Pharmacogenetics of drug metabolizing enzymes and transporters: effects on pharmacokinetics and pharmacodynamics of anticancer agents

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Abstract
There is wide interpatient variability in drug response and toxicity to standard doses of most anticancer medications. Genetic polymorphisms in genes encoding metabolic enzymes, receptors and drug transporters targeted by anticancer medications are often found, in part, to be responsible for the observed variability. Approximately 80% of all sequence variations residing in genes is in the form of single nucleotide polymorphisms or SNPs. The location of SNPs can be in the protein coding sequence, regulatory regions or at exon-intron boundaries of genes. Adverse drug reactions resulting from these sequence variations are due to changes in the activity of the encoded protein (in many instances the protein is non-functional) or perturbations in the level of gene expression. The goal of pharmacogenetic testing is to identify genetic polymorphisms that predispose patients to an adverse drug reaction, thereby allowing the health care provider to make informed decisions pertaining to the type of drug, dosage and dosage scheduling to be administered.

Keywords
pharmacogenetics; single nucleotide polymorphisms; genetic variation; thiopurine S-methyltransferase; thymidylate synthase; dihydropyrimidine dehydrogenase; ATP-binding cassette transporters; multidrug resistance-associated protein; cancer health disparities

Introduction
As an anticancer drug is administered to a patient, it is absorbed and distributed to its site of action. Along the way, the drug can be metabolized by the liver (e.g. prodrug to active drug, active drug to inactive metabolites), translocated from one body compartment to another, bound to targets such as receptors, transporters or enzymes in the tumor itself, and ultimately excreted from the body. Clinically relevant genetic variations can occur in any of the genes involved in these processes. This review will focus on some key pharmacogenetic examples in order to emphasize the role of inherited polymorphisms in the form of SNPs on the pharmacokinetics and pharmacodynamics of anticancer drugs. The underlying message for the reader is that inherited genetic variations affecting anticancer drug response are fairly common occurrences across the patient population and that pharmacogenetic testing for some drugs is now available in the clinical setting, with more likely to follow as next generation sequencing of the human genome further defines genetic variants [1] and technology platforms continue to develop for pharmacogenetic/pharmacogenomic diagnostics [2].
Functional consequences of polymorphisms in genes: an overview

What are single nucleotide polymorphisms?

A single nucleotide polymorphism (SNP) is a DNA sequence variation that occurs when a single nucleotide position in the genome differs between two individuals (A, T, C or G represent possible alleles). Typically one of the alleles (e.g. C) will be the predominant allele, being found in the majority of the population, while the other allele (e.g. T) will represent the minor variant. SNPs represent one type of sequence polymorphism comprising ~78% of all genetic variations in the human genome, while the remaining 22% of sequence variants are of the type known as insertions, deletions, inversions, copy number variants and segmental duplications [3]. It has been estimated that the number of SNPs in the human genome is ~10–30 million [4, 5], and the number of protein coding genes in the human genome has been predicted to range from only ~20,000 to ~35,000 [6, 7]. Consequently, SNPs occur every 100 to 300 nucleotides along the 3 billion nucleotide human genome, approximately 10% of the SNPs can have more than 2 possible alleles, and 1 in every 1200 nucleotides of a gene may be different in any two individuals [4, 5, 8].

Classification of SNPs

SNPs can be classified more or less into 4 major categories, namely those found: i) in the protein coding sequence of genes, ii) in the regulatory regions of genes (e.g. promoter region, 5′ untranslated region, 3′ untranslated region, intronic sequences), iii) at exon-intron boundaries of genes, and iv) in the intergenic regions (intervening genomic segments separating genes). The consequences of these sequence variants can range from no measurable effects on protein function, to changes in the structure and function of the encoded proteins, to perturbations in the level of gene expression. In the latter two scenarios, such SNPs are candidates for drug response-modifying alleles and the inheritance of these alleles by patients receiving standard doses of drug therapy can lead to an adverse drug reaction or chemotherapy failure.

