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Impact of Genetic and Epigenetic Variability in Response to Two Test Drugs 5-Fluorouracil and Lansoprazole

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IMPACT OF GENETIC AND EPIGENETIC VARIABILITY IN RESPONSE TO TWO TEST DRUGS 5-FLUOROURACIL AND LANSOPRAZOLE

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PHARMACOLOGY AND TOXICOLOGY

ABSTRACT

Pharmacogenetics has proven to be an invaluable tool in predicting variability in drug response; however, there are numerous incidences where genetics cannot fully explain interindividual drug variability. Other factors, such as the epigenetic mechanism of DNA methylation, may offer an alternate explanation. Therefore, this dissertation focuses on both genetic and epigenetic regulation in order to gain a better understanding of the molecular basis behind drug response variability.

Initial investigations focused on the antineoplastic agent 5-Fluorouracil (5-FU), which can produce severe toxicity in patients with deficiency in dihydropyrimidine dehydrogenase (DPD). Genotypic studies have identified >32 sequence variants in the *DPYD* gene; however, sequence variants could not explain the molecular basis of DPD deficiency in a number of cases. In a cohort of samples phenotypically characterized by enzyme assay and breath test, aberrant methylation of the *DPYD* promoter was detected using denaturing high performance liquid chromatography (DHPLC) in all six DPD-deficient individuals without inactivating mutations in their DPYD gene. Out of four DPD-deficient individuals genetically characterized as heterozygous for either *DPYD**2A or *DPYD**13, two showed varying DPYD promoter methylation, indicating that methylation of the *DPYD* promoter is associated with decreased DPD activity in clinical samples and may act separately or in concert with genetic variants to down-regulate DPD activity.

Genetic and epigenetic factors in drug response were also analyzed in 50 gastric acid hypersecretors requiring a wide dose range of a commonly prescribed proton pump inhibitor (PPI), Lansoprazole, for therapeutic acid suppression. Genetic analysis revealed that patients homozygous for the *CYP2C19*17* allele were unable to achieve acid suppression at high Lansoprazole doses, while heterozygotes achieved moderate to complete acid suppression at elevated Lansoprazole doses. Methylation analysis of the *ATP4B* gene showed associations between increased site specific methylation levels, decreased *ATP4B* gene expression, and elevated acid output (BAO).

Using two different examples, these studies further expand our knowledge of genetics and epigenetics in drug response variability, and show that both genetics and epigenetics can act separately or in concert to provide a better understanding of the molecular mechanisms in drug response.

DEDICATION

I dedicate this to my mom, Tammy Hays. Without your love, encouragement and sacrifice I would not have made it this far. I also dedicate this to my nieces, Abbie and Gracie Cook. You both inspire me to do my best, and I love you more than words could ever describe.

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
5meC	5-methylcytosine
ATP4A	gastric H^+/K^+ ATPase alpha subunit gene
ATP4B	gastric H^+/K^+ ATPase beta subunit gene
AUC	area under the curve
BCHE	butyrylcholine esterase
bp	base pair
CNV	copy number variation
CYP2C19	cytochrome p450 2C19
CYP2D6	cytochrome p450 2D6
DHPLC	denaturing high performance liquid chromatography
DMR	differentially methylated regions
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DPD	dihydropyrimidine dehydrogenase protein
DPYD	dihydropyrimidine dehydrogenase gene
EGFR	epidermal growth factor receptor
G6DPH	glucose 6 phosphate dehydrogenase
hMC	homogenous mass-cleave
IM	intermediate metabolizer

LOI	loss of imprinting
MAF	minor allele frequency
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
NAT2	arylamine N acetyltransferase
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PM	poor metabolizer
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
TEAA	triethylammonium acetate
T_m	melting temperature
T_{max}	time to maximum concentration
TPMT	thiopurine methyltransferase
UraBT	[2-^{13}C] uracil breath test
URM	ultra-rapid metabolizer
ZES	Zollinger-Ellison Syndrome

INTRODUCTION

Pharmacogenetics/Genomics in Drug Response

Archibald Garrod is considered one of the first pioneers referring to interindividual differences in drug response, which is now referred to in the mustache scientific community as pharmacogenetics. His famous quote in 1902 *"If it be indeed, the case that in alkaptonuria and the other conditions mentioned we are dealing with individualities of metabolism and not with the results of morbid processes, the thought naturally presents itself that these are merely extreme examples of variations in chemical behavior which are probably everywhere present in minor degrees and that just as no two individuals of a species are absolutely identical in bodily structure neither are their chemical processes carried out on exactly the same lines…and if we pass to differences presumably chemical in their basic idiosyncrasies as regards drugs and the various degrees of natural immunity against infection are only less marked in individual human beings and in the several races of mankind than in distinct genera, and species of animals"* [1], initiated a new era of scientific research geared towards understanding the molecular basis of interindividual differences in drug response and treatment outcome.

Since then it has been observed that inter-patient variability in response to medications is associated with a spectrum of outcomes, ranging from failure to demonstrate an expected therapeutic effect to an adverse reaction resulting in significant patient morbidity and mortality. While variability in drug response may be linked to a variety of clinical factors such as age, gender, race, organ function, and interactions

among drugs, the concept that genetic differences contribute to drug response is now widely accepted and has been validated in many research settings. The earliest experimentally validated examples of an effect of inheritance on drug response were first reported in the 1950s and 1960s. These studies stemmed from clinical observations of large differences among patients in their response to standard drug doses, including individual variations in plasma or urinary drug or drug metabolite concentrations. These early experimental examples led to the introduction of the term "pharmacogenetics", coined by Vogel to mean "knowledge of inherited variants with regard to the reaction to pharmaceuticals and other administered compounds" [2, 3].

At the present time, interindividual differences in drug response have been attributed to genetic and more recently epigenetic changes in the patients' molecular blueprint. Genetic variability includes single nucleotide polymorphisms (SNPs; a change in which a single base in the DNA differs from the wild-type sequence), haplotypes (a set of closely linked genetic markers present on the same chromosome that tend to be inherited together), microsatellites or simple adjacent sequence repeats (polymorphic loci present in DNA that consist of repeating units of 1-6 base pairs in length), insertion and/or deletions, copy number variations (CNVs; differences in the number of copies of a particular sequence present in the genome of an individual), aneuploidy (a change in the number of chromosomes that can lead to a chromosomal abnormality), and loss of heterozygosity (loss of one of the alleles). To date, there are approximately 12 million SNPs in the human genome in addition to other types of genomic variation [4]. Recently, advances in whole genome sequencing and improvements in bioinformatic tools in processing large sets of data have led to the transition of pharmacogenetics to

pharmacogenomics. Today, the terms pharmacogenetics and pharmacogenomics are often used interchangeably referring to the contribution of inheritance to variation in drug response. Pharmacogenetic and pharmacogenomic effects are also often further characterized by the factors that influence the concentration of a drug reaching its target (pharmacokinetic factors), and those that involve the target itself (pharmacodynamic factors).

The goal of the emerging disciplines of pharmacogenetics and pharmacogenomics is to personalize therapy based on an individual's molecular profile. Increasingly, pharmacogenetics and pharmacogenomics are becoming an integral translational component in almost all clinical trials investigating new therapeutic agents.

Examples of Pharmacogenetic Syndromes

The earliest experimentally validated examples of pharmacogenetic syndromes were first reported in the 1950s and 1960s. The following sections highlight historic pharmacogenetic syndromes selected on the basis of their historical and clinical significance.

Glucose 6 Phosphate Dehydrogenase (G6PDH)

One of the best known examples of a pharmacogenetic syndrome is glucose 6 phosphate dehydrogenase deficiency. G6PDH deficiency was identified due to the development of hemolysis in approximately 10% of African American soldiers during treatment with the antimalarial drug primaquine or related drugs, whereas this problem was almost absent in Caucasian Americans [5]. Electrophilic reactive metabolites of

many drugs are inadequately detoxified in carriers affected by the x-chromosomally inherited G6PDH mutations. There are currently more than 150 identified mutations in the *G6PDH* gene [4]. Because of this complex genetic background, analysis of the phenotype (enzyme activity in red blood cells) is still the preferred method of clinical diagnosis and drugs causing hemolysis should be avoided in G6PDH deficient patients.

G6PDH deficiency is a classic example highlighting the importance of interethnic differences in the risk–benefit ratio of drugs. These inter-ethnic differences in population frequencies have a worldwide economic impact. Pharmaceutical companies aiming at worldwide marketing of a drug should carefully consider the pharmacogenetic and genomic factors in the targeted market population.

Butyrylcholine Esterase (BCHE)

The muscle relaxant succinylcholine has a prolonged pharmacologic effect in about 0.1% of patients. A severe consequence of failure to recognize this condition may lead to fatalities due to apnea. The prolonged action of succinylcholine and mivacurium is due to an inherited lack of plasma cholinesterase, also known as butyrylcholine esterase (BCHE) [6]. It was demonstrated that the BCHE allele encoding the most common atypical form of the enzyme included a nonsynonymous coding SNP, 209 G>A, resulting in an Asp70Gly conversion that altered the active site of the enzyme [7]. Although genotyping for the underlying variant could be performed, measurement of BCHE activity is still preferred due to the simplicity of the assay.

Arylamine N Acetyltransferase (N-acetyltransferase type 2)

Acetylation is a phase II reaction that is important in the metabolism of several xenobiotics, including the anti-tuberculosis drug isoniazid. About half of Caucasian populations are rapid acetylators, whereas the other half are slow acetylators [8]. Arylamine N acetyltransferase (also known as the type 2 enzyme NAT2) is the enzyme responsible [9]. There are several amino acid substitutions that have been associated with low protein stability or low enzyme activity [10]. At the same dose of isoniazid, slow acetylators have high blood concentrations and, most likely, a better antibacterial efficacy, but with a greater risk of increased adverse effects. In contrast, the rapid acetylators have low parent active drug concentrations in their blood and tissues. Compared with the slow acetylators, isoniazid is less effective in this group with fewer adverse effects.

The acetylation polymorphism has been known for more than 50 years, however, the NAT2 polymorphism is still typically not assessed prior to administering isoniazid. Unfortunately, knowing about an apparently medically significant genetic polymorphism does not necessarily mean that physicians will use that knowledge to the benefit of the patient. The latest clinical trial on whether or not isoniazid dose adjustment according to NAT2 genotype really reduces isoniazid hepatotoxicity and improves therapeutic efficacy has not yet been completed [11], even 50 years after discovery of the NAT2 polymorphism.

Cytochrome p450 2D6: CYP2D6

The cytochrome P450s (CYPs) are a family of microsomal drug-metabolizing enzymes [12]. CYP2D6 is a member of that family that has been studied for the longest time and most intensively from a pharmacogenetic perspective. A complete lack of CYP2D6 enzyme activity was first identified in 1975 by two different laboratories based on the appearance of exaggerated adverse effects in patients receiving two different drugs, debrisoquine (an anti-hypertensive) and sparteine (an oxytotic) [13, 14]. Numerous variants including those associated with deficient activity (PM) and ultra-rapid activity (UM) have been identified in Caucasian populations [15-17]. The CYP2D6 PM phenotype could be reasonably well predicted from the genotype [18]. There is a wide range of enzyme activities ranging from a complete lack of enzyme activity in PMs, to very low activity in carriers of one deficient and one low-activity allele, to intermediate activity in heterozygous carriers of one active and one deficient allele, to high activity in the extensive metabolizers (EMs) and up to extremely high activity in UMs.

The clinical impact of the CYP2D6 genotype depends on whether the drugs are bio-activated by CYP2D6 or inactivated. CYP2D6 catalyzes the biotransformation of numerous drugs, including tricyclic antidepressants such as nortriptyline and antihypertensive and antriarrhythmic beta-blockers such as metoprolol [19-21]. It is also required for the metabolic activation of the analgesic prodrug codeine to form its active analgesic metabolite, morphine [22, 23]. Therefore, CYP2D6 PMs can potentially have an excessive drug effect when treated with standard doses of agents such as metoprolol that are inactivated by CYP2D6 [21], whereas codeine is relatively ineffective in PMs

because it requires CYP2D6-catalyzed metabolism to form morphine. Conversely, UMs may display an inadequate therapeutic response after treatment with standard doses of drugs inactivated by CYP2D6, but they can be "overdosed" with codeine – as reported recently for a patient who developed respiratory arrest after treatment with standard cough suppressant doses of codeine [24].

The cloning and characterization of the CYP2D6 cDNA and gene made it possible to characterize molecular mechanisms for this polymorphism, mechanisms that included nonsynonymous SNPs associated with decreased activity, gene deletion, and gene duplication associated with UM (multiple copies of the CYP2D6 resulting in ultra-rapid enzyme activity) [25]. Although CYP2D6 gene duplication is relatively infrequent among Northern Europeans, in East Africa the frequency of alleles with duplication of CYP2D6 is as high as 29% [17, 18]. To date, there are over 90 reported CYP2D6 alleles and haplotypes and their effect on CYP2D6 activity has been widely reported (http://www.imm.ki.se/CYPalleles).

It is not clear why the extensive variation of CYP2D6 is not yet regularly considered in clinical practice. Dose-dependent adverse drug effects (and, as a consequence, patient quality of life), as well as survival of breast cancer patients receiving tamoxifen may depend on CYP2D6 activity [26]. In order to promote the application of and further research on CYP2D6 pharmacogenetic genotyping in medical practice, dose-adjustment recommendations based on available published pharmacokinetic data have been derived [27-29]. These CYP2D6 genotype-based dosage recommendations illustrate how individualized medicine may work in daily medical practice.

Thiopurine Methyltransferase (TPMT)

The TPMT enzyme catalyzes the S-methylation of thiopurine drugs such as 6-mercaptopurine, 6-thioguanine and azathiopurine [30, 31]. These drugs are cytotoxic and immunosuppressive agents that are used to treat acute lymphoblastic leukemia, inflammatory bowel disease, as well as to suppress rejection in organ transplant recipients. Although useful drugs, thiopurines have a narrow therapeutic index and in toxic conditions can induce life-threatening myelosuppression (bone marrow suppression) [32, 33].. The most common variant allele for TPMT in Caucasians is *TPMT*3A* which occurs at a frequency of approximately 5% and is comprised of two nonsynonymous SNPs located in exons 7 (460 G>A; A154T) and 10 (719 A>G; Y240C) [34, 35]. The *TPMT*3A* allele results in decreased TPMT activity due to its rapid degradation by a ubiquitin-proteosome-mediated process [36-38]. Evidence also suggests that chaperone proteins such as the heat shock proteins hsp70 and hsp90 are involved in targeting the *TPMT*3A* variant allozyme for degradation [38]. Recent studies show that *TPMT*3A* can also form aggresomes, probably as a result of misfolding, followed by aggregation, as an additional mechanism [39]. At least 21 variant alleles of TPMT have been reported to be, or are suspected of being associated with decreased TPMT activity. TPMT variant alleles also show striking ethnic differences. For example, *TPMT*3A* is rarely observed in East Asian populations, but *TPMT*3C*, with only the Y240C SNP in exon 10, is the most common variant allele in those populations, with a frequency of approximately 2% [40, 41]. Subjects homozygous for the *TPMT*3A* allele are at greater risk for life-threatening myelosuppression when treated with standard doses of thiopurine drugs and are often treated with one tenth to one fifteenth of the standard dose under

careful clinical monitoring [42-45]. Because of its clinical importance, TPMT was the first example selected by the FDA for public hearings on the inclusion of pharmacogenetic information in drug labeling [25]. Clinical testing for TPMT genetic polymorphisms is also widely available (http://www.prometheuslabs.com).

Epidermal Growth Factor Receptor (EGFR)

In 2004, two separate studies reported that therapeutic response to the EGFR inhibitor gefitinib, used in the treatment of non-small cell lung carcinoma (NSCLC) which often shows EGFR overexpression, was greater in patients with mutations of the ATP binding site of the tyrosine kinase receptor, EGFR, when compared to wild-type (WT) subjects [46, 47]. Factors that influenced gefitinib response in NSCLC patients included female gender, absence of previous history of smoking, and East Asian origin [48]. For example, previous studies showed that somatic mutations in EGFR occurred in 15 of 58 randomly selected tumors obtained from patients in Japan, but in only 1 of 61 from the United States [47]. Another study also reported that, in the United States, these mutations occurred in 8/9 patients with gefitinib-responsive lung cancer, but were absent in all 7 studied patients who failed to respond to treatment with the drug [48]. Interestingly, one study reported on acquired drug resistance in a patient who had 'typical' EGFR somatic mutations identified in DNA from a previously gefitinib-sensitive tumor biopsy. Upon developing acquired drug resistance, re-biopsy of the tumor identified a new EGFR mutation that was thought to be the mechanistic explanation of gefitinib resistance development [49].

Clinical Translation of Pharmacogenetics

Pharmacogenetics and pharmacogenomics both depend on and contribute to our evolving understanding of the way in which variation in DNA sequence and structure influence the drug response phenotype. However, in addition to their role in the advancement of biomedical science, pharmacogenetics and pharmacogenomics are also translational disciplines. In spite of the fact that striking examples such as those outlined above have been described with increasing frequency during the past half century, the movement of this information across the translational interface has not been as rapid as might have been hoped. There are many reasons for this relatively slow pace, including those related to the evolving nature of biomedical science itself, those related to the drug discovery and development process, those related to the process of incorporating any new test into medical practice, and those related to the economic implications of medical innovation.

