicity. Decreased activity of the UGT1A1 enzyme due to genetic polymorphisms of the UGT1A1 gene resulted in prolonged exposure time to SN-38, a toxic metabolite. This contributed to severe toxicity after irinotecan administration. Moreover, Steiner et al. (2005) reported that a combination of variants of DPYD (IVS14 + 1G>A) and UGT1A1 (UGT1A1*28) increased the risk of severe gastrointestinal and hematological toxicity in patients treated concurrently with 5-FU and irinotecan chemotherapy [68].

UGT1A1*28 is a genetic polymorphism caused by the insertion of repetitions of TA in the (TA)\textsubscript{6}TAA-box (insertion of TA from -53 to -38) at the promoter region. An increased number of TA repeats may inhibit transcription efficiency and reduce enzyme concentrations, leading to the accumulation of SN-38 and irinotecan-induced toxicity [69]. The UGT1A1*28 polymorphism causes an approximate 70% reduction in UGT1A1 enzyme activity [69]. It has also been reported that the incidence of severe irinotecan toxicity is higher in patients with homozygous UGT1A1*28 than in those with either wild-type UGT1A1*1 or heterozygous UGT1A1*28 [69].

The US FDA first approved the irinotecan label and provided the relevant pharmacogenetic information in 2005 [70]. New pharmacogenetic information has subsequently been added to the label regarding the risk of neutropenia in patients with genetic defects in UGT1A1 [71].

We conducted a study by genotyping 306 healthy unrelated volunteers from three major ethnic groups (Malay = 100; Chinese = 104, and Indian= 102) in Malaysia. They were recruited to enable the investigation of UGT1A1*6, *27, and *28 polymorphisms. The UGT1A1*28
variant was detected in all three Malaysian races. We found that 24.5–39.2% of Malaysians were heterozygous for the UGT1A1*28 (6TA/7TA) variant. The Malays and Indians had twice the incidence of homozygous UGT1A1*28 (7TA/7TA) (8 and 8.8%, respectively) compared with the Chinese (only 4.9%). The predicted frequencies of UGT1A1*28 genotypes among the three major races in Malaysia were calculated according to the Hardy–Weinberg equation and reported with 95% CIs. All the genotypes were in agreement with the Hardy–Weinberg equilibrium [72]. In comparison with the allele frequencies reported among Caucasians and some other ethnic groups in Asian countries such as Taiwan and Hong Kong [73-75], the UGT1A1*28 variant was higher in the Malays and Indians, with allele frequencies of 25 and 28%, respectively.

Interestingly, a combination of a heterozygous insertion of the 8TA genotype and an SNP located at -63 (G>C) known as UGT1A1*68 was detected in 0.02% of the Chinese Malaysian population. UGT1A1*68 has been reported previously in Caucasian, Indian, and African-American populations [64, 76].

The genotype and allele frequencies of UGT1A1*6 were higher than those of UGT1A1*27 in the studied populations. The genotype and allele frequencies of UGT1A1*6 and UGT1A1*27 did not differ significantly among the Malay, Chinese, and Indian Malaysian populations. The frequency of UGT1A1*27 was low: less than 3 per cent in Malay and Chinese Malaysians, and not detected at all in the Indian subjects [72]. UGT1A1*27 was not detected in the Caucasian and African-American populations [74]. The incidence of UGT1A1*6 was high among the three major ethnic groups in Malaysia. This is in accordance with the 5–15% frequencies reported by the authors of other studies on the Asian population [74, 75]. Caucasians have a lower frequency of UGT1A1*6 (5%), and it is reported to be a rare variant among this population [75].

Previous studies have shown a correlation between the UGT1A1*6 genotype and an adverse reaction to irinotecan, whereby patients with the UGT1A1*6 variant have a higher level of SN-38. The occurrence of adverse effects has been reported in Asian patients with UGT1A1*6 genotype [74, 77, 78].