Synonymous and non-synonymous SNPs

SNPs residing in the coding sequence of a gene may not necessarily change the amino acid sequence of the encoded protein, especially when the SNP occurs at the third position of the triplet codon due to the degeneracy of the genetic code (Figure 1A). A SNP in which two or more alleles result in the same protein sequence is termed synonymous (also called a silent “mutation”). If the alleles for a particular SNP leads to different amino acid residue substitutions in the polypeptide, they are referred to as non-synonymous which can be further subdivided into missense or nonsense changes (Figure 1A). A missense change results in the coding of a different amino acid (missense changes are sometimes referred to as conservative or non-conservative), while a nonsense change inserts a premature stop codon into the gene. In either case, the translated protein may be rendered inactive or with markedly reduced activity. Non-synonymous changes (via mutation or SNP) appear to account for more than half of the known genetic variations associated with human inherited diseases [9]. Accordingly, numerous computational approaches have been initiated for the purposes of modeling proteins containing non-synonymous SNPs in the hopes of predicting if these sequence variants alter the three dimensional structure and function of the protein [10–13].

Regulatory SNPs

SNPs located in the regulatory regions of genes can have consequences on the regulation of gene expression through effects on transcription factor (TF) binding [14]. Transcriptional regulatory domains (cis-acting elements) residing in the promoter or intronic region of genes
are short sequences (typically ~6–20 bases) which serve as the binding sites for TFs. SNPs that change the recognition site can potentially increase or decrease TF binding efficiency, leading to temporal and spatial alterations in gene expression and/or changes in the level of gene expression. Alternatively, SNPs in the promoter region can impart new TF binding properties, resulting in a gain-of-function. For example, the minor “A” allele of a SNP found in the promoter of the tumor necrosis factor gene creates a de novo binding site for the OCT-1 TF (Figure 1B), leading to increased transcriptional activity [15]. By comparison, the same promoter containing the predominant “G” allele does not bind OCT-1 [15]. Finally, another gene regulatory region that can be affected by SNPs is the 5′ or 3′ untranslated region [14]. These regions are located on either end of the transcribed mRNA molecule and play a role in the post-transcriptional regulation of the mRNA either by translational repression or changes in mRNA stability. The post-transcriptional control is mediated by the binding of regulatory factors (proteins or short 19–21 nucleotide long non-coding RNA molecules known as microRNAs) onto sequence motifs residing in the untranslated region of the mRNA [16, 17]. SNPs targeting these motifs in the 3′ untranslated region have been associated with changes in mRNA stability resulting from alterations in regulatory protein [18] or microRNA binding characteristics [19].

**SNPs affecting alternative splicing**

In addition to modifying the sequence of the encoded protein and affecting gene expression, SNPs can drastically alter the splicing pattern of mRNAs. A typical human pre-mRNA molecule contains an average of 8 exons with internal exons averaging 145 nucleotides in length, and between each exon is an intervening intron that averages >10 times the size of an exon [20]. Alternative splicing of a pre-mRNA molecule is a fundamental mechanism allowing cells to produce structurally and functional distinct proteins from a single gene by catalyzing a ‘cut-and-paste’ reaction that removes introns and joins together different combinations of exons. The end result is the formation of different mature alternatively spliced mRNAs. It has been estimated that ~60–70% of the genes in the human genome have the capacity to generate alternative mRNA species by the mechanism of alternative splicing [20, 21], and that ~80% of the alternatively spliced mRNAs results in changes in the amino acid sequence of the encoded protein [22], providing proteomic diversity to our genome (see review [23]). The control of alternative splicing involves the splicing machinery (comprised of small nuclear ribonucleoproteins and >100 proteins) that recognizes sequence regulatory domains found in the exonic and intronic splicing enhancers, exonic and intronic splicing silencers, the 5′ terminus of an intron defined by an GT dinucleotide motif (splice donor site), and the 3′ splice site end of an intron consisting of an A nucleotide branch point followed by a pyrimidine-rich track and the conserved AG dinucleotide splice acceptor site [24]. SNPs appearing in any of these regulatory domains can result in a disruption of normal splicing leading to the generation of abnormal mRNA species due to exon skipping, exon inclusion or intron retention (Figure 2) [23]. Consequently, the abnormal mRNA species may encode, for example, a truncated protein or significantly altered protein sequence which in either case will likely be non-functional. In the sections below, several examples are provided detailing how SNPs can affect gene products targeted by anticancer drugs, leading to an adverse drug reaction in patients that have inherited the detrimental allele.