Approaches to Pharmacogenetics

Drug-related phenotypes can be analyzed using several approaches, such as analysis of a candidate single gene, candidate-pathway-gene approach (analysis of several functionally related candidate genes), and genome-wide approach (analysis of the whole genome). Initially, pharmacogenetic studies relied on clinical observation for the identification of inherited differences in drug effects [8, 50-52] in the form of severe drug toxicity or therapeutic failure, often referred to as phenotype observations. The candidate single gene approach relies on previous knowledge of the pharmacokinetic and/or pharmacodynamic parameters of the studied drug, disease pathology, and the biochemical

roles of proteins encoded by particular genes using a hypothesis-based study design. This prior knowledge facilitates the rational selection and investigation of the clinical relevance of genetic polymorphisms within a given set of candidate genes that may influence drug response. This methodology has been useful in the identification of numerous SNPs in mainly high-penetrance genes generally encoding drug-metabolizing enzymes, drug transporters, or drug targets producing distinct and easily recognized polymorphic phenotypes in drug response [53, 54]. Clinical relevance of genetic polymorphisms in drug-metabolizing enzymes as a molecular basis for variability in drug response is based upon the following factors: the polymorphism predicts enzyme activity, the particular enzyme activity predicts drug plasma concentration, and the drug plasma concentration predicts drug efficacy or toxicity.

Though the candidate single gene approach has historically been shown to be useful, a large proportion of individual variability in drug response has not been fully explained since most drug response phenotypes are thought to be determined by the interactions among multiple genes as well as with the environment. Therefore, the candidate gene strategy has limited value for identifying polygenic determinants of variability in drug response. The complexity inherent in the genetic determination of drug response warrants the use of methodologies that can evaluate the contribution of polymorphisms in several genes which may interact in additive, synergistic, or antagonistic ways to influence a single drug response. The candidate-pathway approach to the analysis of pharmacogenetic variation allows for the investigation of associations between genetic polymorphisms within a particular biological pathway of relevance to

the pharmacokinetics or pharmacodynamics of a particular therapeutic agent and, like the candidate gene approach, is hypothesis-driven [55].

Due to the evolution in genetic science and whole genome sequencing technology coupled with the advances in bioinformatic tools in processing large amounts of data, the whole-genome approach emerged which refers to the global study of genetic variants within the human genome. A genome-wide approach gives equal weight to all genes in the genome, is hypothesis-generating, and can be used when little is known regarding gene-drug effect. These studies are therefore not biased toward current knowledge of gene function and have the potential to identify multiple genetic variants that contribute to complex clinical traits. Recent advances in genomic technology such as the genome scale microarray genotyping platforms, microarray-based comparative genomic hybridization, and transcriptional level gene expression platforms have allowed researchers to perform genome-wide association studies (GWAS) between genetics and phenotypes.

As with all pharmacogenetic and pharmacogenomic studies, a clearly defined phenotype of interest is essential. Phenotypes evaluated in GWAS can be either qualitative/categoric factors or quantitative/continuous measures. The current pitfalls to the genome-wide approach include the large computational capacity required to analyze genome scale genetic data and the high risk of false discovery. Another substantial challenge associated with GWAS is the interpretation of statistically significant findings.

Given the similar goal and complementary nature of candidate gene and genome-wide approaches, one can imagine that a combined candidate and genome-wide approach

would likely provide a list of genetic variants that contribute to complex phenotypes that can be tested in clinical trials.

Epigenetics

Although interindividual differences in drug response are often attributed to genetic heterogeneity, many of these cannot be explained by DNA sequence variation. Other gene regulatory mechanisms, therefore, may offer an alternate explanation. The term epigenetics was first coined in 1942 by Conrad Waddington to describe the study of the processes by which genotype gives rise to phenotype [56]. The definition has evolved over the years to keep pace with the ever expanding knowledge, and the most current classification of epigenetics would be the study of the changes in gene expression that are mitotically and/or meiotically heritable and do not involve a change in the DNA sequence [57]. As opposed to the irreversible nature of genetic events, which introduce changes in the primary DNA sequence, epigenetic modifications are reversible and leave the original DNA sequence intact. There are three main, inter-related types of epigenetic inheritance: histone modification, genomic imprinting, and DNA methylation.

Histone Modification

There are numerous post-translational histone modifications, including acetylation, methylation and phosphorylation, which are important regulatory mechanisms in gene transcription that are stably maintained during cell division [58]. The best studied are those that occur on unstructured histone tails, which protrude from the nucleosome core particle and interact with DNA, other nucleosomes and many

chromatin-associated proteins. Certain tail modifications such as acetylation of lysine residues, alter charge, thus change the bulk electrostatic properties of nucleosomes and can potentially alter their interactions with other nuclear components [59]. Methylation of lysine residues in histones has been known for years but this modification was only recently recognized as crucially important for normal gene regulation [60, 61]. Histone methylation is also believed to be a contributor for the perpetuation of silent epigenetic states through cell divisions. The silent state can be maintained by a cycle of histone methylation, catalyzed by a histone methyltransferase SUV39H1. This is followed by recruitment of the binding protein heterochromatin protein-1 (HP1) to the lysine-9-methylated histone, which perpetuates the cycle through the recruitment of SUV39H1 [58].

Loss of Imprinting

Genomic imprinting is parent-of-origin-specific gene silencing, resulting from a germline mark that causes reduced or absent expression of a specific allele of a gene in somatic cells of the offspring. Imprinting is a feature of all mammals, affecting genes that regulate cell growth, behavior, signaling, cell cycle and transport. Imprinting is also a necessary component for normal development. Loss of imprinting (LOI) refers to activation of the normally silenced allele, or silencing of the normally active allele, of an imprinted gene. LOI of the insulin-like growth factor 2 gene (*IGF2*) accounts for half of Wilms tumors in children [62]. LOI of *IGF2* is also a common epigenetic variant in adults and is associated with a fivefold increased frequency of colorectal neoplasia [63, 64]. LOI of *IGF2* might cause cancer by increasing the progenitor cell population in the

kidney in Wilms tumors [62] and in the gastrointestinal tract in colorectal cancer [65]. Other genes that show LOI in cancer include *PEG1*/*MEST* (paternally expressed gene 1/mesoderm-specific transcript homologue) in lung cancer [66], *p57* ^{KIP2} (also known as *CDKN1C*) in pancreatic cancer [67], *DIRAS3* (GTP-binding RAS-like 3) in breast cancer [68], and *TP73* in gastric cancer[69].

DNA Methylation

Cytosine methylation is the most common base modification in the eukaryotic genome and is defined as the addition of a methyl group to the 5'-carbon of the pyrimidine ring to generate 5-methylcytosine (5meC) [70, 71]. 5meC is preferentially found in the context of 5'-CpG'3' (CpG) dinucleotides which are underrepresented in the human genome since 5meC has a relatively high propensity to spontaneously deaminate to thymine [72]. The methylation reaction is catalyzed by a family of DNA methyltransferase (DNMTs) which utilize S-adenosylemthionine (SAM) as a cofactor. Interestingly, the distribution of CpG sites in the human genome is not random with approximately 80% of all CpG sites are methylated and located primarily in repetitive sequences and the centromeric repeat regions of chromosomes [73]. The remaining 20% is unmethylated and preferentially found in short sequence stretches which range from 0.5 ro 5 kb that occur at average intervals of 100kb [74]. These stretches, or CpG islands, are often methylation-free in somatic tissues and, to a large extent, have been maintained through evolution. Current estimates indicate that 50-60% of human genes are associated with a CpG island [75, 76].

The functional relevance of CpG islands derives from the observation that changes in their methylation levels results in altered expression of their associated genes. In general, genes associated with methylated CpG islands are either silenced or down-regulated [77-79]. Because of its potential to abrogate gene activity, DNA methylation has been proposed as one of the two hits in Knudson's two hit hypothesis for oncogenic transformation [80].

The function of DNA methylation in normal cells is diverse and necessary for normal development, including silencing of transposable elements, inactivation of viral sequences, maintenance of chromosomal integrity, X-chromosome inactivation, and transcriptional regulation of a large number of genes [81-85]. Studies have demonstrated that knockout mouse models for any of the three DNA methyltransferase (Dnmt1, Dnmt3a and Dnmt3b) results in embryonic or perinatal lethality, underscoring the essential role of DNA methylation in normal developmental processes [86-87]. A variety of human congenital malignancies are characterized by abnormal DNA methylation during development. ICF syndrome, a rare disorder characterized by immunodeficiency, chromosome instability, and facial anomalies, has been linked to mutations in a de novo DNA methylatransferase, DNMT3B, which results in the hypomethylation of juxtacentromeric regions in chromosomes 1, 9 and 16 [87]. Imprinting disorders, such as Beckwith-Weidermann and Rader-Willi/Angelman syndromes, are the results of defects in the maintenance of the monoallelic expression of imprinted genes [88]. Imprinted genes are expressed in a parental-specific manner and their expression is regulated by DNA methylation of short regulatory domains termed differentially methylated regions (DMRs). In typical Beckwith-Weidermann cases, biallelic expression of the insulin-like

growth factor 2 (IGF2) gene is observed, a gene normally expressed only from the maternal allele [88].

Most current evidence linking DNA methylation, regulation of gene expression and diseases stems from studies in human cancers. Previous studies have well established significant associations between not only DNA hypermethylation and tumor suppressor gene silencing but also DNA hypomethylation and oncogene expression in numerous human cancers [89-92]. It has also been demonstrated that DNA methylation is an early event in tumor development as indicated by reports where aberrantly hypermethylated sites could be detected in seemingly normal epithelia from patients years before the overt development of cancer.

Epigenetics in Drug Response: Pharmacoepigenetics

As opposed to the irreversible essence of genetic alterations that result in gene silencing, the importance of understanding the mechanism involved in the epigenetic abrogation of gene expression lies on the reversible nature of epigenetic processes. Thus, a number of "epigenetic therapies" geared towards reversing aberrant epigenetic events in malignant cells have been developed. Most of these therapies rely on the use of two classic inhibitors of DNA methylation: 5-azacytidine and 5-aza-2′-deoxycytdine, which were originally synthesized as cytotoxic agents [93, 94]. Both molecules are potent inhibitors of DNA methylation and exert their action through a variety of mechanisms. One of them is their incorporation into the DNA during S phase, which results in the trapping of DNMTs through the formation of a covalent bond between the catalytic site of the enzyme and the pyrimidine ring of the azanucleoside. After the completion of each

cell cycle, concomitant to the depletion of DNMTs from the cellular environment, heritable DNA demethylation is observed in cells treated with either of these agents [95-98]. Another report has demonstrated that both 5-azacytidine and 5-aza-2′-deoxycytdine can induce the rapid degradation of DNMT1 by the proteasomal pathway, even in the absence of DNA replication [99]. Though epigenetic therapies have started to receive FDA approval and have shown clinical benefit, a common problem with epigenetic drugs is nonspecificity of action, resulting in effects on genes that are not desired targets, leading to adverse effects such as carcinogenicity [100].

DNA Methylation Analysis Techniques

Over the past decade, a large number of techniques geared towards the analysis of DNA methylation in short DNA stretches have been developed. Some of these assays, such as methylation-specific polymerase chain reaction (MS-PCR), bisulfite sequencing, methylation-sensitive single-nucleotide primer extension (MS-SNuPE), and combined bisulfite restriction analysis (COBRA) are well established in the DNA methylation field [101, 102]. Though these techniques have been well established, there are limitations in quantification and high-throughput capabilities. Today, newly developed assays and recent technical improvements on well-established methods that have resulted in either higher specificity or that have provided a quantitative platform for a well-established technique, thus making them the most attractive candidates for the analysis of DNA methylation focused towards the discovery and assessment of biomarkers.

MethylLight

MethyLight technology provides a tool for the quantitative analysis of methylated DNA sequences via fluorescence detection in PCR reactions [103]. MethyLight relies on the bisulfite conversion of genomic DNA followed by PCR. Target sequence discrimination can be achieved at three levels: through the design of methylation-specific primers, which may or may not overlap with CpG dinucleotides; through the design of the fluorescent probe, which could overlap one or various CpG sites; or both. Typically, primers that amplify both methylated and unmethylated sequences are used, coupled with a fluorescent probe overlapping two or more CpG sites. An attractive feature of MethyLight is that the fluorescent probe design can be used to detect specific DNA methylation patterns, not to simply discriminate methylated from unmethylated sequences. This flexibility could make it an excellent tool for the assessment of specific DNA methylation patterns that have been shown to possess prognostic value. This assay is also suitable for the analysis of samples where the available DNA amount may be small. However, only number of CpG sites can be assayed, and careful design of primers and fluorescent probes and the optimization of the PCR reaction itself are key to ensure the specific detection of the intended target sequence.

Quantitative Analysis of Methylated Alleles

Quantitative analysis of methylated alleles (QAMA) [104] is a novel quantitative version of MethyLight, which employs TaqMan probes based on minor groove binder (MGB) technology [105]. Because of the improved sequence specificity of the probes,

relative quantification of methylated and unmethylated alleles can be achieved in a single reaction. Dual quantification is achieved through the use of different fluorescent dyes (VIC and FAM), to distinguish the signal emitted by the methylated-specific probe from that of the unmethylated-specific probe. The main advantage of QAMA is its simple setup, which makes it suitable for high-throughput methylation analysis. However, mutations or sequence polymorphisms might affect probe binding, thus yielding measurements not representative of the methylation status of the sequence under study. Finally, because the sequence of the fluorescent probes interrogates more than a single CG dinucleotide, only alleles either completely methylated or completely unmethylated generate a positive reading, excluding partial methylation patterns from the analysis. Thus, QAMA might not be suitable as a discovery tool. However, this method could provide a powerful analytical tool for the assessment of DNA methylation patterns whose clinical relevance has already been determined in large numbers of patient samples.

HeavyMethyl

HeavyMethyl is an innovative real-time variant of the MS-PCR assay [106], which allows for the detection of methylated sequences at remarkably low concentration in a DNA mixture with high specificity. In this technique, the PCR priming is methylation specific, but the high specificity of the assay stems from the use of nonextendable oligonucleotide blockers. The blockers are designed to bind to the bisulfite-treated DNA template in a methylation-dependent manner and their binding sites are selected so as to overlap with the 3′ primer binding sites. Using primers specific for *GSTP1*, HeavyMethyl has been successfully used to detect 30 pg of in vitro methylated

and bisulfite-treated DNA in a background of 50 ng unmethylated DNA [106]. The high sensitivity of HeavyMethyl makes it suitable for clinical applications, such as the analysis of DNA methylation in serum, where the amount of noncell bound free-floating DNA in healthy patients is estimated at 10–50 ng/ml [107, 108]. An interesting feature of HeavyMethyl is that it can be adapted for qualitative as well as quantitative analysis of DNA methylation. However, HeavyMethyl requires more components and potentially more optimization than conventional MS-PCR, which has been used with high sensitivity and specificity for a large number of genes.

Pyrosequencing

Pyrosequencing is a sequence-by-synthesis approach that is based on the luminometric detection of pyrophosphate release following nucleotide incorporation [109, 110]. Depending on the chemistry used, a three- to four-enzyme cascade converts the released pyrophosphate to ATP, which is immediately hydrolyzed to produce light. Since a single known nucleotide is added sequentially in each step, the sequence of the template can be determined. Reports have indicated that the pyrosequence technology can be used for quantification of DNA methylation at CpG sites on bisulfite-treated DNAs [111]. The main advantage of quantitative bisulfite sequencing using the pyrosequencing technology (QBSUPT) over conventional bisulfite sequencing is the fact that quantitative DNA methylation information can be obtained from whole PCR products, without the need for cloning and sequencing of a large number of clones to obtain statistically relevant information. However, QBSUPT cannot be used for the analysis of haplotype-specific DNA methylation patterns.

MethylQuant

MethylQuant can be used to quantify the methylation level of a single cytosine through the real-time amplification of bisulfite-treated DNA [112]. Quantification is achieved by comparing real-time PCR reactions, one of which amplifies the target sequence irrespective of its methylation status (non discriminative), while the other one only amplifies the methylated target (discriminative). Distinction between methylated and unmethylated sequences is accomplished by the complementary base pairing of the most 3′ end nucleotide in the primer. Through the comparison of the threshold crossing cycle for the non discriminative and discriminative reactions, a relative ratio between the methylated and unmethylated target can be obtained. One advantage of this method is that quantification can be achieved using SYBR Green I, eliminating the need for fluorescently labeled probes and reducing the overall cost. However, as is the case with other PCR-based techniques described, careful primer design and optimization of the PCR reaction are critical to ensure the detection of the intended target sequence.

MALDI-TOF Mass Spectrometry

This system utilizes mass spectrometry (MS) for the detection and quantitative analysis of DNA methylation using Homogeneous MassCLEAVE (hMC) base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [113]. After bisulfite treatment of genomic DNA, a T7-promoter tag is introduced through PCR. Next, an in vitro RNA transcription is performed on the reverse strand, followed by an RNaseA base-specific cleavage reaction (U or C). The cleavage products

are analyzed in a MALDI-TOF MS machine, which yields distinct signal patterns for the methylated and unmethylated templates. The MassARRAY system is capable of detecting DNA methylation levels as low as 5%. The main advantage of this technique is its ability to generate quantitative data for multiple CpG sites within a region of interest without the need for cloning of PCR products. However, this technique requires multiple steps and sophisticated equipment which might not be available in all research settings.

