Silent Polymorphisms Speak: How They Affect Pharmacogenomics and the Treatment of Cancer

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Abstract
Polymorphisms in the human genome contribute to wide variations in how individuals respond to medications, either by changing the pharmacokinetics of drugs or by altering the cellular response to therapeutic agents. The goal of the emerging discipline of pharmacogenomics is to personalize therapy based on an individual’s genotype. Due to the relatively large frequency of single-nucleotide polymorphisms (SNP) in the human genome, synonymous SNPs are often disregarded in many pharmacogenomic studies based on the assumption that these are silent. We have shown recently that synonymous SNPs in ABCB1 (P-glycoprotein), which is implicated both in determining drug pharmacokinetics and multidrug resistance in human cancer cells, can affect protein conformation and function. We discuss the importance of polymorphisms in drug metabolizing enzymes and transporters in anticancer therapy and suggest that synonymous polymorphisms may play a more significant role than is currently assumed. [Cancer Res 2007;67(20):9609–12]© 2007 American Association for Cancer Research.

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Should Synonymous Single-Nucleotide Polymorphisms Be Ignored?
Clinical observations beginning in the 1950s suggested that individuals exhibit differences in their responses to drugs and that these variations could be inherited (1). Numerous studies over the next few decades clearly established that genetic factors influence the heterogeneity of individual responses to medications with respect to both toxicity and efficacy (1). Although it was recognized that medical practice based on population responses did not reflect the best treatment for an individual (2), the difficulties associated with identifying the genetic determinants of drug sensitivity and toxicity in individual patients were almost insurmountable (1). The dramatic expansion in the development of functional genomics, bioinformatics and high-throughput screening that followed the completion of the human genome project has, however, led many authorities to predict that the dream of personalized medicine may soon be a reality. Analysis of the sequence of the human genome has shown that the extent of genetic variation in the human population is far greater than had been estimated (3), and the most common sequence variation is the single-nucleotide polymorphism (SNP). SNPs (which by definition have a minor allele frequency of >1%) are major determinants of variations in disease susceptibility, response to medication, and toxicity (4). As SNPs occur at a frequency of ~1 per 100 to 1000 bp, several strategies have been advocated to systematically or rationally reduce the number of SNPs that need to be studied. Risch, for example, classified SNPs into five types that constitute a hierarchy of importance (see Table 2 in ref. 4). One casualty of this approach has been the synonymous SNPs (which do not alter amino acid residues) based on the assumption that these are “silent” and do not affect protein expression or function.

Genetic studies have indicated for several years that synonymous mutations can have significant “fitness consequences” and that there is a bias (enforced by evolution) in the use of redundant codons (5). Thus, for example, a comparative study of five mammals found 200 sites of “extreme conservation” among the species that have very few synonymous mutations (at least thrice fewer synonymous compared with nonsynonymous mutations; ref. 6). The authors speculated that the synonymous codons are under evolutionary selection because they overlap mRNA processing motifs. These could include splice sites and sites for micro-RNAs.

However, there is very little data available to prove or disprove this hypothesis, and as we shall see, other explanations for conservation of synonymous mutations are plausible. The Ka/Ks test, based on the conjecture that synonymous substitutions (Ks) usually occur much more frequently than nonsynonymous ones (Ka), is often used to predict the protein-coding regions of genomic sequences (7). The underlying assumption is that synonymous substitutions would have negligible effects on the phenotype and be under reduced selection pressure. However, Xing and Lee carried out a systematic study on 925 transcript-confirmed alternatively spliced exons and found that the Ka/Ks test failed in a significant proportion of cases (8). Very recently, we have reported that synonymous SNPs alter the interaction of the ABC transporter ABCB1, with its substrates and inhibitors plausibly mediated by translational pauses induced by rare codons, because the synonymous SNPs that produce the phenotype all use relatively rare codons (9). Concomitantly, Diatchenko and coworkers have provided evidence that synonymous SNPs can affect protein expression (and thus function) by alterations in the stability of the mRNA (10). In addition, it has been shown that a synonymous SNP of the corneodesmosin gene leads to increased mRNA stability, and haplotypes that have this SNP are more likely to develop psoriasis (11). These studies suggest that excluding synonymous mutations in pharmacogenetic studies may be too simplistic and based on naive assumptions (Fig. 1).
How Synonymous SNPs of Multidrug Resistance 1 Affect Protein Folding and Function

The human multidrug resistance 1 (MDR1) or ABCB1 gene codes for P-glycoprotein, a plasma membrane protein that is clinically important because cancer cells that overexpress this protein evade chemotherapy and exhibit the MDR phenotype (12). P-glycoprotein is also a key player in the absorption, distribution, metabolism, excretion, and toxicity (ADMET) system used to described pharmacokinetics and pharmacodynamics of drugs (13). The most common SNPs in human MDR1 are the synonymous 1236C>T and 3435C>T changes and the nonsynonymous 2677G>T change. Moreover, the 1236C>T/2677G>T/3435C>T combination constitutes the most common haplotype in Chinese, Indian, and Malay populations, representing 31% to 49% of the population. The fact that some SNPs and/or haplotypes represent a large fraction of the population (or certain subpopulations) raises the question of what selective advantages these polymorphisms might confer.