**Polymorphisms in the thiopurine S-methyltransferase gene**

The thiopurine prodrugs 6-mercaptopurine (6-MP) and azathioprine are clinically employed in the treatment of acute lymphoblastic leukemia and as an immunosuppressant, respectively [25, 26]. Both of these agents undergo enzymatic conversion via the purine salvage pathway to the active antimetabolite 2′-deoxy-6-thioguanosine 5′-triphosphate, which is incorporated into DNA resulting in target cell cytotoxicity. Conversely, the thiopurine prodrugs can be
inactivated through methylation by the enzyme thiopurine S-methyltransferase (TPMT). Any disturbance in the metabolic balance between prodrug activation and inactivation, such as a reduction in TPMT activity, can lead to life-threatening bone marrow toxicity and myelosuppression.

The activity of TPMT is influenced by the presence of SNPs where there exist at least 24 TPMT alleles, but only a handful of which have known clinically relevant effects [27, 28]. Large inter-individual variability across the patient population can be observed in terms of the rate of thiopurine methylation and inactivation. Alleles for low- and high-activity TPMT are inherited in an autosomal co-dominant fashion, resulting in a trimodal distribution within the Caucasian population consisting of 89% possessing high (normal) enzyme activity, 11% with intermediate activity and 0.3% with low activity [29–31].

The wild-type (predominant) allele is designated as TPMT*1 and exhibits full enzymatic activity while the variant alleles (TPMT*2 – TPMT*24) have been associated with slightly to drastically reduced activities relative to the predominant allele [28]. Variant alleles TPMT*2 (G238C; Ala80Pro), TPMT*3A (G460A/A719G; Ala154 hr/Tyr240Cys), TPMT*3B (G460A; la154 hr) and TPMT*3C (A719G; Tyr240Cys) involve non-synonymous coding polymorphisms that result in alterations in the sequence of the encoded protein (Figure 3), and account for >80% of individuals with low TPMT activity [27, 29]. Patients homozygous for the TPMT*3A allele have negligible TPMT activity and consequently are at increased risk for life threatening myelosuppression upon treatment with standard doses of thiopurines [32, 33]. To circumvent the severe hematopoietic toxicity, TPMT activity-deficient patients can be treated with one-tenth of the conventional dose. Patients with TPMT*3B have a 9-fold reduction and those with TPMT*3C display a <2-fold reduction in enzyme activity compared to TPMT*1-expressing individuals [34, 35].

The molecular basis for the reduced TPMT activity associated with variants TPMT*2 and TPMT*3A appears to be a consequence of enhanced proteolytic degradation of the proteins encoded by these variant alleles [36]. Moreover, the degradative process is facilitated by the chaperone proteins heat shock protein 70 and heat shock protein 90, which have been demonstrated to preferentially bind and target the TPMT*3A protein to the ubiquitin-proteasome [37]. By comparison, the wild-type protein encoded by TPMT*1 is much less physically associated with the heat shock proteins [37].

Based on compelling pharmacogenetic evidence over the past decades, the FDA has included genotyping information in the drug label for 6-MP and recommends the usage of genotyping to guide dosing [38, 39]. The implementation of TPMT genotyping allows oncologists to be informed of patient risk for developing chemotherapy-associated toxicities with thiopurine treatment. Thus, genotype-tailored dosing for the individual patient holds the promise of minimizing adverse drug reactions.

5-flourouracil treatment and polymorphisms of the thymidylate synthase and dihydropyrimidine dehydrogenase genes

The pyrimidine analog 5-flourouracil (5-FU) is an antimetabolite used as an adjuvant or in the palliative treatment of a number of tumors, including pancreatic, head and neck, and breast cancers. This antimetabolite represents a mainstay as a chemotherapeutic drug for colorectal carcinomas, which is one of the leading causes of cancer mortality in the U.S. with approximately 100,000 new cases and >50,000 deaths each year [40]. 5-FU is readily transported into tumor cells (and normal cells) where it undergoes anabolic conversion into a number of active cancer-killing species [41]. One of the active metabolites, 5-flouro-2 4 deoxyuridine-5 4 monophosphate (FdUMP), in combination with 5,10-
methylene tetrahydrofolate forms a stable ternary complex with the enzyme thymidylate synthase [42]. The ensuing suicide complex inhibits normal synthesis of deoxynucleotides, resulting in an imbalance of deoxynucleotides, DNA damage and tumor cell cytotoxicity [41]. Thymidylate synthase is ordinarily responsible for the reductive methylation of the pyrimidine nucleotide deoxynucleotide to produce deoxynucleotide, with the methyl group coming from the methyl donor 5,10-methylenetetrahydrofolate.