Patients with the UGT1A1*28 genotype are reported to be four times more likely to experience side effects from irinotecan than people who do not have the variant [79]. Some studies have shown that a combination of two polymorphic variations in the same gene may increase the risk of adverse effects. A higher incidence of neutropenia has been observed in patients who have a combination of UGT1A1*28 and UGT1A1*6 genotypes; such patients have a lower glucuronidation capacity for SN-38, which leads to the accumulation of this toxic metabolite [80]. This suggests that the gene does affect the pharmacokinetics of irinotecan, and it is recommended that patients be genotyped for its commonly encountered variants.
The allelic types and the frequency of \textit{UGT1A1}*28 among the Indian Malaysians in this study were similar to those in the Caucasian population [78]. However, there was a low \textit{UGT1A1}*28 allele frequency in the Chinese Malaysians (17%), which was similar to the frequencies reported in the Chinese in Singapore, Taiwan, and Japan (16, 14.3, and 9.7%, respectively) [73-75]. In our previous studies on the genetic polymorphisms of \textit{CYP2C9} and \textit{CYP2D6}, the Indian Malaysians showed a pattern of allele frequencies similar to that commonly found in Caucasians [8, 81], whereas the Malay and Chinese Malaysians were intermediate between East Asians and Caucasians with respect to \textit{CYP2D6} allelic frequencies [8]. In this study, a combination of the TA8 genotype and \textit{UGT1A1}*68 (G>C) was found in 0.02% of the Chinese Malaysian population. Interestingly, \textit{UGT1A}*68 (G>C) has been reported in the Indian population [64]. This suggests heterogeneity and an inter-ethnic difference for \textit{UGT1A1}*28 in Malaysia.

With the high prevalence of genetic variants of \textit{UGT1A1} among the three ethnic groups, and increasing incidence of colorectal cancer [82], genotyping of \textit{UGT1A1}*28, \textit{UGT1A1}*6, and \textit{UGT1A1}*27 is appropriate for patients prescribed with irinotecan in Malaysia. Because there may be inter-ethnic differences in the distribution of allelic variants of \textit{UGT1A1}, it is important to know the prevalence of such variants to predict which population is likely to be at risk of adverse effects [72].

**Sustainable technologies for pharmacogenetics research and clinical practice**

Sensitive, robust, and specific diagnostics are required to translate pharmacogenetics into clinical practice. Diagnostic tests should be made available to patients at reasonable cost to facilitate their wide usage and implementation. We developed simple yet innovative genotyping tests based on the concepts and principles of the PCR. The tests were developed using existing or evolving technologies, and low cost per test was ensured. Improved clinical decisions based on these new and improved diagnostic tools and techniques should lead to better health outcomes, while contributing to the sustainability of healthcare systems.

We adopted a PCR-based approach for the detection of SNPs, and designed primers that specifically differentiated genetic sequences.

**PCR genotyping analysis**

In 1983, Kary Mullis developed an elegant enzymatic method for amplifying DNA \textit{in vitro} and termed it PCR. The invention has revolutionized molecular biology. To commemorate his efforts, Mullis was awarded the 1993 Nobel Prize in Chemistry. Since then, PCR has evolved into an indispensable tool in molecular biology, largely owing to its simplicity, sensitivity, speed, and robustness. These qualities have propelled the method beyond traditional genetics into fields such as forensic science, agriculture, and molecular diagnostics.
One of the most important factors that influences the outcome of PCR is the primer design. Even though PCR has powerful capabilities that can be exploited, its usefulness could be hampered by bad primer design because an assay is only as good as its primers. The goal of primer design is to strike a balance between the efficiency and specificity of the amplification process. Many variables are taken into consideration for good primer design. Often the variables are adjusted to fit the requirements of the experiment to be carried out. Such variables include primer length, melting temperature (T_m), secondary structures, repeats, 3′-end stability, and homologous cross dimers.