To determine whether this haplotype affects the MDR phenotype, we studied the three SNPs individually and as a haplotype in a transient expression system. All variants were expressed at equivalent levels at the cell surface and showed comparable function when we measured the transport of several fluorescent substrates using flow cytometry. There was, however, a reduction in the extent of reversal of transport by the P-glycoprotein modulators verapamil and cyclosporine A in the haplotype. Current dogma would have had us predict that this change in phenotype would be caused by the nonsynonymous SNP. The surprising observation, however, was that the nonsynonymous SNP alone had no effect; our next question was how the synonymous SNPs 1236C>T and 3435C>T alter the interaction of P-glycoprotein with some modulators (albeit in combination with a nonsynonymous SNP or with each other). Splice variants and mRNA stability could explain our observation, but a full-length mRNA was found and its levels and P-glycoprotein protein levels and localization were unchanged in the haplotype. Alternative amino acids could, in theory, be introduced as substitutes for the amino acids encoded by the haplotype protein. However, protein sequencing of the wild-type P-glycoprotein and the haplotype by mass spectrometry resulted in identification of 82 peptides (representing 37% of the P-glycoprotein sequence by amino acid count). Each of these sequences was identical to the sequence of haplotype P-glycoprotein. Moreover, several different peptides encoded by the synonymous SNP (3435C>T), which is the key polymorphism linked to the functional change in P-glycoprotein, were sequenced and found to be unchanged. These observations suggested that

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**Figure 1.** Synonymous SNPs and changes in protein structure and function. Synonymous SNPs have often been called silent, i.e., unable to affect functional changes. However, recent reports indicate that there are several mechanisms by which synonymous mutations could bring about such changes. These may have important implications in biology and in the diagnosis and treatment of human cancers and other diseases.
splice variant alterations in mRNA stability and encoded amino acid substitutions were unlikely explanations for the P-glycoprotein phenotype. The use of the conformation-sensitive antibody against P-glycoprotein, UIC2, and limited trypsin digestion showed that there were differences between the wild-type and the haplotype P-glycoproteins, suggesting that it was plausible that there were subtle but measurable differences in the conformation at sites where drugs and modulators interact (9).

How does one explain the fact that proteins with identical primary sequences could exhibit different conformations, leading to a functional change? The moniker “silent” to describe a synonymous mutation or SNP comes from Anfinsen's principle that the amino acid sequence of a protein alone determines the three-dimensional structure of a protein and, hence, its function. Genetic and biochemical studies have, however, suggested for a long time that such a view may be too simplistic. There is clear evidence that synonymous codons are not used randomly, that preferred codons correlate strongly with the relative abundance of the corresponding tRNAs, and that natural selection acts on synonymous mutations (see ref. 14 and references therein). There is a rapidly growing list of synonymous mutations that lead to human diseases (for list, see ref. 15). Studies also show that proteins fold while being translated and that “cotranslational folding” leads to different intrapeptide contacts during folding from those that occur during refolding of the covalently intact but unfolded polypeptide (16). It has, moreover, been suggested that, in addition to the thermodynamic control of protein folding, there may also exist a kinetic control and that translational pauses may be necessary for proteins to fold correctly (17). The kinetics could be influenced by codon bias because the rate of translation would be rapid over mRNA stretches that use frequent codons and slower when rare codons are used. Although a few studies in cell-free translation systems support these ideas, similar studies are extremely difficult to design and interpret using in vivo systems.

P-glycoprotein has two significant advantages as a model system for monitoring subtle changes in tertiary structure as a consequence of changes in codon usage. First, P-glycoprotein transports a broad range of compounds (12), which suggests structural flexibility at the transport substrate site may be favored and not eliminated by the quality control machinery of the cell. Second, the large repertoire of transport substrates and modulators allows numerous combinations to assay for subtle changes in the conformation of the “active” site. Our observations that cyclosporine A and verapamil (which reverse the MDR phenotype in P-glycoprotein) were less effective for the haplotype (where the synonymous codons change from frequently used to rarely used) and that substitution of an even rarer codon accentuated the phenotype (9) suggest that the most likely explanation is a change in the local rate of translation due to the use of rare codons.

Cancer Chemotherapy and the Promise of Pharmacogenomics

The recognition that an individual's genetic make up influences both the response to a medication and toxicity (pharmacogenetics) is not new, but applying the whole genome approach to this problem (pharmacogenomics; ref. 1) may allow the application of individualized medicine in the clinic. The promise of pharmacogenomics lies in maximizing the efficacy of a medical intervention while minimizing the toxicity. It is widely accepted that such a need is most pronounced in anticancer treatments due to the inherent toxicity of the drugs used and the wide variability in individual responses (18). Ratain and coworkers have, for example, presented a very detailed analysis of the potential effect of pharmacogenomics using irinotecan, 5-fluorouracil, and epidermal growth factor receptor inhibitors as specific examples (18). There is also the recognition that many emerging anticancer agents will not affect cures but act as disease-stabilizing agents (19). Traditional drug development strategies rely on defining the maximum tolerated dose based on the assumption that the medication will be given over relatively short durations. Disease-stabilizing agents, on the other hand, require long-term (often life long) adherence and require a paradigm shift in determining the maximum effective dose rather than the maximum tolerable dose (20). Pharmacogenomics would have incalculable benefits in such instances and could easily justify the additional costs associated with personalized medicine that is a cause of concern for many health economists (21).