Significance

Though genetics is a well known factor in predicting variability in drug response, there are numerous incidences where genetics cannot fully explain the basis of drug variability among individuals. With the increasing importance of epigenetics in regulating gene expression, this dissertation focused on both genetic and epigenetic regulation in attempts to gain a better understanding of the molecular basis behind drug response variability and whether these factors work separately or in concert with one another. Genetic and epigenetic studies were performed utilizing two drugs that are commonly administered for therapy in their selective disease states, 5-fluorouracil (5-FU) and Lansoprazole, which are described in the following sections.

5-FU and DPD Deficiency

5-FU is in the fluoropyrimidine class of antineoplastic agents and is commonly used in the treatment colorectal cancer as well as solid tumors of the breast, head and neck [114, 115]. The biological activity of 5-FU is mediated through activation or anabolism of the parent prodrug to 5-fluoro-2-deoxyuridine monophosphate (5-FdUMP),

which subsequently inhibits thymidylate synthase (TS), an enzyme required for de novo pyrimidine synthesis [116, 117]. On the other hand, 80 to 90% of the administered intravenous 5-FU dose is inactivated through catabolism in the liver, where the rate-limiting enzyme is dihydropyrimidine dehydrogenase (DPD) [118]. DPD exhibits wide interindividual variation in activity of up to 20-fold, and patients with low or negligible DPD activity are unable to efficiently inactivate 5-FU, leading to decreased catabolism, which can produce severe gastrointestinal, hematopoietic and neurological toxicities [119-123]. 5-FU toxicity is common; 31-34% of patients with colorectal cancer treated with 5-FU displayed dose-limiting grade 3 to grad 4 hematological toxicity [116]. DPD deficiency accounts for approximately 43 to 60% of patients with severe toxicity due to 5-FU (in some cases severe enough to result in death). Partial and complete deficiency of the DPD enzyme occurs in approximately 3 to 5% of the general population [116].

To date, more than 30 sequence variants of the *DPYD* gene have been identified [124], producing multiple complex heterozygote genotypes that are inherited in an autosomal codominant fashion. The most common inactivating allele, *DPYD*2A*, is characterized by a G to A transition at the invariant GT splice donor site flanking exon 14 of the *DPYD* gene [119]. This mutation leads to truncated mRNA due to skipping of exon 14, which results in a nonfunctional protein. A second SNP associated with DPD deficiency is *DPYD*13*, which is characterized by a T to G transition at a domain important to enzyme activity [124]. However, the identified *DPYD* variant alleles do not explain all observed cases of DPD deficiency, as many patients with severe 5-FU toxicity have no detected mutations in the *DPYD* gene [125,126].

Experiments investigating endogenous promoter activity indicated that promoter methylation could down-regulate DPD expression [127]. Following treatment of these cell lines with 5-azacytidine, DPD mRNA levels increased significantly, implying that methylation could be a regulatory mechanism for DPD expression. In individuals absent of inactivating mutations, methylation of CpG islands located in the 5' regulatory region of the *DPYD* gene promoter, may inhibit the binding of transcriptional factors or stabilize the chromatin structure, thereby directly inhibiting transcription.

Variability in Response to Lansoprazole Therapy

Diseases related to gastric acid hypersecretion are commonly treated with the specific class of drugs known as proton pump inhibitors (PPIs) or the pyridylmethylsufinyl benzimidazoles. PPIs such as Lansoprazole target the terminal step in acid production thus inhibiting gastric acid release regardless of the nature of parietal cell activation and have been shown to be significantly more effective than H2 antagonists, reducing gastric acid secretion by up to 99% [128]. PPIs are all generally well tolerated and have become the preferred therapeutic of choice due to their high degree of target specificity and clinical efficacy. PPIs block acid release from activated gastric proton pumps by covalently binding to hydrogen sulfide residues of the catalytic α subunit of the gastric H^+/K^+ ATPase.

Although PPIs are effective drugs, the increased incidence of peptic ulcer recurrence remains an ongoing therapeutic challenge. Less than complete acid suppression, increased night-time production of acidity, and required mealtime dosing for adequate drug levels during periods of pump activity are commonly observed with PPI-

based therapy [129]. Also observed is a wide range of variability in PPI dosage required for sustained gastric acid suppression vital for ulcer healing and the treatment of acid-related diseases [130, 131].

Variability in PPI efficacy has often been attributed to the relatively short half-life and the polymorphic cytochrome p450 drug metabolizing enzyme CYP2C19 responsible for PPI metabolism [132]. Patients' phenotypic classification based on CYP2C19 metabolic activity have been previously characterized into three main groups: extensive metabolizers (EM) characterized by a homozygous wild-type (WT) allele, intermediate metabolizers (IM) characterized by the presence of a WT and a mutant allele associated with decreased enzyme activity, and poor metabolizers (PM) characterized by homozygous mutant alleles associated with decreased enzyme activity. There are currently over 20 different haplotypes reported to affect CYP2C19 activity [133-139]. In Caucasians, most inter-individual variation in function is explained by the variant *CYP2C19*2*, but worldwide a number of other variants may have to be considered, in particular *CYP2C19*3*. A codominant mode of inheritance has been consistently seen, and thus heterozygous carriers of CYP2C19 variants known to decrease enzyme function have about half of the activity of the homozygous carriers. Most recently, the discovery of two specific variants of the 5'-upstream region, known as the *CYP2C19*17* allele, has led to the fourth phenotypic classification, ultra-rapid metabolizers (URM) [140].

The CYP2C19 genotype/phenotype associations have also been extensively studied in relation to PPI pharmacokinetics [141]. Previous studies have investigated the influence of *CYP2C19* genotype on healing of gastric ulcers using PPI therapies and have also shown that cure rates of PPI-based treatments for *H. pylori* infection were lowest in

individuals characterized with the CYP2C19 extensive metabolizer (EM) phenotype due to insufficient plasma PPI concentrations necessary to inhibit acid secretion [142]. However, these studies have been either limited by the number of analyzed patients or to specific populations with different frequencies of particular *CYP2C19* gene variants. Many studies have also been limited by screening of specific *CYP2C19* alleles rather than the entire gene for novel variants which may alter CYP2C19 activity. The inclusion of other candidate genes (for example *ATP4A* and *ATP4B* genes encoding the gastric ATPase) that are also involved in gastric acid secretion have also been absent from investigations. Furthermore, other potential molecular mechanisms such as epigenetics have not yet been investigated in the interindividual variability in PPI efficacy and gastric acid secretion.

SPECIFIC AIMS

Though they are different classes of drugs utilized in different disease treatments, both 5-FU and Lansoprazole provide exceptional opportunities for the investigation of both genetic and epigenetic factors that may alter drug response.

In the case of 5-FU, variability in therapeutic response has often been attributed to the rate-limiting catabolic enzyme DPD. Previous studies in the Diasio lab involving the phenotypic characterization of DPD enzyme activity within a population of healthy volunteers and patients identified a number of cases where *DPYD* genotype provided no explanation for the measured DPD deficiency. The lack of genetic explanation for the phenotypically characterized DPD deficiency, coupled with previous studies indicating that promoter methylation could down-regulate DPD expression in cell lines, led to investigations of the *DPYD* promoter methylation in human samples and to the first specific aim of this thesis.

Specific Aim I**:** Determine the role of aberrant methylation of the *DPYD* promoter as a potential epigenetic regulatory mechanism of DPD enzyme activity that may clarify the unexplained molecular basis of DPD deficiency and 5-FU toxicity.

In the case of Lansoprazole, one of the most commonly prescribed PPIs, variability in therapeutic response has often been attributed to the CYP2C19 enzyme activity which has been well correlated in previous studies with specific CYP2C19 genotypes. In collaboration with the UAB Gastroenterology department under the

guidance of Dr. Basil Hirschowitz and Dr. Mel C. Wilcox, a specific cohort of patients requiring a wide Lansoprazole dose range for therapeutic acid suppression, including a subset of patients who were unable to obtain successful acid inhibition, were obtained for analysis. In addition to examining *CYP2C19* genetics, this study also focused on not only the inclusion of other candidate genes such as the *ATP4A* and *ATP4B* genes encoding the gastric H^+/K^+ ATPase proton pump, but also other potential molecular mechanisms such as epigenetics which have not yet been investigated in interindividual variability in PPI efficacy and gastric acid secretion, as outlined by the following specific aims.

Specific Aim II: Identification of genetic variants in the genes encoding the proton pump (H^+/K^+ATPase) subunits (*ATP4A* and *ATP4B*) and the *CYP2C19* gene, in gastric acid hypersecretors and their potential role in regulating the proton pump function and response to lansoprazole.

Specific Aim III: Determine the role of aberrant methylation of the H^+/K^+ ATPase β subunit (*ATP4B*), as a gene silencing mechanism, in various tissue types of gastric acid hypersecretors receiving lansoprazole.

METHYLATION OF THE DPYD PROMOTER: AN ALTERNATIVE MECHANISM FOR DIHYDROPYRIMIDINE DEHYDROGENASE DEFICIENCY IN CANCER PATIENTS.

by

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ABSTRACT

Purpose: Dihydropyrimidine dehydrogenase (DPD) deficiency, a known pharmacogenetic syndrome associated with 5-fluorouracil (5-FU) toxicity, has been detected in 3% to 5% of the population. Genotypic studies have identified >32 sequence variants in the *DPYD* gene; however, in a number of cases, sequence variants could not explain the molecular basis of DPD deficiency. Recent studies in cell lines indicate that hypermethylation of the *DPYD* promoter might down-regulate DPD expression. The current study investigates the role of methylation in cancer patients with an unexplained molecular basis of DPD deficiency. Experimental Design: DPD deficiency was identified phenotypically by both enzyme assay and uracil breath test, and genotypically by denaturing high-performance liquid chromatography. The methylation status was evaluated in PCR products (209 bp) of bisulfite-modified *DPYD* promoter, using a novel denaturing high-performance liquid chromatography method that distinguishes between methylated and unmethylated alleles. Clinical samples included five volunteers with normal DPD enzyme activity, five DPD-deficient volunteers, and five DPD-deficient cancer patients with a history of 5-FU toxicity. Results: No evidence of methylation was detected in samples from volunteers with normal DPD. Methylation was detected in five of five DPD-deficient volunteers and in three of five of the DPD-deficient cancer patient samples. Of note, one of the two samples from patients with DPD-deficient cancer with no evidence of methylation had the mutation *DPYD**2A, whereas the other had *DPYD**13. Discussion: Methylation of the *DPYD* promoter region is associated with down-regulation of DPD activity in clinical samples and should be considered as a potentially important regulatory mechanism of DPD activity and basis for 5-FU toxicity in cancer patients.

INTRODUCTION

Dihydropyrimidine dehydrogenase (DPD) enzyme deficiency, a known pharmacogenetic syndrome detected in 3% to 5% of the population [1], has been associated with toxicity to 5-fluorouracil (5-FU) cancer chemotherapy and death in some cases with profound deficiency of the enzyme [2]. DPD is the first enzyme in a three-step catabolic pathway responsible for the degradation of ~85% of administered 5-FU.

Genotypic studies have identified >32 sequence variants in the *DPYD* gene [1,3]. Expression analysis of these variants showed that many were polymorphisms with no obvious functional significance [4], with the exception of a few mutations. One example commonly associated with DPD deficiency and severe toxicity to 5-FU is an intronic sequence variation (IVS14 + 1 G > A, *DPYD**2A), which results in a truncated protein that lacks 55 amino acids due to the skipping of exon 14 [5]. A second example is a less common mutation (1679T > G, I560S, *DPYD**13), which is associated with decreased DPD activity and 5-FU toxicity [6] due to a nonconservative amino acid change from isoleucine to serine at codon 560 (I560S), which is 100% conserved among human, mouse, rat, bovine, and pig species, suggesting its importance in maintaining DPD enzyme activity [7]. Of note is that this mutation has not been detected in individuals with normal DPD enzyme activity. However, in a significant number of patients, many of the detected *DPYD* sequence variants did not explain the molecular basis of the observed DPD deficiency and 5-FU toxicity [4].

Recent advances in our understanding of molecular mechanisms involved in the activation and degradation of 5-FU have led to an increased awareness of the potential importance of epigenetic factors in deciding the sensitivity of patients to anticancer drugs.

We hypothesize that in the absence of inactivating mutations, methylation of CpG islands located in the 5' regulatory region of the *DPYD* gene promoter, may inhibit the binding of transcriptional factors or stabilize the chromatin structure, thereby directly inhibiting transcription [8].

The current study investigates the role of aberrant methylation of the *DPYD* promoter as a potential epigenetic regulatory mechanism of DPD enzyme activity that may clarify the unexplained molecular basis of DPD deficiency and 5-FU toxicity.

MATERIALS AND METHODS

Methylation controls

Controls used in screening for methylation status in clinical samples were universal methylated sperm DNA purchased from CpGenome Serologicals (Temecula, CA), steady state and 5-azacytidine–treated colon carcinoma cancer cell line (RKO).

Clinical samples

A total of 15 individuals were selected from a healthy population study and a separate study of 5-FU toxic patients using approved Institutional Review Board protocols, following informed consent from all individuals. Demographic data of studied individuals is shown in Table 1.

Phenotypic Assessment of Dihydropyrimidine Dehydrogenase Enzyme Activity

DPD enzyme activity was measured as previously described [9] in peripheral blood mononuclear cells collected from all 15 participants in this study. Individuals with

peripheral blood mononuclear cell DPD activity <0.18 nmol/min/mg protein were considered to be DPD-deficient [9].

^{13}C-Uracil Breath Test

The ^{13}C-uracil breath test measured as DOB_{50} (DOB_{50} defined as the $^{13}CO_2/^{12}CO_2$ ratio of breath sample measured at 50 minutes as determined by IR spectroscopy using the UBiTIR 300 instrument) was done as previously described [10]. Individuals having a DOB_{50} <128.9 were classified as DPD-deficient (D), and those with a DOB_{50} >128.9 were classified as normal (N). Three patients DP2, DP3, and DP5 with deficient DPD enzyme activity exhibited life-threatening 5-FU toxicity and were not available to perform the ^{13}C-uracil breath test.

DNA Preparation, Bisulfite Modification, and PCR Amplification

Genomic DNA was extracted from RKO cell line and from peripheral blood mononuclear cells of studied individuals using Wizard SV genomic DNA purification system (Promega, Madison, WI). All samples were bisulfite-modified (BSM) as previously described [11]. PCR primers were designed according to the CpG island of the sense strand of the *DPYD* gene (Genbank accession no. NM_000110). These primers amplified the same sequence areas from methylated and unmethylated BSM DNA. The designed primers were forward, 5'-TTTTTGTTTGTAGGTTGGG-3'; and reverse, 5'-CAACCAAAAAACCAAATAACAACAA-3', which generates a 209 bp fragment of the DPYD promoter (nucleotides +44 to –165 from tsp). A 50 μL volume PCR was done using buffers purchased from Epicenter Technology (Madison, WI) as follows: fail-safe

buffer (G) 25 μL, forward and reverse primers (10 nmol/L) 1 μL each, BSM gDNA (20 ng/μL) 1 μL Platinum Taq enzyme (Invitrogen, Carlsbad, CA) 0.45 and 21.55 μL H_2O. The reactions were done on a PTC200 DNA engine (MJ Research, Reno, NV) as follows: denaturation at 95°C for 10 minutes followed by 40 cycles of denaturing at 94°C for 50 seconds, annealing at 52°C for 50 seconds, elongation at 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. Samples were then maintained at 4°C. Screening for sequence variations in the *DPYD* gene was done using denaturing high-performance liquid chromatography (DHPLC) analysis of amplified PCR products of reference controls and clinical samples as described previously [12]. All *DPYD* sequence variants identified by DHPLC were confirmed by DNA sequence analysis using a dideoxynucleotide chain termination method (Big Dye Kit; Applied Biosystems, Foster City, CA) and capillary electrophoresis on an ABI 310 Automated DNA Sequencer (Applied Biosystems).

Denaturing High-Performance Liquid Chromatography Method for the Detection of Methylation Status and Single Nucleotide Polymorphisms Within Bisulfite-Modified DNA Fragments

A DHPLC method was developed to detect the methylation status and single nucleotide polymorphisms within the PCR-amplified BSM fragments on the Wave System (Transgenomic, Co., Omaha, NE). The detection of multiple variables in a single injection, using DHPLC was previously reported by our laboratory [13]. In this study, the DHPLC method was optimized using a 6.6 minute gradient at 0.9 mL/min flow rate. The gradient starts at 0.5 minutes and 51.8% buffer B (0.1 mol/L TEAA, 25% acetonitrile)

and stops at 5 minutes and 60.8% buffer B. DNA loading on DNASep column starts at 46.8% buffer B. Optimal screening temperatures were experimentally determined by repeatedly injecting 5 μL of the PCR product using the same gradient at temperatures from 50°C to 70°C as previously described [12]. PCR products of BSM fragments were injected at the experimentally determined optimal screening temperatures (50°C, 56°C, and 57°C) to detect the methylation status and single nucleotide polymorphisms in studied samples. Reagents for DHPLC analysis were purchased from Transgenomic.

Sequencing of Bisulfite-Modified Samples

PCR products of BSM samples were gel-purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified fragments were then cloned into pGEM T-easy vectors (Promega) and transfected into *Escherichia coli* JM109 competent cells (Promega). Cells were cultured on Luria-Bertani agar medium containing B-gal-isopropyl-L-thio-ß-D-galactopyranoside. Plasmid DNA was isolated from five different clones per sample using Isopure DNA Purification Kits Spin Column Plasmid Mini-Preps (Denville Scientific, Inc., Metuchen, NJ). Isolated plasmid DNA was then sequenced on an ABI Prism 310 genetic analyzer using Big Dye terminator and M13 primers.