There is a growing view that alterations in thymidylate synthase levels may be associated with 5-FU resistance in cancer cells [43]. The UTR of the human thymidylate synthase gene contains a variable number of tandem repeats (VNTR) polymorphism comprising of two (2R allele), three (3R allele) or more 28-base pair repeated sequences [44–46] (Figure 4). Early in vitro and clinical studies demonstrated that the expression of reporter gene constructs containing the 3R allele was 2 times higher than constructs containing the 2R allele [45], and the 3R allele was associated with higher thymidylate synthase mRNA and protein levels in colorectal/gastrointestinal cancers [47, 48]. From a treatment standpoint, colorectal cancer patients homozygous for the 3R genotype have a poorer antitumor response to 5-FU [47, 49–51]. It should be noted that the relationship between 2R/3R genotype and 5-FU response outcome appears to extend beyond colorectal carcinomas and includes for example acute lymphoblastic leukemia [52]. More recent studies indicate that the prediction of 5-FU response outcome may be more nuanced than simply genotyping a patient for the 2R and 3R alleles by polymerase chain reaction. The appearance of a G/C polymorphism in the 3R VNTR appears to stratify even further the response outcome of 5-FU-treated colorectal cancer patients (Figure 4), with the “3G” SNP in the 3R locus being associated with high thymidylate synthase levels and poor chemotherapy response and the “3C” SNP associated with low thymidylate synthase levels and a more positive treatment outcome [53, 54]. Taken together, evidence is compelling for the routine implementation of thymidylate synthase genotyping to identify patients who are likely to benefit from 5-FU treatment. At that the present time, however, the FDA has yet to recommend genotyping of the thymidylate synthase gene for patients scheduled to receive 5-FU treatment [38].

The bioavailability of 5-FU in patients is poor as the liver enzyme dihydropyrimidine dehydrogenase rapidly degrades up to ~80% of the administered antimetabolite to dihydrofluorouracil [55, 56]. Cancer patients with a deficiency in dihydropyrimidine dehydrogenase are at risk of developing life-threatening neurotoxicity and myelosuppression due to excessive anabolic conversion of undegraded 5-FU to active metabolites. In ~25–50% of the patients suffering from severe 5-FU toxicity, the associated dihydropyrimidine dehydrogenase deficiency can be traced back to an inherited SNP (G-to-A transition) in the invariant GT splice donor site contained in an intron that flanks exon 14, leading to exon skipping and an inactive encoded enzyme [57–61] (Figure 5). It has been estimated that ~3% of the population are heterozygous and 0.1% are homozygous for the inactivating A allele [61–63]. Additional polymorphisms and/or mutations potentially associated with reduced dihydropyrimidine dehydrogenase activity have been identified [60, 64]. To date, more than 40 variants are known to exist, including frameshift mutations, nonsense mutations, intronic mutations and 27 non-synonymous SNPs or mutations [60, 64]. Given the prevalence of the above pharmacogenetic data, the FDA has recommended genotyping of the dihydropyrimidine dehydrogenase gene in patients receiving 5-FU treatment [38].

**Polymorphisms in drug transporters**

Cancer cells exposed to anticancer agents have the capacity to develop resistance by employing a number of mechanisms such as decreased drug importation, alterations in target protein, increased metabolism/detoxification of the drug, and increased drug efflux [65].
ATP-binding cassette (ABC) transporter family plays a critical role in the regulation of drug absorption, distribution and excretion. At this time, there are at least 48 family members, subdivided into 7 subfamilies (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG), ranging in protein size from several hundred to several thousand amino acids [66]. The over-expression of specific members of the ABC transporter family in cancer cells has been associated with increased anticancer drug efflux resulting in multidrug resistance, a major obstacle for successful cancer chemotherapy [67–69]. Given that genetic polymorphisms in ABC transporter genes have the potential to alter protein function and/or expression, it is not surprising that numerous studies have been initiated in the search for sequence variants linked to drug responsiveness in patients.