Finally, the risks and costs associated with adverse drug reactions are not trivial. A metaanalysis of 33 studies between 1966 and 1996 showed that, even compensating for heterogeneity among studies and biases, a conservative estimate would place fatal adverse drug reactions between the fourth and sixth leading cause of death in the United States (22). A large part of these arise because of SNPs, occurring singly or as haplotypes, in drug targets or key drug metabolizing enzymes or transporters and cannot be predicted in the absence of genotyping. Irinotecan provides a good example (23) of how pharmacogenetics may be used to manage the marked interpatient variability in cancer therapy due to the complex pathways that involve both metabolic enzymes and transporters (24). The active moiety of irinotecan, SN-38, undergoes glucuronidation by uridine diphosphoglucuronyl transferase (UGT)-1A1, and the metabolites are effluxed via membrane transporters, such as P-glycoprotein and MRP1. Studies have found that polymorphisms in the genes coding for P-glycoprotein and UGT-1A1 influence irinotecan pharmacology and treatment outcomes (see ref. 18 and references therein). These findings are directly applicable in the clinic, and they are now being incorporated into the patient package insert by the Food and Drug Administration (25). Significantly, many of the SNPs in the MDR1 gene have diverse effects on human physiology and disease states. For example, SNPs in the MDR1 gene are associated with the response of transplant patients to administration of cyclosporine A or FK506 (26), and the incidence of Crohn's disease, inflammatory bowel disease (26), or Parkinson's disease may be related to the frequency of the specific haplotypes. Moreover, an association between SNPs in the promoter region of MDR1 and colorectal cancer has been documented (27), suggesting a role for these polymorphisms in cancer progression and not only in the treatment outcomes mediated by MDR1.

Implications for Disease Prediction and Response to Treatment

Our study of the 1236C>T/2677G>T/3435C>T haplotype of MDR1, described above, adds an additional dimension to the role of SNPs in individual treatment outcomes. We showed that synonymous SNPs in MDR1 could affect P-glycoprotein protein function (9). There have been several reports of associations between synonymous SNPs and human disease. For example, a synonymous SNP in the apoE gene is associated with a higher risk of developing Alzheimer's disease (28), and a recent study shows that SNPs in the low-density lipoprotein receptor-related protein 6 gene are
associated with late-onset Alzheimer’s disease (29). The variant is a haplotype that includes a synonymous SNP in exon 18. Our studies suggest that protein conformational changes could be contributing to clinically important phenotypes.

It is noteworthy that the model system we used was the human ABC transporter P-glycoprotein, which is implicated in MDR during chemotherapy. This is a significant obstacle to the successful treatment of cancer, because chemotherapy is the only treatment option for almost half of all cancers, and ~90% of those develop MDR. In the 20 years since the discovery of P-glycoprotein, there has been a concerted effort to develop inhibitors of P-glycoprotein to be used in conjunction with chemotherapeutic agents. Although multiple generations of MDR reversal agents have entered clinical trials, none have made it into clinical practice.

Unmanageable side effects and toxicity have been the major causes of failure for these agents (12). In addition, it is extremely difficult to design studies wherein P-glycoprotein function against the chemotherapeutic agent has been clearly shown. Furthermore, variation in the MDR1 gene is high, with over 35 reported SNPs, and there is also considerable variability in the frequency of individual SNPs as well as haplotypes across different populations and ethnic groups. Moreover, the literature on the "functional" effects of SNPs in the MDR1 gene is replete with contradictory findings (for review, see ref. 12). One reason for this may be lack of standardized methodology and assays. Furthermore, as our study illustrates, SNPs often result in very subtle functional outcomes. For example, the haplotype in 1236C>T/2677G>T/3435C>T did not result in a change in substrate transport per se but affected the inhibition of transport by a small subset of modulators (9).

Thus, not many studies published to date allow a prediction of whether the cancer cells carrying the relevant haplotype of P-glycoprotein would be expected to respond to the P-glycoprotein inhibitor being tested.

Conclusions

It is important to note that, although the alterations in P-glycoprotein related to synonymous polymorphisms seem to be subtle, the biological outcomes are significant. In addition to predicting altered pharmacokinetics of drugs, it may be necessary to define the effect of the more common P-glycoprotein SNPs and haplotypes on MDR modulators and a repertoire of anti-cancer drugs in vitro studies. Similarly, attention should be given to the synonymous polymorphisms in the secondary transporters and various types of ATPases, which may affect the absorption and/or excretion of drugs. These approaches would help to design clinical studies with more stringent inclusion-exclusion criteria that include genetic profiling. Such studies are more likely to meet with success than the current more broad-based approaches.

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