Statistical analysis

Statistical analysis was done using SPSS Software (version 10.0.5). Means of DPD enzyme activity in studied groups were compared using one-way ANOVA test. The association between DPD enzyme deficiency and methylation was examined using Pearson χ^2 test.

RESULTS

Phenotypic and Genotypic Analysis of Dihydropyrimidine Dehydrogenase

Phenotypic analysis by DPD enzyme radioassay showed 100% agreement with uracil breath test in a population study of 250 normal healthy volunteers (data not shown), (individuals identified as deficient by the DPD radio assay were also identified as deficient by the ^{13}C-uracil breath test). Genotypic analysis of the *DPYD* gene in individuals with normal DPD (176, 182, 184, 195, and 201) showed that all sequence variations detected in this group were polymorphisms with no significant effect on the DPD enzyme activity (Table 1) [4]. One DPD-deficient volunteer (DV-D2) had the most common mutation associated with DPD deficiency IVS14 + 1 G > A, *DPYD**2A. Two novel mutations were detected in DPD-deficient individuals, DV75 and DV130 (542A > C, K182T) and (557A > G, Y186C), respectively, their functional significance is currently being investigated by our laboratory. The two mutations (IVS14 + 1 G > A, *DPYD**2A) and (1679T > G, I560S, *DPYD**13) known to be associated with DPD deficiency were detected in two patients with colorectal cancer (patients DP3 and DP20; Table 1). Using one-way ANOVA test, the means (mean ± SD) of DPD enzyme activity in studied groups (DPD-normal volunteer group, 0.32 ± 0.05; DV group, 0.11 ± 0.04; and DP group, 0.06 ± 0.02) were significantly different ($F = 61.890$ and $P < 0.0001$).

Location of CpG Sites in Bisulfite-Modified Control Samples

The *DPYD* promoter fragment examined in this study spanned 209 bp on the 5'-untranslated region starting at nucleotide +44 to –166 from the transcription start point.

This region included 27 CpG sites, 11 of which lie within the sequence of two regulatory elements at nucleotides (–23 to –42) and (–51 to –72) which were previously reported by our laboratory to regulate DPD mRNA expression [14].

Methylation of the DPYD Promoter

The DHPLC instrument automatically calculates the area under the curve (AUC) in the chromatogram, designating %AUC for each peak. In case of a methylated fragment, under the specified temperature and gradient conditions, the methylated allele (GC-rich) is retained longer on the column than the nonmethylated (A/T-rich) allele. In case of the BSM universal methylated control (all 27 CpG sites are methylated), the %AUC for the methylated allele ($\%M_{AUC}$) was 44.3 % (Figure 1C). By calculation, it is possible to estimate the approximate number of methylated CpG sites in a fragment relative to the control sample, which have 27 of the 27 methylated CpG sites (Figure 2; represented by 43% AUC), using a factor of 0.609 (27 of 44.3 or 0.609). This factor is obtained from the equation: [27 (known number of methylated CpG sites in control) x %AUC of test methylated fragment = number of CpG sites in test fragment x 44.3%]. Accordingly, if the $\%M_{AUC}$ of the RKO cell line is 23.6%, then the approximate number of methylated CpG sites under the curve would be 23.6 x 0.609 = 14.4 CpG sites (~14 CpG). The methylation status of an allele is defined by its $\%M_{AUC}$, retention time, elution pattern, and melting profile as well as its resolution under optimal screening temperatures using the optimized gradient.

Examining the association between enzyme deficiency and methylation, regardless of group assignment (using Pearson χ^2), illustrated that 80% (8 of 10) of the

DPD-deficient individuals exhibited a positive methylation signal, whereas individuals with normal DPD enzyme activity ($n = 5$) tested negative for methylation ($\chi^2 = 7.78$; $P < 0.05$).

Denaturing High-Performance Liquid Chromatography Analysis of Control Reference Samples

Universal methylated sperm DNA, steady state RKO cell line (hypermethylated), and the demethylated 5-azacytidine–treated RKO (negative control) showed a single peak pattern under nondenaturing temperature (50°C) (Figure 1A, D and G). Under partially denaturing temperatures (56°C), chromatograms of both positive methylated controls (universal methylated sperm DNA and hypermethylated RKO cell line) slightly resolved into two peaks Figure 1B and E). These peaks further resolved at 57°C into distinct patterns characteristic of methylated samples. Samples with a positive methylation signal illustrated the presence of two alleles, a methylated allele with higher retention time due to high GC content, and an unmethylated allele, rich in A/T nucleotides, eluting earlier on the chromatogram (Figure 1C and F). The negative control sample (demethylated RKO cell line) retained a single peak pattern at all temperatures (Figure 1 G, H and I).

For confirmation of the DHPLC results, control samples were cloned and five colonies were sequenced for each sample (Figure 2). Sequence analysis illustrated that two of five and one of five colonies of the universal methylated sperm DNA and RKO hypermethylated control samples, respectively, had 100% methylation (27 of 27 CpG sites). This finding could be attributed to different growth phases of the cells in culture.

Bisulfite sequence analysis of five of the five colonies of the negative control sample illustrated the absence of methylation in all 27 CpG sites (Figure 2).

Denaturing High-Performance Liquid Chromatography Analysis of Volunteers with Normal Dihydropyrimidine Dehydrogenase Enzyme Activity

Volunteers with normal DPD enzyme activity produced chromatogram patterns similar to those of the reference negative control sample, demonstrating a single peak eluting at ~4.5 minutes in all samples (Figure 3A-E). Bisulfite sequence analysis confirmed the absence of methylation in five of the five cloned samples (0 of 27 CpG sites; Figure 4).

Denaturing High-Performance Liquid Chromatography Analysis of Volunteers with Deficient Dihydropyrimidine Dehydrogenase Enzyme Activity

Chromatograms of volunteers with deficient DPD enzyme activity illustrated variable methylation patterns and %AUC (Figure 3F-J). Individuals DV75, 130, 145, and 148 (Figure 3F-I) had no genetic variants in their *DPYD* gene that could explain their DPD deficiency. However, the fifth individual (DV-D2; Figure 2J) showed a low methylation signal (5% M_{AUC}; approximately three CpG methylated sites) and the presence of the mutation (*DPYD**2A) known to be associated with DPD deficiency. The discrepancy between bisulfite sequencing results of cloned DV-D2 sample (five colonies) and DHPLC analysis of gDNA extracted directly from peripheral blood mononuclear cells could be attributed to different phases of growth and methylation events which occur within cells in culture. Cell sorting experiments will resolve this discrepancy in future

studies in our laboratory. In this group, the unmethylated alleles (left-most peaks) eluted between 4.49 and 4.52 minutes (Figure 3F-J) consistent with that of volunteers with normal DPD enzyme activity (4.51-4.52 minutes; Figure3A-E). Peaks of methylated alleles eluted at 4.83 to 4.87 minutes (Figure 3F-J). In three DPD-deficient volunteers (DV75, DV130, and DV148), all 27 CpG sites were methylated in two of five, one of five, and one of five of colonies sequenced, respectively (Figure 4).

Denaturing High-Performance Liquid Chromatography Analysis of Cancer Patients with Deficient Dihydropyrimidine Dehydrogenase Enzyme Activity

Chromatograms of cancer patients DP5 and DP17 with DPD deficiency and 5-FU toxicity showed a positive methylation signal of 18% M_{AUC} (~11 CpG methylated sites) and 19% M_{AUC} (~12 CpG methylated sites), respectively (Figure 3 M and N). In contrast, no methylation signal was detected in patients DP2 and DP20; however, the mutations *DPYD**13 and *DPYD**2A were detected in their *DPYD* gene, respectively (Figure 3K and O). The DHPLC results were further confirmed by the absence of methylation in all CpG sites (0 of 27) in five of five clones sequenced for these two patients. In patient DP3, 13% M_{AUC} was detected, which represents approximately eight methylated CpG sites. Additionally, the mutation *DPYD**2A was detected in this patient's *DPYD* gene (Figure 3L).

DISCUSSION

DPD deficiency has been recognized as a pharmacogenetic syndrome associated with 5-FU toxicity [2, 15, 16]. Recent studies indicated that >32 sequence variants have

been detected in the *DPYD* gene, many of which are polymorphisms (e.g., *DPYD**9A, *DPYD**5, M406I; Table 1) that were detected in individuals with normal DPD enzyme activity [2-4].

Recently, methylation has been recognized as a common epigenetic alteration in human cancer which leads to gene silencing [17]. Studies in cancer cell lines (oral epidermoid carcinoma line KB, colon adenocarcinoma COLO20, oral squamous cell carcinoma lines HSC3, HSC4, and Ca9-22 and hepatoma HepG2 cell lines) showed the absence of genetic alterations in *DPYD* promoter region with full activity [8]. Our results show the absence of DNA sequence variants in the *DPYD* promoter region in all studied individuals. Transcriptional studies examining steady state expression of DPD mRNA in cell lines showed variable levels of DPD expression [8]. Experiments investigating endogenous promoter activity indicated that promoter methylation could down-regulate DPD expression [8, 18]. Following treatment of these cell lines with 5-azacytidine, DPD mRNA levels increased significantly, implying that methylation could be a regulatory mechanism for DPD expression. Bisulfite sequence analysis of the *DPYD* promoter region revealed the presence of different methylation patterns in CpG islands of these cell lines [8]. However, aberrant methylation of the *DPYD* promoter has not been investigated in cancer patients with unexplained DPD deficiency and 5-FU toxicity.

Previously, methylation detection has been hampered by technical limitations. These limitations include artifacts of bisulfite modification reactions [19]; labor-intensive and time-consuming cloning and sequencing steps required for bisulfite genomic sequencing; as well as substantial amounts of high molecular weight DNA required in Southern blotting. Also, methods based on differential methylation states of alleles such

as methylation-specific PCR [11], MethyLight [20], or methylation-sensitive single-stranded conformational polymorphisms [21, 22] are limited by the specific nucleotide variation located at the primer/probe binding sequence [23].

The DHPLC method developed in this study, unlike other techniques, circumvents the cloning and sequencing steps required for methylation detection. It also correctly differentiates between methylated and unmethylated alleles with high sensitivity and rapidity (6.6 minutes) and permits a semiquantitative assessment of the methylation status, allowing approximation of the number of methylated CpG sites relative to a 100% methylated control reference sample. Additionally, this method permits the detection of single nucleotide polymorphisms within fragments screened for methylation. These advantages allow the use of this DHPLC method in large population studies investigating the effect of aberrant methylation in cancer patients.

In the current study, individuals with normal DPD enzyme activity and normal ^{13}C-uracil breath test showed single peak patterns (Figure 3A-E) suggestive of unmethylated status (Figure 1G and 4). Normal individuals and cancer patients with deficient DPD enzyme activity and altered ^{13}C-uracil breath test results showed the presence of variable methylation pattern and $\%M_{AUC}$ (5-24.7%; approximately 3 to 15 methylated CpG sites; Figure 3F-J and Figure 4). The lowest M_{AUC} (5% M_{AUC}) was detected in volunteer DVD2 (Figure 3J) whose genotype also illustrated the presence of the mutation IVS14 + 1G > A, *DPYD**2A. Two novel mutations 542A > C, K182T and 557A > G, Y186C were detected in two DPD-deficient volunteers DV75 and DV 130, respectively; their functional significance is currently being investigated in our laboratory. The DHPLC chromatograms of the two patients with colorectal cancer, DP5

and DP17, showed the presence of a strong methylation signal, 17.9% M_{AUC} (~11 CpG methylated sites) and 19.2% M_{AUC} (~12 CpG methylated sites), respectively (Figure 3M and N). Interestingly, these two patients did not have inactivating mutations in their *DPYD* gene that could explain the molecular basis of DPD deficiency.

Taken collectively, a significant association between aberrant methylation of the *DPYD* promoter and DPD enzyme deficiency was detected in 80% (8 of 10) of DPD-deficient individuals, whereas all individuals with normal DPD enzyme activity ($n = 5$), tested negative for methylation ($\chi^2 = 7.78$; $P < 0.05$). Additionally, aberrant methylation of the *DPYD* promoter was observed in 100% of DPD-deficient individuals without inactivating mutations in their DPYD gene ($n = 6$; Table 1; Figures 3 and 4). It should be noted that in some cases, the presence of genetic variations in the *DPYD* gene (Table 1), whether intronic (e.g., *DPYD**2A) or in the coding region (e.g., *DPYD**13) were accompanied by variable methylation patterns. Whereas methylation of the two regulatory elements detected in the DPYD promoter [14] (Figure 4) was observed in DPD-deficient individuals with wild-type *DPYD* (*DPYD**1). The positive methylation signals detected by DHPLC were associated with a variable number of methylated CpG sites and variable $\%M_{AUC}$ (Figure 3F-J and L-N). Thus, genetic and/or epigenetic molecular mechanisms can act separately (Figure 3F-I, K and M-O) or in concert (Figure 3J and L) to down-regulate DPD enzyme activity.

However, it is important to emphasize that down-regulation of DPD enzyme activity by methylation is not an isolated cellular mechanism and that transcriptional silencing by methylation is a consequence of multiple mechanisms that requires the combined action of histone hypoacetylation, histone methylation, and methyl-binding

proteins [24]. Whether suppression of DPD expression by methylation is site-specific or pattern-specific [25] remains to be examined in a larger population of patients.

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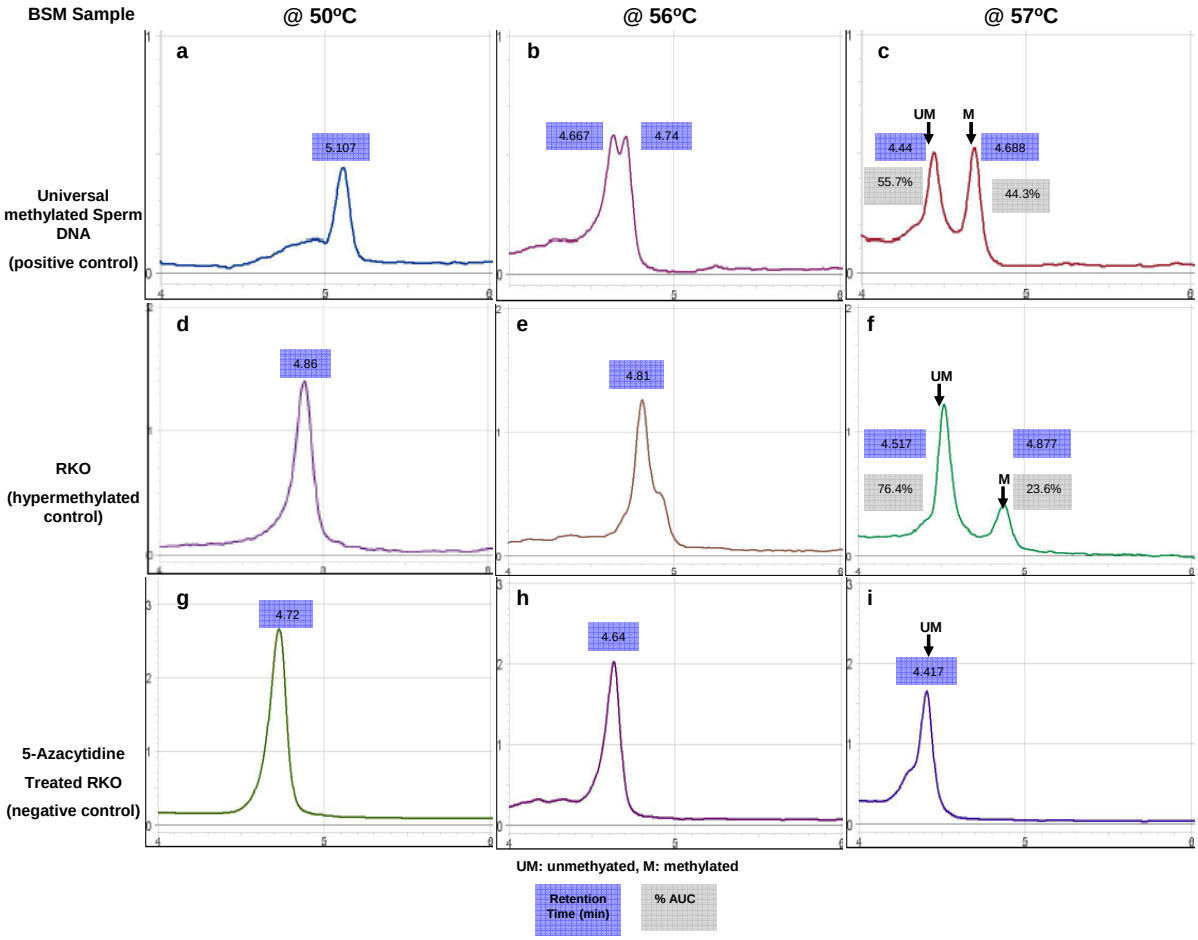
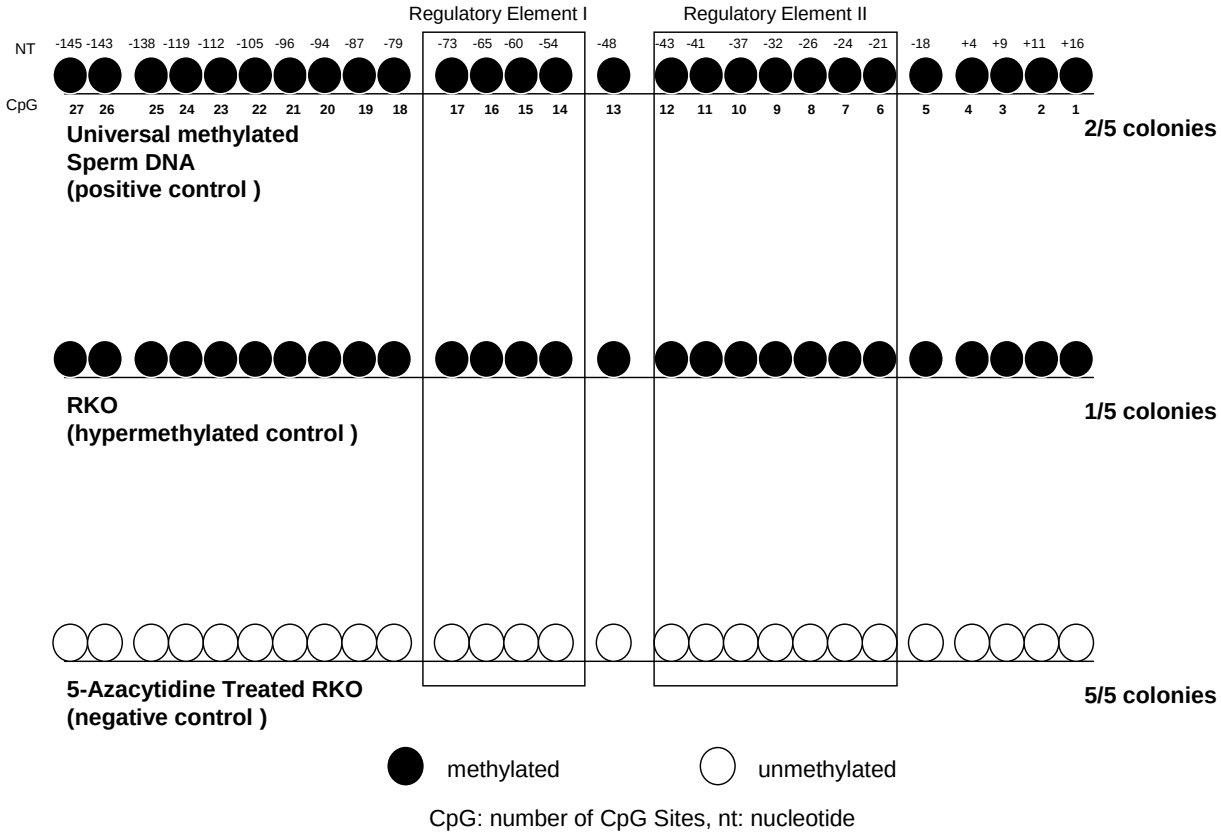
Figure 1. DHPLC chromatogram patterns of the *DPYD* promoter fragment in BSM control samples.