So, do polymorphisms in ABC transporters modify patient response to anticancer agents? Taken as a whole, the majority of clinical studies to date has been relatively small and performed as single institutional studies as opposed to large-scale multi-institutional undertakings, and consequently lack sufficient statistical power to draw consistent conclusions. The clinical consequences of transporter sequence variations in a number of different cancers have been either inconclusive or contradictory and thus remain to be fully delineated [43, 70–76]. It is clear that future large-scale clinical investigations are warranted pertaining to genetic polymorphisms and ABC transporter genes. Nonetheless, there are noteworthy examples (see below) that merit additional scrutiny.

**Multidrug resistance 1 (MDR1) gene**

The MDR1 gene (also known as ABCB1) encodes P-glycoprotein and represents one of the most extensively studied anticancer drug resistance transporters in pharmacogenetics. For example, the ABCB1 variant C3435T has been associated with decreased drug efflux activity, and a favorable clinical outcome for locally advanced breast cancer patients on preoperative anthracycline antibiotics [77]. Of interest is the finding that the C-to-T transition in exon 26 does not change the amino acid sequence of P-glycoprotein (i.e. synonymous SNP), rather the T allele is linked to lower MDR1 gene expression in intestinal enterocytes compared with the C allele [78]. A similar positive clinical outcome associated with the C3435T variant has been reported for acute myeloid leukemic (AML) patients, where expression of the T allele was lower in patient AML blast cells compared to the corresponding C allele [79]. It remains to be definitively determined from these studies whether treatment outcome is a consequence of polymorphic transporters in the cancer cells themselves (pharmacodynamic effect), in the intestinal enterocytes mediating drug excretion (pharmacokinetic effect), or both.

**Breast cancer resistance protein (BCRP) gene**

The BCRP gene (also known as ABCG2) was first identified in a mitoxantrone-resistant breast cancer cell line that did not over-express either ABCB1 or ABCC1 [80]. In a later prospective pilot study, ovarian and small cell lung cancer patients with a non-synonymous C421A (Gln141Lys) variation of the BCRP gene were shown to have elevated blood levels of the orally administered anticancer drug topotecan, a topoisomerase I inhibitor [81]. The authors of the pilot study subsequently demonstrated in vitro that the A allele was associated with a 30% decrease in efflux transport of topotecan compared to the C allele, and speculated that the increased oral bioavailability of topotecan is a reflection of decreased intestinal excretion by the C421A variant [81]. In addition to the observed pharmacokinetic effects, it is noteworthy that the C421A variant has been linked to increased risk and poor survival of diffuse large B-cell lymphoma [82]. Clearly, additional larger-scale investigations are warranted to confirm these findings.
Conclusions and future directions

It has been estimated that over half of all adverse drug reactions can be traced to the expression of polymorphic genes. Anticancer drugs typically have a very narrow therapeutic index, and the use of these agents at maximal tolerated doses renders a subgroup of individuals at higher risk to life-threatening toxicities due to the inheritance of specific polymorphisms in genes encoding target proteins and drug metabolizing enzymes. This review has served to highlight select prototypical examples, namely polymorphisms in the: i) TPMT gene leading to thiopurine toxicity, ii) dihydropyrimidine dehydrogenase gene resulting in adverse reactions to 5-FU, and iii) thymidylate synthase gene giving rise to 5-FU treatment failure. Additionally, genetic variation in ABC transporter genes such as MDR1 and BCRP can affect cancer risk and clinical outcome to anticancer agents. A thorough characterization of all genetic polymorphisms in the human genome coupled with an understanding of their role in clinical endpoints should facilitate the rational selection of cancer chemotherapeutic drug(s) and dosage for the purposes of individualizing patient treatment regimens (i.e. personalized or targeted medicine).