Oligo Software, which is freeware developed by Teemu Kuulasmaa (2002) that comprises Oligo Analyzer and Oligo Explorer software, (T. Kuulas, University of Kuopio, Kuopio, Finland (http://www.uku.fi/~kuulasma/OligoSoftware)) was used to aid primer design for the PCR in this study [83].

The flexibility of PCR is illustrated by its applications: reverse-transcription PCR (RT-PCR), and nested, multiplex, long-range, and allele-specific PCR. RT-PCR is the amplification of RNA samples after transcription to complimentary DNA (cDNA), and can be used to determine the expression level of target genes. Nested PCR increases the sensitivity and specificity of PCR by conducting two rounds of amplification, with the second amplification using products from the first amplification as templates. Multiplex PCR is simply the combined amplification of different targets in a single reaction using multiple primer pairs, which obviates the need to run multiple reactions to assay different targets. Long-range PCR enables the amplification of long fragments of DNA of more than 20 kb using specialized polymerases. Finally, allele-specific PCR is run with specially designed primers that can differentiate between alleles, and is particularly useful in molecular diagnostics [84].

Allele-specific PCR was first described by Newton et al., (1989), and was originally known as the amplification refractory mutation system [85]. The method is based on the observation that under appropriate PCR conditions, oligonucleotides with a mismatch at the 3′-residue do not bind to the template and extend. Therefore, only a primer that perfectly complements the 3′-end will bind and amplify. The concept is illustrated in Figure 5.
Figure 5. Basic concept of allele-specific polymerase chain reaction (PCR)

Haplotype analysis

SNPs are the most common type of polymorphism and are found in every 200–300 bp in the human genome [86]. The abundance of SNPs makes it possible to generate a dense map using association analysis to find variations that may be relevant in determining individual responses to drugs or susceptibility to complex diseases [86]. However, a single gene offers little information and may be difficult to use owing to problems with validating the SNP, elucidating its frequency in a population, and understanding its impact on phenotype and function [87].

The use of haplotypes is said to increase the power to detect important variants that affect the phenotype by 15–50%. A haplotype is a set of polymorphic alleles located along the same chromosome that are inherited together as one unit [88]. The haplotype is important because it provides information on chromosome recombination that can be used to find disease-causing variations based on linkage disequilibrium. Linkage disequilibrium is a measure of the degree of association between variants, and it depends on their location because variants that are closer to each other are more likely to be inherited together [88]. When SNPs are in high linkage disequilibrium with each other, knowledge of the status of an SNP at one locus can infer the status of SNPs in other loci. Haplotyping coupled with association studies can reveal a pattern in the genome’s complex architecture. When an SNP of clinical relevance is identified, the typing of an SNP in that haplotype set makes the diagnosis and management of diseases easier and more effective. Multiplex PCR, which facilitates haplotype analysis, is used for the identification of SNPs.
Validation of PCR genotyping test

Validation determines the accuracy and specificity of the test, whereas verification involves comparison of the existing performance specification. Before a test can be used for diagnostics, an appropriate workflow must be adopted for validation and verification. An example is depicted in Figure 6. The requirement for or the usefulness of the test need to be determined prior to development. The factors that influence decision for test development include the clinical significance as well as the prevalence of the defective alleles in the population of interest. Critical parameters that may affect performance, and all necessary control measures and limitations must be considered. The method must be validated for its accuracy, specificity and sensitivity. An appropriate standards or internal controls must be included for the purpose of quality control as recommended by FDA in Pharmacogenetic Tests and Genetic Tests for Heritable Markers. A sensitive method will be able to distinguish the target signal from other components. Interference and carryover (cross-contamination), which cause false-positives, should be identified. An optimized process for verification and validation can improve product quality, reduce costs, and shorten the development cycle. Ongoing validation should be performed as a surveillance audit of a test method.

![Diagram of assay validation and verification]

Figure 6. Assay validation and verification.
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