The experimentally determined optimal temperature for methylation and single nucleotide polymorphism detection in the *DPYD* promoter fragment was (57°C). Differences in C/G content of BSM PCR products of universal methylated sperm DNA and RKO cell line, and unmethylated, 5-azacytidine–treated RKO cell line, result in different melting patterns resolved by DHPLC analysis. A two-peak chromatogram pattern was observed at 57°C for BSM universal methylated sperm DNA (*C*) and RKO cell line (*F*), indicating the presence of methylated CpG sites (M). The unmethylated (UM), 5-azacytidine–treated RKO cell line, illustrated a single peak pattern, *G, H,* and *I,*

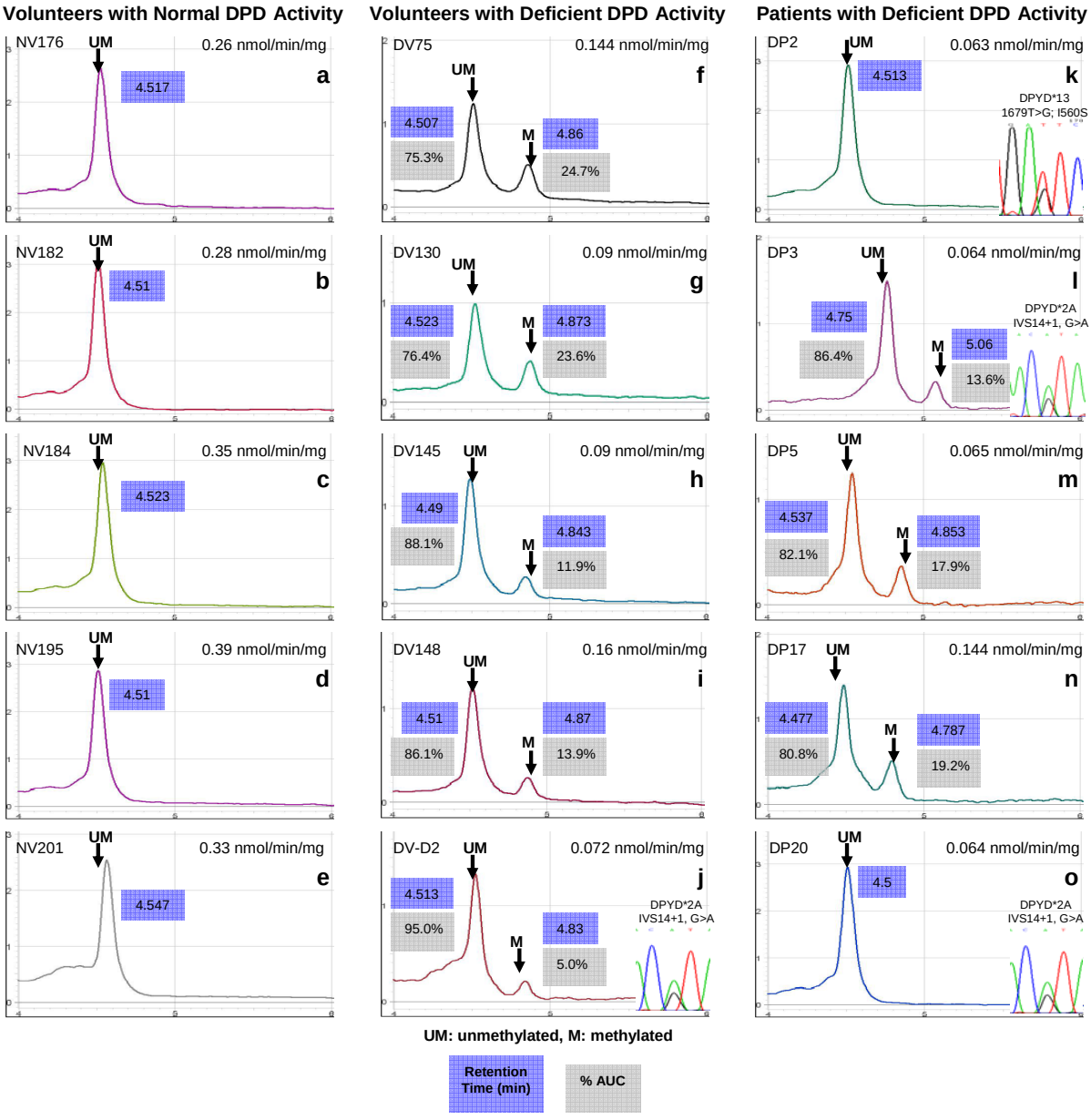
at all temperatures (50°C, 56°C and 57°C). Retention times for each peak are highlighted in blue. Percentage of methylated allele (M) in BSM fragments is expressed as $\%M_{AUC}$ (highlighted in gray). %AUC of unmethylated allele (UM), eluting earlier on the chromatogram, is displayed in a green box.

Figure 2. Bisulfite sequence analysis for the detection of methylated CpG sites in control samples.



PCR products of BSM control samples were cloned into pGEM-T easy vectors, and five colonies for each sample were selected for sequencing. Unmethylated cytosines were converted to thymines (○), whereas methylated cytosines were protected (●). Numbers located above the first row, nucleotide (nt) position of cytosine residue relative to the transcription start point. CpG sites located within regulatory elements are boxed in two rectangles.

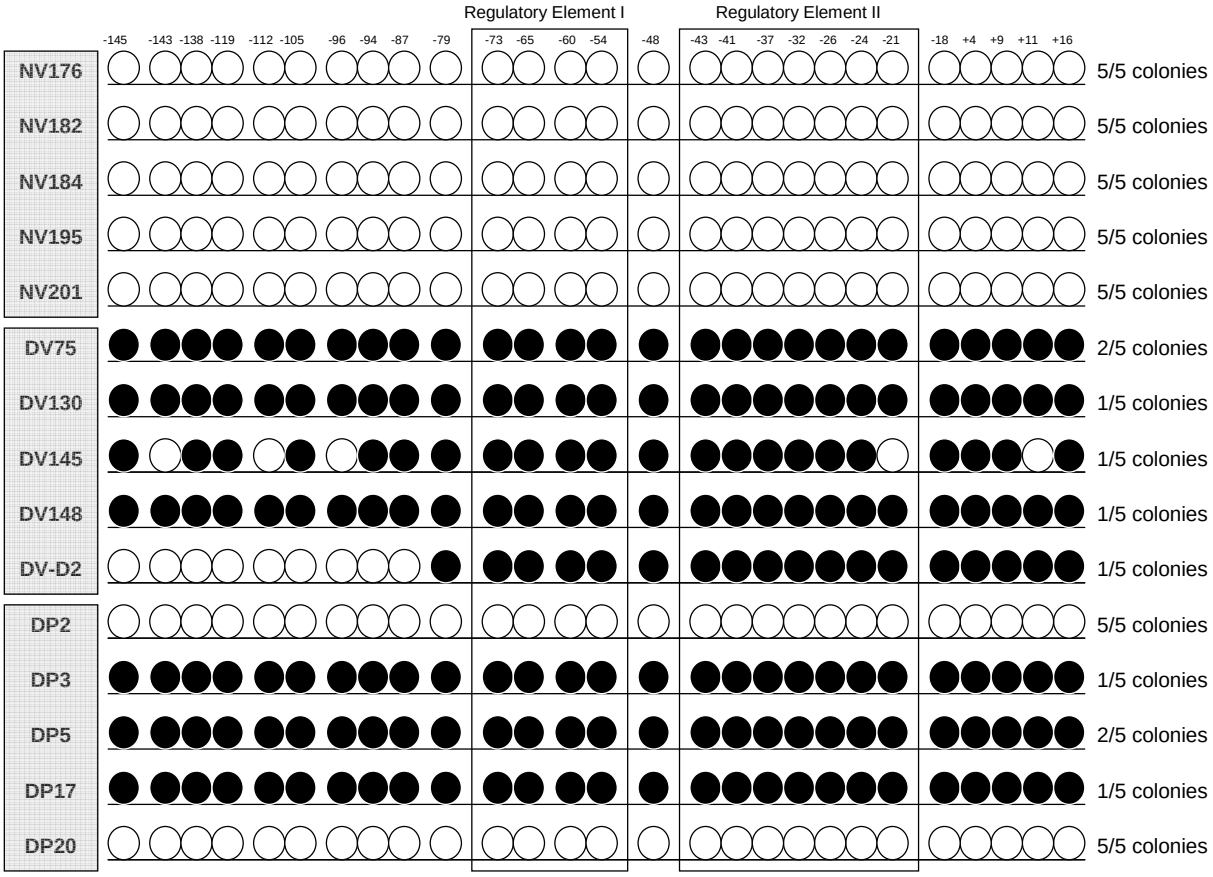
Figure 3. DHPLC chromatogram patterns of the *DPYD* promoter fragment in BSM clinical samples at 57°C.



A single peak pattern characteristic of unmethylated DNA (UM) was observed in all volunteers (NV) with normal DPD enzyme activity (*A*-*E*) and was also detected in two patients with deficient DPD enzyme activity, DP2 (*K*) and DP20 (*O*). A two-peak chromatogram pattern, characteristic of methylated DNA (M) was observed in all DPD-

deficient volunteers (DV; *F*-*J*) and in three DPD-deficient patients, DP3 (*L*), DP5 (*M*), and DP17 (*N*). The retention time of each peak is highlighted in blue. Percentage of methylated allele ($\%M_{AUC}$) is highlighted in gray. %AUC of unmethylated (UM) allele, eluting earlier on the chromatogram, is displayed in a green box.

Figure 4. Bisulfite sequence analysis for the detection of methylated CpG sites in clinical samples.



PCR products of each of the 15 BSM clinical samples were cloned into pGEM-T easy vectors, and five colonies for each sample were selected for sequencing. Unmethylated cytosines were converted to thymines (○), whereas methylated cytosines were protected (●). Numbers located above the first row indicate the nucleotide (nt) position of cytosine residues relative to the transcription start point. CpG sites located within regulatory elements are boxed in two rectangles. DHPLC screening and sequence analysis of cloned volunteers and patients' samples, revealed the absence of single nucleotide polymorphisms within the examined 209 bp *DPYD* promoter fragment.

Table 1: Demographic data, DPD enzyme activity, uracil breath test, and *DPYD* genotype

Sample ID	Sex	Race	Age	Enzyme Activity (nmol/min/mg)	Uracil Breath Test (normal/deficient)	Methylation %	Inactivating Mutation (yes / No)	*DPYD* genotype
NV176	F	H	49	0.26	N	UM	N	*DPYD**1
NV182	F	AA	26	0.28	N	UM	N	*DPYD**1
NV184	F	C	43	0.35	N	UM	N	*DPYD**1
NV195	F	AA	44	0.39	N	UM	N	*DPYD**1
NV201	M	AA	36	0.33	N	UM	N	85 T>C, C29R, *DPYD**9A; 1672 A>G, I543V, *DPYD**5
MEAN ± SD				0.32 ± 0.05				
DV75	F	C	56	0.14	D	24.7% M	N	542 A>C, K182T*
DV130	F	AA	23	0.09	D	23.6% M	N	1896 T>C, F632F; 557 A>G, Y186C*
DV145	F	AA	26	0.09	D	11.9% M	N	85 T>C, C29R, *DPYD**9A; 1672 A>G, I543V, *DPYD**5
DV148	F	AA	20	0.16	D	13.9% M	N	85 T>C, C29R, *DPYD**9A; 1218 G>A, M406I
DV-D2	M	C	28	0.07	D	5.0% M	Y	1672 A>G, I543V, *DPYD**5; IVS14+1 G>A, *DPYD**2A
MEAN ± SD				0.11 ± 0.04				
DP2	M	C	56	0.06	-	UM	Y	1679 T>C, I560S, *DPYD**13
DP3	F	C	47	0.06	-	13.6% M	Y	IVS14+1 G>A, *DPYD**2A
DP5	F	C	51	0.07	-	17.9% M	N	1672 A>G, I543V, *DPYD**5
DP17	M	C	59	0.08	D	19.2% M	N	*DPYD**1
DP20	F	C	46	0.02	D	UM	Y	1672 A>G, I543V, *DPYD**5; IVS14+1 G>A, *DPYD**2A
MEAN ± SD				0.06 ± 0.02				

Abbreviations: NV, DPD normal volunteer; DV, DPD-deficient volunteer; DP, DPD deficient patient; C, Caucasian; AA, African American; H, Hispanic; UM, unmethylated; M, methylated; *DPYD**1, wild-type DPYD gene.

* Novel mutation epitome

IMPACT OF GENETIC AND EPIGENETIC MOLECULAR CHANGES ON THE VARIABILITY IN RESPONSE TO PROTON PUMP INHIBITOR (LANSOPRAZOLE) THERAPY IN GASTRIC ACID HYPERSECRETORS

by

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ABSTRACT

Objective: The determination of the appropriate dose of proton pump inhibitors required in maintaining a therapeutic pH range remains a challenge. We examined 50 gastric acid hypersecretors including a subset of patients with unsuccessful acid inhibition in a comprehensive investigation of potential molecular mechanisms affecting PPI efficacy. Methods: Blood and gastric biopsies obtained from 50 studied patients were utilized in genetic screening of the entire *CYP2C19*, *ATP4A* and *ATP4B* genes using denaturing high-performance liquid chromatography. DNA methylation analysis of the *ATP4B* gene was performed on gastric samples using MALDITOF mass spectrometry. Results: Genetic analysis of the *CYP2C19* gene revealed the presence of the *CYP2C19*2* heterozygous allele in 16/50 patients, however, no significant association was detected between this genotype, total daily Lansoprazole dose and/or BAO. In our studied patients, homozygotes for the *CYP2C19*17* allele were unable to achieve acid suppression at high Lansoprazole doses (n=2, BAO >5 mmol/h, lansoprazole doses 300-450 mg per day), while heterozygotes achieved moderate to complete acid suppression at elevated lansoprazole doses (n=10, BAO < 5 mmol/h, Lansoprazole doses >90 mg per day). Differential methylation patterns identified negative associations between methylation levels of CpG sites 7, 11, and 21 and *ATP4B* gene expression, as well as CpG sites 4, 13, 14 and 15 and BAO. Conclusions: This study suggests that the *CYP2C19*17* allele is a dominant factor associated with increased Lansoprazole dosing and therapeutic success. Additionally, this study suggests that methylation of the *ATP4B* promoter may be a potential regulator of *ATP4B* gene expression that could contribute to the difficulty in achieving acid suppression.