There are a number of potential obstacles to be addressed in our pursuit of targeted medicine. The implementation of next generation sequencing platforms will be needed to expedite the identification of rare SNPs and grow the SNP database from the International HapMap Project [83]. Accordingly, our ability to precisely classify alleles linked to adverse drug reactions will be enhanced as a result of the additional genomic information. Moreover, technology platforms are under continual development in our quest for the ‘$1000 genome’ [84], and the lessons learned from these endeavors will enable pharmacogenetic and pharmacogenomic testing to be more reliable, accurate and accessible. Additional hurdles will need to be overcome such as greatly increasing the number of patients in pharmacogenetic studies in order to strengthen the statistical power of associating polymorphisms to variable drug responses in the clinical setting. This should be realized as sequencing costs for genotyping continue to descend. Another area that needs further investigation and emphasis is cancer health disparities. For example, African Americans have a higher incidence and/or mortality for breast, lung, prostate and colon cancers compared to non-Hispanic white women [85–89]. For at least some of these cancers, the disparities persist even after accounting for cultural, socioeconomic and access to health care differences [87, 89]. A major biological component for the disparities appears to be due in part to population-based polymorphisms residing in genes targeted by anticancer drugs [86, 90].

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LITERATURE CITED


Figure 1. Functional consequences of SNPs

(A) SNPs residing in the protein coding sequence of a gene can lead to synonymous or non-synonymous changes. The latter modifies the amino acid sequence (missense) of a protein or results in early termination of protein translation (nonsense), both potentially producing a non-functional protein. (B) SNPs in the promoter or regulatory enhancer regions of a gene can lead to changes in transcriptional activity. In the human tumor necrosis factor (TNF) gene promoter, the predominant G allele at nucleotide position -376 does not bind the OCT-1 transcription factor. The minor A allele at position -376 creates a de novo binding site for the OCT-1 TF, leading to increased transcriptional activity. Lines represent introns, boxes are exons. Exons and introns are not drawn to scale.
Figure 2. SNP leading to inappropriate mRNA splicing

Alternative splicing of a gene generates variable mRNAs, a process by which functionally diverse protein isoforms can be expressed in the cell. The 5′ and 3′ ends of the intronic regions (lines) of genes contain conserved GT and AG dinucleotide motifs serving as splice donor and acceptor sites, respectively (top panel; correct splicing). A SNP (G-to-A transition) occurring in the GT splice donor site of an intron following exon 2 leads to inappropriate slicing (bottom panel; inappropriate splicing). The consequences of inappropriate splicing can be skipping of exon 2 or retention of the intron containing the A allele SNP. In both cases, the encoded protein is likely to be non-functional. Lines are introns, boxes are exons.
Figure 3. Polymorphisms in the thiopurine S-methyltransferase gene
The predominant allele TPMT*1 encodes an enzyme with full activity. Minor alleles TPMT*2, TPMT*3A, TPMT*3B and TPMT*3C contain one or two non-synonymous coding SNPs leading to changes in amino acid sequence of thiopurine S-methyltransferase. The encoded proteins derived from these minor alleles have significantly reduced to no enzyme activity. Lines represent introns, closed boxes are the translated regions, and open boxes are the untranslated regions of the gene. Exons and introns are not drawn to scale.
Figure 4. 3R and 2R alleles in the thymidylate synthase gene

The 5′-untranslated region of exon 1 of the thymidylate synthase (TYMS) gene contains a 28-base pair repeat polymorphism (variable number of tandem repeats), mainly in the form of two repeats (2R) or three repeats (3R). Contained within the second repeat is a C/G SNP, producing the following alleles: 2R/2G, 2R/2C, 3R/3G and 3R/3C. Colorectal cancer patients genotyped as having one or two copies of the 3R/3G allele exhibit a poor response to 5-FU treatment. Lines represent introns, closed boxes are the translated regions, and open boxes are the untranslated regions of the gene. Arrows represent the 28-base pair repeats found in the 5′-untranslated region. Exons and introns are not drawn to scale.
Figure 5. Polymorphism in the dihydropyrimidine dehydrogenase gene
The dihydropyrimidine dehydrogenase (DPYD) gene contains a G/A SNP in the splice donor site of an intron flanking the 3′-end of exon 14. Individuals inheriting the A allele have inappropriate splicing of the DPYD gene, leading to the skipping of exon 14 and the translation of a nonfunctional enzyme. Cancer patients with a deficiency in DPYD cannot properly degrade standard doses of 5-FU and are at risk of developing life-threatening neurotoxicity and myelosuppression. Lines represent introns, boxes are exons. Exons and introns are not drawn to scale.