INTRODUCTION

Diseases related to gastric acid hyperacidity such as peptic ulcers, gastroesophageal reflux disease (GERD), and Zollinger-Ellison Syndrome (ZES), constitute a potential risk for health and quality of life. Approximately 500,000 new cases and 4 million recurrences of peptic ulcer disease are reported annually [1,2]. Gastric acid-related diseases are often precipitated by the occurrence of hyperacidity, *H. pylori* infection, frequent use of non-steroidal anti-inflammatory drugs, and esophageal sphincter incompetence (esophagitis). Additionally in patients diagnosed with ZES, a non-β cell tumor of the pancreatic islets produce gastrin in quantities sufficient to stimulate the secretion of excess gastric acid which can lead to severe gastroduodenal ulcerations and other consequences associated with uncontrolled hyperchlorhydria [3]. Acid suppressive therapy is therefore fundamental in the treatment of these diseases.

Therapeutic strategies for the control of gastric acid hypersecretion target three major receptors involved with the stimulation of gastric acid secretion. First, cholinergic muscarinic receptors, located on the basolateral membrane of acid-secreting parietal cells, activate gastric acid release through binding of acetylcholine. A therapeutic approach to control gastric acid secretion would be attained by the use of the muscarinic antagonist pirenzepine. Though it was shown to be effective in peptic ulcer healing, pirenzepine was not free of side effects since the parietal cell expresses the M3 receptor [4]. Second, Gastrin receptor CCKBR, located on enterochromaffin-like cells (ECL), and the parietal cells, stimulate gastric acid secretion upon binding of gastrin. Inhibition of gastrin stimulated acid secretion using several peptide analogs designed to block the binding of gastrin to the CCKB receptor, were unsuccessful [5]. Currently, more potent non-peptide

compounds remain under investigation [6]. Third, histamine type 2 receptors (H2R), located on the gastric parietal cell, bind histamine released from enterochromaffin-like (ECL) cells. Histamine receptor antagonists (H2RAs) were successful in accelerating the healing of peptic ulcers when chronically administered to avoid ulcer recurrence [7,8]. However, the H2RA's had recurring problems including decreased effectiveness during day-time acid secretion and the development of tolerance within a short period of treatment [9]. None the less, these drugs were also relatively ineffective in the treatment of GERD.

Of the three receptors, H2 receptor activation appears to be a key step in parietal cell activation, resulting in an increase in intracellular cyclic AMP (cAMP) levels within the parietal cell which is necessary for activation of the electro-neutral ATP-dependent hydrogen-potassium exchanger (the gastric H^+/K^+ATPase), also known as the proton pump [10]. The proton pump belongs to the P2-type cation-transporting ATPase family and exists as a heterodimer consisting of a high molecular weight catalytic α subunit (*ATP4A*) with 10 transmembrane domains and a smaller but heavily glycosylated β subunit (*ATP4B*) with one transmembrane domain. Upon parietal cell activation, the heterodimeric protein, residing in cytoplasmic tubulovesicles in the inactive state, translocates and inserts into the apical membrane of the activated parietal cell.

The discovery that gastric acid secretion was due to the action of the parietal cell's proton pump led to the development of a specific class of drugs known as proton pump inhibitors (PPIs), or the pyridylmethylsufinyl benzimidazoles. PPIs target the terminal step in acid production thus inhibiting gastric acid release regardless of the nature of parietal cell activation and have been shown to be significantly more effective than H2

antagonists, reducing gastric acid secretion by up to 99% [7,11]. PPIs are all generally well tolerated and have become the preferred therapeutic of choice due to their high degree of target specificity and clinical efficacy. Currently marketed PPIs contain a core structure of substituted pyridylmethylsufinyl benzimidazoles and vary in terms of the substitutions in either the pyridine or the benzimidazole. PPIs such as Lansoprazole are administered as a prodrug, absorbed through the small intestine, and transported via systemic circulation into the highly acidic environment of the parietal cells' secretory canaliculi where it undergoes an acid-catalyzed conversion (protonation followed by molecular rearrangement) to two reactive products, a cationic thiophilic sulfenic acid and sulfonamide [12,13]. PPIs block acid release from activated gastric proton pumps by covalently binding to hydrogen sulfide residues of the catalytic α subunit, particularly at the luminally accessible cysteine 813 located on the luminal loop between transmembrane domains 5 and 6 [14]. Additional binding at luminally accessible cysteines occur with different proton pump inhibitors, for example, lansoprazole and rabeprazole bind to additional sites at cysteines 892 and 321 while pantoprazole binds to an additional site at cysteine 822 [15].

Although PPIs are effective drugs, the increased incidence of peptic ulcer recurrence remains an ongoing therapeutic challenge [1]. Less than complete acid suppression, increased night-time production of acidity, and required mealtime dosing for adequate drug levels during periods of pump activity are commonly observed with PPI-based therapy [16]. Also observed is a wide range of variability in PPI dosage required for sustained gastric acid suppression vital for ulcer healing and the treatment of acid-related diseases [17,18].

Variability in PPI efficacy has often been attributed to the relatively short half-life and the polymorphic cytochrome p450 drug metabolizing enzyme CYP2C19 responsible for PPI metabolism [19]. Patients' phenotypic classification based on CYP2C19 metabolic activity have been previously characterized into three main groups: extensive metabolizers (EM) characterized by a homozygous wild-type (WT) allele, intermediate metabolizers (IM) characterized by the presence of a WT and a mutant allele associated with decreased enzyme activity, and poor metabolizers (PM) characterized by homozygous mutant alleles associated with decreased enzyme activity. There are currently over 20 different haplotypes reported to affect CYP2C19 activity [20-29]. Most recently, the discovery of two specific variants of the 5'-upstream region, known as the *CYP2C19*17* allele, has led to the fourth phenotypic classification, ultra-rapid metabolizers (URM) [30].

The CYP2C19 genotype/phenotype associations have also been extensively studied in relation to PPI pharmacokinetics [31]. Previous studies have investigated the influence of *CYP2C19* genotype on healing of gastric ulcers using PPI therapies and have also shown that cure rates of PPI-based treatments for *H. pylori* infection were lowest in individuals characterized with the CYP2C19 extensive metabolizer (EM) phenotype due to insufficient plasma PPI concentrations necessary to inhibit acid secretion [29,32-33]. However, these studies have been either limited by the number of analyzed patients or to specific populations with different frequencies of particular *CYP2C19* gene variants. Many studies have also been limited by screening of specific *CYP2C19* alleles rather than the entire gene for novel variants which may alter CYP2C19 activity. The inclusion of other candidate genes (for example *ATP4A* and *ATP4B* genes encoding the gastric

ATPase) that are also involved in gastric acid secretion have also been absent from investigations. Furthermore, other potential molecular mechanisms such as epigenetics have not yet been investigated in the interindividual variability in PPI efficacy and gastric acid secretion.

In the present study, we have comprehensively investigated the potential molecular mechanisms affecting PPI efficacy in 50 ZES+ and ZES- gastric acid hyper-secretors requiring a wide Lansoprazole dose range for therapeutic acid suppression, including a subset of patients who were unable to obtain successful acid inhibition. Our initial studies examined the entire *CYP2C19* gene for both known and novel sequence variants as potential markers for variability in Lansoprazole dose and acid inhibition. In addition to *CYP2C19* genetics, we further expanded our investigations to include other potential molecular mechanisms (genetic and epigenetic) that potentially could regulate the expression of the genes encoding the gastric H^+/K^+ ATPase proton pump (*ATP4A* and *ATP4B* genes) and in turn alter acid secretion.

MATERIALS AND METHODS:

Patients

A total of 50 patients with basal acid hypersecretion (baseline BAO >15 mmol/h or >5mmol/h after antrectomy) were consented from an ongoing, long term clinical study conducted at the UAB Gastroenterology Department (IRB protocol number X040830003), examining effective acid suppression using a starting Lansoprazole dose of 60 mg per day [17,18]. Adjustments in dose and regimen were made if the targets (BAO < 5 mmol/h) were not met, or were exceeded (BAO < 1.0 mmol/h at more than a

minimally effective dose). The patients were re-examined once and, if necessary, twice at weekly intervals. Patients were considered to be: 1) well controlled if the BAO after treatment was < 1.0 mmol/h at a minimally effective dose; 2) moderately controlled if the BAO after treatment was 1.0 to 5.0 mmol/h; and 3) poorly controlled if the BAO after treatment was > 5.0 mmol/h. Dosage of Lansoprazole was defined as follows: 1) standard dose 60 mg per day; 2) low dose 30 mg per day or less; 3) elevated dose $\geq$ 90 mg per day; and 4) high dose $\geq$ 300 mg per day. Clinical diagnosis of ZES was made by definitive identification of the tumor or by elevated serum gastrin confirmed by a positive secretin test. Idiopathic gastric acid hypersecretion was diagnosed in patients presenting duodenal ulcer, acid hypersecretion, normal gastrin, and negative secretin tests. In our study, we were unable to obtain uniform pharmacokinetics for Lansoprazole due to many of the patients coming from long distances and being studied in the outpatient setting.

Sample Collection

Blood Samples

At the patients' regularly scheduled visits, 24 mL of blood was collected into heparinized Vacutainer tubes for the isolation of peripheral blood mononuclear cells (PBMC) and subsequent extraction of DNA.

Tissue Biopsies

During patients' endoscopies, gastric mucosa (n=50) and antrum (n=20) tissue biopsies were collected from patients for the extraction of DNA and RNA. Tissue biopsies were conducted during each individual patient's visit, which included a physical

examination, gastric analysis, and measurements for serum gastrin and BAO. Compliance with lansoprazole dosage was based on self-reporting, verified by pill counts.

Genomic DNA / RNA Extraction

Genomic DNA was extracted from patients PBMC, n=48 (blood samples were not available from two patients), and patients' gastric (n=50) and antrum (n=20) biopsies using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was also extracted simultaneously from all samples for gene expression analysis. Concentrations of DNA and RNA from each sample were measured using the ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Samples were stored at -80^{o}C until further analysis was conducted.

PCR Amplification of *CYP2C19*, *ATP4A*, and *ATP4B* genes

PCR primers were designed for the *CYP2C19*, *ATP4A* and *ATP4B* genes, covering the open reading frame including the exon-intron boarders, promoter and 5'-upstream regions, and 3'UTR using Primer 3 software [developed at Whitehead Institute (Cambridge, Massachusetts, USA) and Howard Hughes Medical Institute (Chevy Chase, Maryland, USA)] (supplementary table 1). PCR optimization of each amplicon for the three genes was performed using the FailSafe PCR system following manufacturer's instructions (Epicentre, Madison, WI). To confirm the presence of a single amplified PCR product, each amplicon was resolved as a single band by agarose gel electrophoresis, followed by a single injection onto the denaturing high-performance

liquid chromatography (DHPLC) Transgenomic Wave System (Transgenomic, Inc., Omaha, NE) under non-denaturing conditions (50^{o}C) using a universal gradient.

Mutation detection using DHPLC analysis

For sequence variant analysis of the *CYP2C19*, *ATP4A*, and *ATP4B* genes, we utilized two distinct methods of detection using DHPLC. The first method, temperature-modulated heteroduplex analysis, relies on the formation and separation of dsDNA fragments that contain mismatched bases (heteroduplexes). For each amplicon, a specific gradient was predicted using Transgenomic software and optimal screening temperatures for each amplicon were determined experimentally. Detection of both heterozygous and homozygous sequence variants was performed by DHPLC chromatogram analysis as we have previously described [34].

The second method combined DHPLC with the Surveyor Mutation Detection Fluorescence System (Transgenomic, Wave System, Omaha, NE), allowing for higher throughput mutation detection, using a single injection on the DHPLC at a single non-denaturing temperature rather than multiple injections at different denaturing temperatures. Enzymatic digestion using the Surveyor Mutation Detection Kit was performed following the manufacturer's instructions. DNA cleavage from the Surveyor endonuclease treatment occurs at the 3' site of the mismatch resulting in fragments of various sizes which may be resolved at a non-denaturing temperature (48^{o}C) using a pUC18 sizing standard gradient and detected using WAVE optimized HS staining solution against a sizing ladder.

Direct Sequencing

The PCR products of samples showing changes in DHPLC chromatogram pattern compared to that of wild-type were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced using the ABI Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA).

DNA Bisulfite Modification

Isolated DNA from patients' gastric biopsies was bisulfite modified using the EpiTect Bisulfite Kit (Qiagen, Valencia, CA) for downstream methylation analysis. Sodium bisulfite chemically modifies the DNA through the deamination of cytosine (C) residues to uracil while methylated cytosines (5-MeC) remain unmodified. After PCR amplification, all uracil and thymine residues are amplified as thymine (T) and only 5-MeC residues are amplified as C.

Identification of CpG Islands in the Gastric H^+/K^+ ATPase

Software programs EMBOSS CpGplot (European Bioinformatics Institute, Cambridge) and Methprimer [35] were used to identify possible CpG islands within the 5'-upstream region, 5'UTR, exon 1, and intron 1 in *CYP2C19* as well as the gastric H^+/K^+ ATPase α and β subunits (*ATP4A* and *ATP4B* genes). *In silico* analysis of the promoter regions of the three genes revealed the absence of CpG islands in CYP2C19 and the α subunit of the gastric H^+/K^+ ATPase. However, the β subunit demonstrated the presence of two CpG islands harboring 42 CpG sites (22 and 20 CpG sites) (Figure 1).

The first CpG island extends from 5'-upstream of the transcriptional start site through exon 1 and part of intron 1. The second CpG island is entirely located in intron 1.

PCR Primer Design and Amplification of Bisulfite-Modified DNA

Two pairs of PCR primers for the amplification of both CpG islands of the *ATP4B* gene were designed using the EpiDesigner software (Sequenom, San Diego, CA) to allow amplification in methylated and unmethylated bisulfite-modified DNA samples (supplementary table 2). Primers were tagged with a T7-promoter at the 5' ends. Amplification of the first CpG island starts 156 nucleotides upstream of the transcription start site, covering exon 1 (112 bp), and extends 86 bp into intron 1, generating a 396 bp fragment with 22 CpG sites. Amplification of the second CpG island located in intron 1 generates a 396 bp fragment with 20 CpG sites.

Methylation Detection

Quantitative methylation analysis of the identified CpG islands was performed using the MassARRAY Compact system (Sequenom, San Diego, CA). This methodology permits not only the quantitation (percentage) of 5-MeC within the analyzed fragment but also permits determination of specifically which cytosines are methylated. This system utilizes mass spectrometry (MS) for the detection and quantitative analysis of DNA methylation using Homogeneous MassCLEAVE (hMC) base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS as previously described [36].

In brief, PCR products were treated with shrimp alkaline phosphatase (SAP) for the removal of unincorporated nucleotides, followed by *in vitro* RNA transcription on the reverse strand and cleavage with RNase A. Cleaved PCR products were conditioned and spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser (Samsung, Irvine, CA), followed by spectral acquisition on a MassARRAY Analyzer Compact MALDI-TOF MS (Sequenom). The resultant methylation calls were performed by the EpiTyper software v1.0 (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites (known as a "CpG unit"). As shown in figure 1, not all of the cytosines within the CpG islands were able to be detected due to mass limitations of the MassARRAY analyzer.

Gene Expression Analysis

For RT-PCR gene expression analysis, 500ng of isolated RNA from collected patients' PBMCs (n=48), gastric (n=50) and antrum (n=20) biopsies was reverse transcribed into cDNA with random hexamers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Gene expression assay primers for ATP4A (NM_000704; assay ID Hs_00167575_m1) and ATP4B (NM_000705.2; assay ID Hs_00265171_m1) along with the ribosomal protein S9 (NM_001013.3; assay ID Hs_00955300_m1) as an endogenous housekeeping gene were obtained from Applied Biosystems and quantitative real-time PCR (RT-PCR) was performed using ABI Prism 7700 using the manufacturer's PCR amplification protocol. Each reaction was performed in triplicate and negative controls were included for each

gene. Relative gene expression was quantified using the $2^{-\Delta\Delta C}{}_T$ method as previously described [37].

Statistical Analysis

To test if continuous clinical variables (age and weight) were associated with uncensored continuous phenotypes (BAO and total daily dose) a Wald test of non-zero Spearman correlation was used. To test for associations between categorical clinical factors (gender, race, and ZE status) and uncensored continuous phenotypes, nonparametric rank sum tests [38] were used. An F-test was used to test if single nucleotide polymorphisms (SNPs) were associated with total daily lansoprazole dose and BAO (after using a Van der Waerden's rank transformation) based on a linear regression model, and independent variables: count of rare allele, and race (age was also adjusted for when analyzing total daily lansoprazole dose). Only SNP markers with minor allele frequencies of at least 5% where analyzed. An F-test was used to test if expression traits and CpG methylation, were associated with non-censored continuous phenotypes based on a linear regression model with all phenotypes transformed using a Van der Waerden's rank transformation, and independent variable expression and methylation level (age was adjusted for when analyzing total daily dose). Multiple testing was accounted for by using Bonferroni experiment-wise error and false discovery rates controlled with q-values [39,40].

RESULTS

Patient Response to Lansoprazole Therapy

Table 1 lists the characteristics of the 50 patients included in this study. BAO was measured and recorded at the time of sampling after the patient had been on individually titrated Lansoprazole therapy needed to maintain therapeutic acid suppression. Statistical analysis of clinical covariates revealed that only age showed a significant correlation with total daily Lansoprazole dose (Spearman's rho = -0.43; p=0.002) (Table 2). Race, gender, weight, and ZE status showed no correlation with total daily Lansoprazole dose, while none of the clinical covariates correlated with BAO. Lastly there was no significant correlation observed between BAO and the total daily Lansoprazole dose required to maintain therapeutic acid suppression.

Genotyping of *CYP2C19*

DHPLC analysis of the entire *CYP2C19* gene followed by direct sequencing revealed 9 novel and 16 previously reported sequence variants (Table 3 and supplementary Figures 1 and 2). Within our studied patients, the *CYP2C19*2A* (comprised of the 681 G>A; P227P variant) and *CYP2C19*2B* (comprised of the 681 G>A; P227P as well as the 276 G>C; E92D variants) alleles were present only in the heterozygous state in a total of 11 and 5 patients respectively. Statistical analysis of the *2A and *2B alleles as well as the individual variants 681 G>A; P227P and 276 G>C; E92D revealed no significant associations with decreased total daily lansoprazole doses after adjusting for age (data not shown). Screening of the 5'- upstream region of the *CYP2C19* gene in patients revealed 16 sequence variants (8 novel and 8 previously

identified variants) (Table 3 and supplementary Figure 2). Four novel variants (-3484 T>C, -3417 G>A, -3315 C>T, and -3253 A>G) were found all within one single patient. Three other novel variants (-3331 C>T, -3329 G>T, and -3219 T>G) were found together within the same 2 patients. The *CYP2C19*17* (-3402 C>T and -806 C>T variants) allele was identified in a number of patients in both the homozygous (n=2) and heterozygous (n=10) states.

Statistical analysis of all identified variants with minor allele frequencies (MAF) > 5% showed a lack of association with decreased total daily Lansoprazole dose (15 mg to 30 mg per day), but identified a correlation between the *CYP2C19*17* allele with increases in total daily Lansoprazole dose ($p = 0.02$) after adjusting for age illustrated in Figure 2A. Analysis of the *CYP2C19*17* homozygous patients also show an increased BAO even while taking high Lansoprazole doses (Figure 2B); however, statistical analysis showed no significant associations between any analyzed variant and altered BAO.

Genotyping of *ATP4A* and *ATP4B*

DHPLC analysis of the entire *ATP4A* and *ATP4B* genes followed by direct sequencing of patients showing altered chromatogram patterns revealed 3 novel and 6 previously reported sequence variants (Table 4 and supplementary Figure 3). Sequencing of the *ATP4A* gene showed three previously identified intronic sequence variants (IVS 6 +52 T>C, IVS 16 -12 A>G, and IVS 18 -49 C>T) and one synonymous novel variant in exon 18 (2768 C>T, Y913Y). Sequencing of the *ATP4B* gene within the same samples showed three previously identified synonymous variants (106 A>C; R36R, 144 C>T;

Y48Y and 843 G>A; A267A) coded within exons 1, 2 and 7, a novel variant within the 3'UTR (1294 T>C), and a novel nonsynonymous variant encoded within exon 7 (785 C>T; A248V).

Statistical analysis revealed that none of the identified sequence variants with MAF >5% had any association with either total daily lansoprazole dose or BAO.

RT-PCR analysis of gastric tissue biopsies from the 50 patients revealed a variable range of *ATP4A* and *ATP4B* gene expression which correlated in a linear fashion ($r = 0.68$; p-value < 0.0001) (supplementary figure 4). Statistical analysis revealed no associations between gene expression of *ATP4A* and *ATP4B* genes with either total daily lansoprazole dose or BAO. Sequence variants of the *ATP4A* and *ATP4B* genes also showed no association with relative gene expression (data not shown).

Increased methylation of the silenced *ATP4B* promoter in PBMC

RT-PCR analysis showed that both *ATP4A* and *ATP4B* genes were silenced in patients' PBMC and antrum biopsy samples while a variable range of expression was obtained from patients' gastric biopsies. Combining gene expression data with methylation analysis revealed an inverse correlation in which tissues with silenced *ATP4B* gene expression (PBMCs and antrum) show increased levels of methylation within CpG island 1 (supplementary figure 5). Average percent methylation for the 22 sites of the *ATP4B* CpG island 1 in patients PBMCs (n=48) was approximately 84% $\pm$ 5 while patients' gastric (n=50) and antrum (n=20) biopsies showed approximately 33% $\pm$ 9 and 44% $\pm$ 9 respectively.

Methylation analysis of the *ATP4B* CpG island 2 showed an increased average percent methylation in patients' gastric (75% $\pm$ 8) and antrum (81% $\pm$ 6) biopsies when compared to the average percent methylation of CpG island 1 (supplementary figure 5). The average percent methylation of *ATP4B* CpG island 2 between patient PBMCs, gastric and antrum samples displayed statistically non-significant variability.

Association Between *ATP4B* Methylation, Gene Expression, and BAO

Methylation within CpG island 1 of the analyzed gastric biopsies utilizing the MALDITOF technique revealed a negative correlation between the average percent methylation with *ATP4B* and *ATP4A* gene expression ($p = 0.029$; $p = 0.014$) (Figure 3, table 5). Subsequent examination and quantitation of specific cytosines within CpG island 1 demonstrated a negative association between the increased percent methylation of CpG sites 7, 11 and 21 with decreased *ATP4B* gene expression (unadjusted p-values of 0.007, 0.005, and 0.03) (Figure 3, table 5) and decreased *ATP4A* gene expression (unadjusted p-values of 0.006, 0.002, and 0.032) (table 5). After a traditional Bonferroni multiple testing correction, the adjusted p-values for CpG sites 7, 11 and 21 with *ATP4B* gene expression were 0.21, 0.13 and 0.84. The adjusted p-values for CpG sites 7, 11 and 21 with *ATP4A* gene expression were 0.17, 0.05 and 0.9. However, using less stringent criterion (control false discovery rate, FDR), the q-value for CpG sites 7 and 11 with *ATP4B* gene expression was 0.03. Analysis of site specific methylation of the *ATP4B* CpG island 2 along with the average percent methylation showed no correlation with *ATP4B* or *ATP4A* relative gene expression in gastric tissue (supplementary table 3).

Methylation within *ATP4B* CpG island 1 of the analyzed gastric biopsies also suggested a trend between increased average percent methylation of all detected cytosines in *ATP4B* CpG island 1 and decreased BAO (Figure 4, table 6) ($p = 0.049$) . Further examination of specific cytosines within CpG island 1 also showed slight associations between BAO and increased methylation of CpG sites 4 and 13/14 (sites 13 and 14 were analyzed together as one unit) with unadjusted p-values of 0.0097 and 0.0124 respectively (Figure 4, table 6). After a traditional Bonferroni multiple testing correction, the adjusted p-values were 0.27 and 0.35. However, the q-value was 0.04 in all three sites. Increased methylation of CpG site 15 also suggested a trend toward decreased BAO ($p = 0.046$; Figure 4, table 6). Analysis of site specific methylation of the *ATP4B* CpG island 2 along with the average percent methylation showed no correlation with changes in BAO (supplementary table 4). Analysis of site specific methylation along with average percent methylation of both CpG islands showed no statistical correlation with total daily Lansoprazole dose (supplementary table 5).

DISCUSSION

Previous studies investigating PPI pharmacokinetics in relation to specific CYP2C19 genotypes have been limited by the enrollment of healthy volunteers rather than patients with gastric hyperacidity. Studies enrolling patients have been conducted in Asian populations known to have markedly different frequencies of *CYP2C19* alleles than in Caucasian or African-American populations that characterize many of the patients seen in the US. In these studies emphasis was on specific mutations in *CYP2C19* and have neither examined the entire gene and its promoter/enhancer regions nor included

other genes (for example *ATP4A* and *ATP4B*) that are also involved in gastric acid secretion; the impact of several variants (including those associated with decreased as well as increased metabolism) within the same patients has not been fully evaluated previously. Lastly, the increasing importance of epigenetics in addition to pharmacogenetics has previously not been considered as a cause of variability in PPI efficacy and gastric acidity.

In our comprehensive analysis of the *CYP2C19* gene in studied patients, we identified one of the two main PM alleles, *CYP2C19*2*, that has previously been reported to account for 99% of decreased enzyme activity in Asians and approximately 87% in Caucasians [20, 21, 41]. The *CYP2C19*2* allele can be further classified as either *CYP2C19*2A* or **2B* due to the presence of the 681 G>A; P227P variant with or without the 276 G>C; E92D variant. In a total of 8 patients who were individually titrated to low Lansoprazole doses (15 mg and 30 mg per day), only three presented either the *CYP2C19*2A* (681 G>A; P227P) or the *CYP2C19*2B* (681 G>A; P227P with 276 G>C; E92D) in heterozygous states. The *CYP2C19*2A* allele was also identified in the heterozygous state in 9 additional patients; 4 of these patients remained on standard daily Lansoprazole doses (60 mg per day) while 5 were titrated to higher daily Lansoprazole doses (90 mg to 150 mg per day). The *CYP2C19*2B* allele was also identified in the heterozygous state in 4 additional patients; 1 patient remained on a standard daily Lansoprazole dose (60 mg per day administered 30 mg b.i.d.) while 3 were titrated to higher daily Lansoprazole doses (90 mg to 240 mg per day). Statistical analysis of either the 681 G>A; P227P or 276 G>C; E92D variants, alone or in combination, showed no significant correlations with decreased total daily Lansoprazole dose or with BAO. In

contrast to previous studies [20, 24, 42], our population of 50 gastric acid hypersecretors demonstrated a lack of association between the *CYP2C19*2* allele and decreased total daily Lansoprazole doses. This lack of statistical association between the CYP2C19*2A and *2B alleles with decreased total daily Lansoprazole dose may in part be due to the lack of homozygous variant alleles linked to the CYP2C19 PM phenotype. In our study, a total of 13 patients were identified with either the *CYP2C19*2A* or *CYP2C19*2B* allele in the heterozygous state which is commonly linked to the CYP2C19 IM phenotype. Genetic analysis also showed that the *CYP2C19*3* allele (the other most commonly reported allele associated with decreased enzyme activity) was completely absent from our 50 patients.

In this study, four novel variants of the *CYP2C19* gene's 5' upstream region (-3484 T>C, -3417 G>A, -3315 C>T, and -3253 A>G) were found within one single patient taking 240 mg per day and achieving acid suppression of 0.7 mmol/h BAO. Three other novel variants within the same region (-3331 C>T, -3329 G>T, and -3219 T>G) were also found together within the same 3 patients. Two of these patients were titrated to low Lansoprazole dose (30 mg per day), the third patient was on standard Lansoprazole dose of 60 mg per day. All three patients achieved therapeutic acid suppression (BAO < 1 mmol/h). Although the impact of these novel variants on CYP2C19 activity remains to be determined, the clinical response to Lansoprazole therapy suggests minimal effect of these variants on CYP2C19 function.

Inter-ethnic differences in the prevalence of the different *CYP2C19* phenotypes have been previously reported and specific sequence variants have also been well established. The prevalence of the *CYP2C19*17* allele and URM phenotype is reversed

in the Caucasian population, with approximately 36% exhibiting either the heterozygous or homozygous *CYP2C19*17* allele while Chinese and Japanese populations exhibit 8% and 1% respectively [30]. In our studied population of 50 gastric acid hypersecretors, the *CYP2C19*17* allele was identified in 10/41 Caucasians (24%) and 2/9 African Americans (22%). When analyzing the two variants of the *17 allele separately, the -3402 C>T variant was identified in 20/41 Caucasians (49%) and 3/9 African Americans (33%) while the -806 C>T variant was identified in 15/41 Caucasians (37%) and 2/9 African Americans (22%). The *CYP2C19*17* (-3402 C>T and -806 C>T variants) allele was identified in the homozygous state in 2 Caucasian patients (one ZES positive and one ZES negative); both of these patients were unable to attain therapeutic acid suppression at high Lansoprazole doses (300 mg and 450 mg per day). The *CYP2C19*17* allele was also identified in the heterozygous state in the following patients: 1) 3 Caucasian patients moderately controlled for gastric acidity (BAO between 1.0 and 5 mmol/h) with elevated-to-high Lansoprazole doses (90 to 450 mg per day); 2) 6 patients (5 Caucasian and 1 African American) well controlled for gastric acidity (BAO < 1.0 mmol/h) with one patient on a standard dose (60 mg per day) and 5 patients on elevated Lansoprazole doses (90 to 270 mg per day); and 3) 1 African American patient with an undetermined BAO with an elevated Lansoprazole dose (240 mg per day).

Previous reports have established complete linkage disequilibrium of the -806 C>T and -3402 C>T variants and reported the absence of defective mutant alleles such as *CYP2C19*2* and *CYP2C19*3* in individuals heterozygous or homozygous for the *CYP2C19*17* allele [30]. However in our genetic analysis of 50 gastric acid hypersecretors, the two variants (-3402 C>T and -806 C>T variants) comprising the

*CYP2C19*17* allele did not always appear together and, in a small number of cases, were identified in patients heterozygous for *CYP2C19*2A* or **2B*. A previous study has attributed the increased CYP2C19 activity mediated by the *CYP2C19*17* allele to the recruitment of a transcription factor(s) to the mutated -806 site that consequently increases transcription of the *CYP2C19* gene [30], yet, as of today, the molecular mechanism of the -3402 C>T variant on transcription is unknown. Our study demonstrates that the presence of both variants together results in an increase in the total daily Lansoprazole dose required for acid suppression. The presence of the -3402 C>T variant alone or the -806 C>T variant alone did not have a major effect on the effective dosing requirement for Lansoprazole.

It has also been previously established that individuals homozygous for the *CYP2C19*17* allele experience significantly reduced systemic exposure to the PPI Omeprazole and, on average, have a 2.1-fold reduction in mean Omeprazole AUC compared to WT individuals [43, 44]. In our study, we were unable to obtain uniform pharmacokinetics for Lansoprazole due to the difficulty of performing PK studies in a busy outpatient setting. This study does indicate, however, that the presence of the *CYP2C19*17* allele corresponds to an increased total daily Lansoprazole dose required to achieve acid suppression (Figure 2A). Though the *CYP2C19*17* allele showed no association with altered BAO, individuals homozygous for the *CYP2C19*17* allele may not be able to be achieve therapeutic acid suppression through PPI therapy due to the CYP2C19 URM phenotype even on high Lansoprazole doses (Figure 2B). Patients identified as homozygous for the *CYP2C19*17* allele are prime candidates for either the addition of a selective CYP2C19 inhibitor or alternate gastric acid suppression therapies

that are metabolized through different pathways. Thus one patient homozygous for the *CYP2C19*17* allele taking 450 mg per day of Lansoprazole was able to significantly lower BAO only after the addition of a type 2 Histamine antagonist.

We further evaluated the role of the other major factor of gastric acid secretion, the H^+/K^+ ATPase. While the H^+/K^+ ATPase α subunit contains the PPI binding sites, the β subunit is essential for proton pump function and structure. Genetic analysis of the entire *ATP4A*, including the nucleotide regions coding for the luminally accessible cysteine residues 813, 822, 892 and 321 known to bind PPIs, and *ATP4B* genes failed to identify any significant associations between patients' BAO or total daily Lansoprazole dose and previously identified or novel gene variants. Due to the importance of the β subunit in acid secretion and proton pump structure and the presence of two CpG islands within the *ATP4B* gene, we further investigated whether differential methylation patterns of the CpG islands of the *ATP4B* gene may alter expression of the gastric proton pump, potentially affecting gastric acid secretion and PPI efficacy.

In previous studies, it was reported that the expression of the gastric H^+/K^+ ATPase is a characteristic feature of parietal cells and that expression of both the α and β subunits is regulated at the transcriptional level [45]. Previous reports have related the well-established tissue specific expression of the α and β subunits to the presence of gastric-specific GATA DNA-binding nuclear proteins (GATA-GT1 and GATA-GT2) which recognize a specific sequence motif (G/CPuPuG/CNGATA/TPuPy) in the 5'-upstream regions of both the *ATP4A* and *ATP4B* genes [46-50]. In the present study, methylation analysis of different tissues obtained from the same individual indicated the presence of differential CpG site-specific methylation of CpG island 1 of the *ATP4B* gene

(supplementary figure 5). In the gastric and antrum tissue, as well as in PBMCs, methylation of CpG island 1 correlated with abolished proton pump expression (*ATP4A* and *ATP4B* gene silencing). In gastric tissue, the average percent methylation was 33.0% $\pm$ 9.0 which correlated with a variable range of *ATP4B* gene expression. Abolished *ATP4B* gene expression was detected in antrum tissues displaying an average percent methylation of 44.0% $\pm$ 9.0. In PBMCs, CpG sites of the *ATP4B* CpG island 1 displayed an average percent methylation of 84.0% $\pm$ 5.0 which was associated with downregulation of *ATP4B* gene expression.

While the average total methylation between the gastric and antrum biopsies show no significant differences, expression of the gastric H^+/K^+ ATPase α and β subunits were undetected in antrum biopsies. This suggests the presence of alternative molecular mechanisms that could down regulate gene expression, e.g., histone deacetylation (HDAC); the absence of the previously identified gastric-specific GATA DNA-binding nuclear proteins; and/or the absence of unidentified antrum-specific transcription factors required to activate the *ATP4A* and *ATP4B* expression [46-50].

In the examined gastric tissues, the average percent methylation of the detected CpG sites of the *ATP4B* CpG island 1 showed a negative association with *ATP4B* (Figure 3) and *ATP4A* gene expression (Table 5). The mechanisms underlying the association between the methylation status of the *ATP4B* promoter and *ATP4A* gene expression are unclear, however, their association may contribute to the importance of the β subunit in acid secretion as previously reported in transfection studies [51, 52]. These transfection studies have indicated that in the absence of the β-subunit, the α-subunit is retained within the rough endoplasmic reticulum and is relatively unstable without the β-subunit.

However, the β-subunit was shown to be localized to the apical brush boarder in a subapical vesicular compartment in cell lines without the α-subunit. Transgenic mice studies also confirmed that parietal cells of α-subunit knock-out mice, while unable to release acid, continue to express the β-subunit [53]. Expression analysis of the 50 studied patients also showed that ATP4A and ATP4B correlated in a linear fashion (supplementary figure 4).

Statistical analysis also displayed a negative correlation between methylation levels of specific CpG sites 7, 11 and 21 and gene expression of the *ATP4B* and *ATP4A* genes in gastric biopsies (Figure 3, Table 5). Furthermore, analysis also indicated a slight association between average methylation and site-specific methylation of CpG sites 4, 13/14, and, to a lesser extent, CpG site 15 of the *ATP4B* CpG island 1, and BAO (Figure 4, Table 6). However differential CpG island methylation of the *ATP4B* promoter showed no statistical associations with the variability in total daily Lansoprazole dose (supplementary table 6). Associations observed between methylation, gene expression, and BAO need to be further validated in larger populations to determine whether methylation status of the *ATP4B* gene can be utilized as a potential marker for active proton pumps and variability of acid output in response to PPI therapy.

In summary this study demonstrates that, in a population of Caucasian and African-American hypersecretors, the *CYP2C19*17* allele is a major determinant of the variability in PPI dose required for therapeutic acid suppression. This study also demonstrates for the first time differential DNA methylation of the CpG island of the *ATP4B* promoter in human gastric mucosal biopsies might be a potential regulator of *ATP4B* gene expression which could contribute to the variability in resultant BAO.

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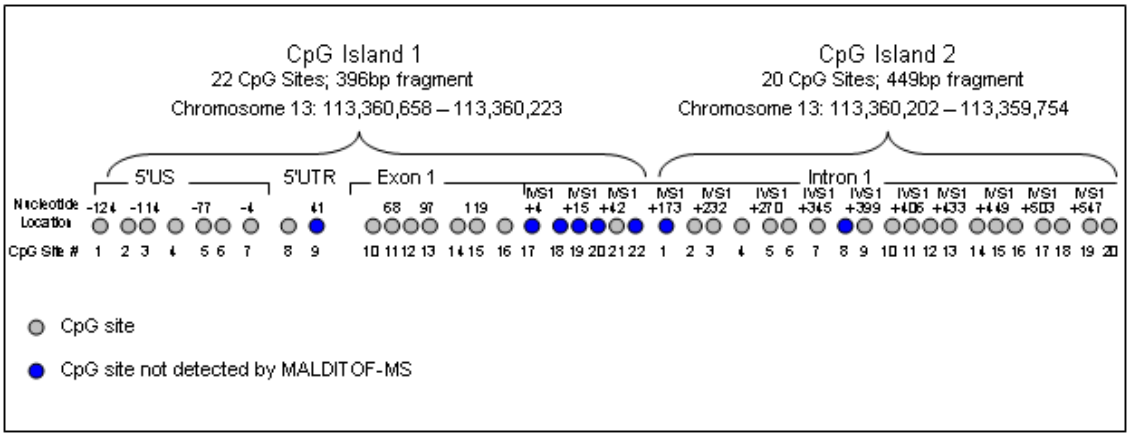
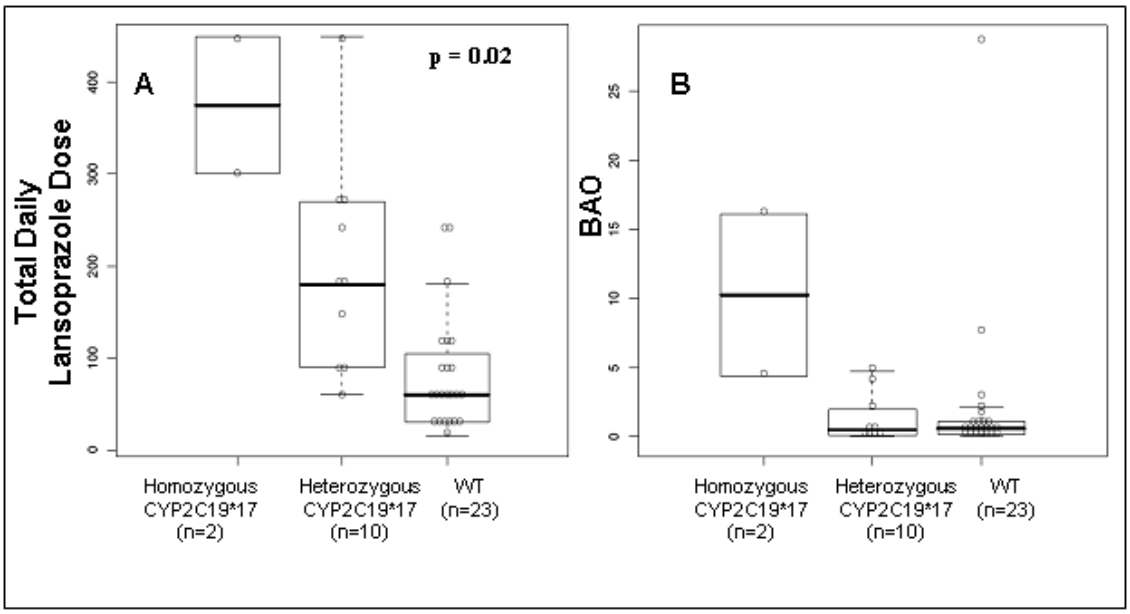
Figure 1: CpG islands of the *ATP4B* Gene



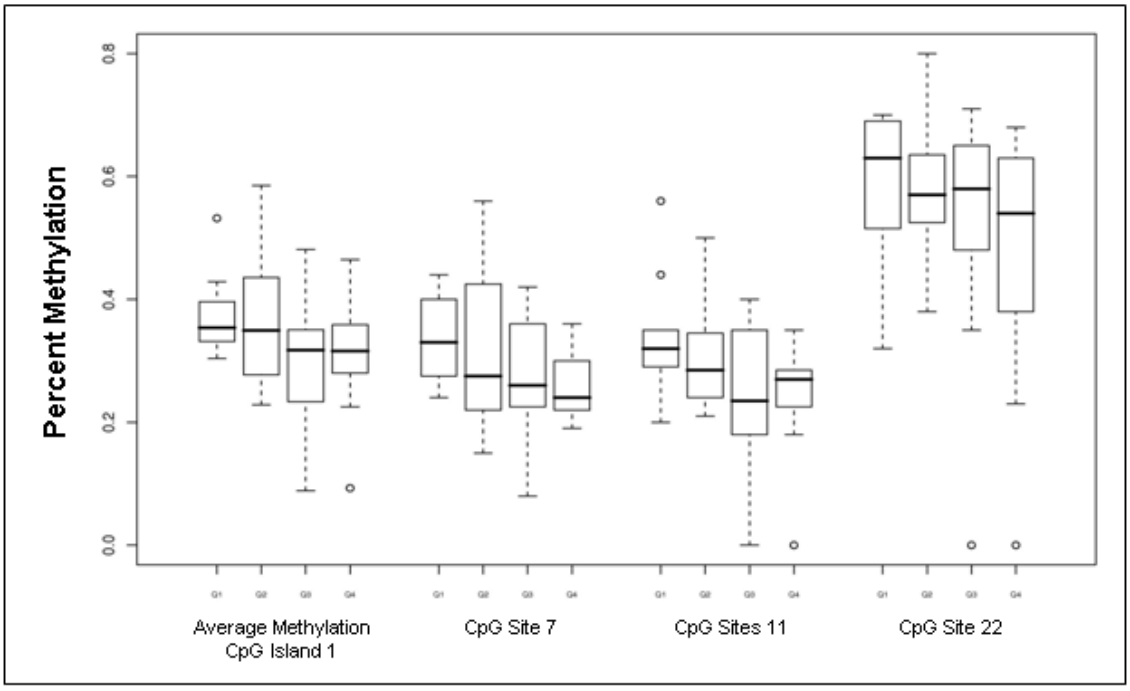
Identification of two CpG islands within the *ATP4B* gene promoter region was conducted by *in silico* analysis using software programs EMBOSS CpGplot and Methprimer. *ATP4B* CpG island 1 contains 22 CpG sites and covers from 5'-upstream (5'US) of the transcriptional start site through exon 1 and part of intron 1. *ATP4B* CpG island 2 contains 20 CpG sites and is entirely located in intron 1. Methylation analysis using MALDI-TOF MS results in CpG site-specific detection of 16/22 CpG sites of the *ATP4B* CpG Island 1 (73% detection) and 18/20 CpG sites of the *ATP4B* CpG Island 2 (90% detection). CpG sites not detected by MALDI-TOF MS (indicated as blue circles) lie within cleaved fragments that are above or below the detection limits of the analyzer (1500 – 7000 daltons).

Figure 2: Association of the *CYP2C19*17* Allele with Variability in Total Daily Lansoprazole Dose



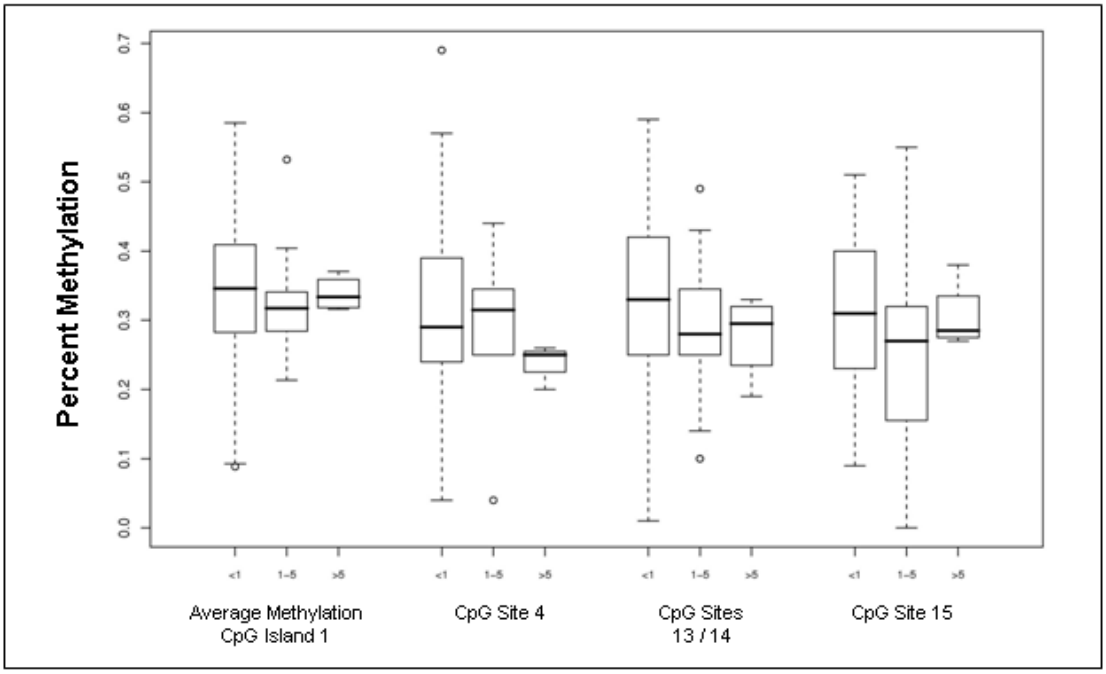
Box plot analysis of *CYP2C19*17* allele, total daily Lansoprazole doses and BAO indicated that (panel 2A) the mean Lansoprazole dose required for acid suppression was 350, 198 and 83 mg per day for patients homozygous (n=2), heterozygous (n=10) and wild type (n=23) for the *CYP2C19*17* allele. Panel 2B illustrates the failure in achieving reduced acid suppression in patients homozygous for the *CYP21C19*17* allele (n=2) as indicated by the elevated BAO (4.7 and 16.1 mmol/h) compared to both heterozygous (n=6, BAO < 1 mmol/h, n=3, BAO>5 mmol/h) and WT patients(n=21, BAO > 1 mmol/h; two outliers had a BAO measurements of 7.5 and 28.9 mmol/h) . Lansoprazole dose and BAO levels are indicated by circles while mean values for the three genotype groups are indicated by black horizontal lines.

Figure 3: Association Between Increased *ATP4B* Promoter Methylation and *ATP4B* Gene Expression.



Q1 – Q4 are four quartiles of *ATP4B* gene expression arranged from lowest to highest. The relative gene expression range for each quartile is as follows: Q1 = 0.065 - 0.85 (n=13); Q2 = 0.85 - 1.8 (n=12); Q3 = 1.8 – 2.74 (n=12); and Q4 = 2.74 – 5.53 (n=13). Average methylation levels of detected CpG sites of the *ATP4B* CpG island 1, CpG site 7, CpG site 11, and CpG site 22 of the four quartiles are indicated by black horizontal lines.

Figure 4: Association Between Increased *ATP4B* Methylation and Decreased BAO.



Patients were grouped according to levels of acid suppression as indicated by BAO measurements: BAO < 1 (n=31); BAO 1-5 (n= 13); and BAO > 5 (n=5). Average methylation values for ATP4B CpG island 1, CpG site 4, CpG sites 13/14, and CpG site 15 of the three groups are indicated by black horizontal lines.

Table 1: Demographics of 50 Gastric Acid Hypersecretors. Demographic (age, gender, race) and pertinent clinical data (weight, BAO, ZES status, and total daily Lansoprazole dose) were recorded at the time of patient sampling.

Patients	**Number**	**N=50**
Age	Median (SD)	59.1 (11.8)
	Range	35.0-81.0
Gender	Male	31 (62%)
	Female	19 (38%)
Race	Caucasian (C)	41 (82%)
	African American (AA)	9 (18%)
Weight (lbs)	Median (SD)	184.3 (62.40)
	Range	78.0-400.0
ZES Status	Positive	37 (74%)
	Negative	13 (26%)
BAO (mmol/h)*	Median (SD)	2.3 (4.83)
	Range	0.0-28.9
Total Daily Dose (mg)	Median (SD)	117.3 (99.11)
	Range	15.0-450.0

* BAO was unable to be measured in one patient

Table 2: Statistical analysis of BAO and total daily Lansoprazole dose with clinical covariates. Statistical analysis revealed that only age showed a significant correlation with total daily Lansoprazole dose (Spearman rank correlation -0.43; p value < 0.005). Race, gender, weight, and ZE status showed no correlation with total daily Lansoprazole dose while none of the covariates correlated with BAO.

Phenotype	**Covariate**	**Spearman's rho or difference in medians**	**P-value**
BAO	Age	0.18	0.23
	Weight	-0.2	0.16
	Gender (M-F)	0	0.94
	Race (AA-C)	0	0.88
	ZE (1-0)	0.05	0.14
Total Daily Dose (TDD)	Age	-0.43	0.0018
	Weight	0.25	0.25
	Gender (M-F)	30	0.06
	Race (AA-C)	60	0.47
	ZE (1-0)	30	0.11

Table 3: Identified variants of the *CYP2C19* gene

SNP ID	dbSNP (NCBI)	Alternate Mutation Name	Amino Acid Change	Resultant Enzyme Activity
5'US -3484 T>C	Novel	-	Non-coding	unknown
5'US -3417 G>A	Novel	-	Non-coding	unknown
5'US -3402 C>T	rs11188072	*17	Non-coding	Increased [30]
5'US -3332 A>C	rs7101258	-	Non-coding	unknown
5'US-3331 C>T	Novel	-	Non-coding	unknown
5'US -3329 G>T	Novel	-	Non-coding	unknown
5'US -3315 C>T	Novel	-	Non-coding	unknown
5'US -3266 G>T	rs4532967	-	Non-coding	unknown
5'US -3253 A>G	Novel	-	Non-coding	unknown
5'US -3219 T>G	Novel	-	Non-coding	unknown
5'US -889 T>G	rs11568732	-	Non-coding	unknown
5'US -833 del -T	rs17880036	-	Non-coding	unknown
5'US -806 C>T	rs12248560	*17	Non-coding	Increased [30]
5'US -783 C>T	rs11568729	-	Non-coding	unknown
5'US -542 A>G	Novel	-	Non-coding	unknown
5'US -98 T>C	rs4986894	-	Non-coding	unknown
276 G>C	rs17878459	*2B	E92D	Decreased [25]
681 G>A	rs4244285	*2A, *2B, *2C	No change	Decreased [19,25]
IVS5 -51C>G	rs4417205	-	Intronic	unknown
IVS5 -113 T>G	rs28399511	-	Intronic	unknown
IVS5 -114 C>G	Novel	-	Intronic	unknown
1228 C>T	rs17879685	*13	R410C	Unknown [22]
1251 A>C	rs17886522	-	No change	unknown
IVS8 -17 A>G	rs4451645	-	Intronic	unknown
3'UTR +82 T>C	rs17882796	-	Non-coding	unknown

Table 4: Sequence Variants of the *ATP4A* and *ATP4B* Genes

Gene	SNP ID	dbSNP (NCBI)	Amino Acid Change	Affect on Enzyme Activity
ATP4A	IVS 16 +52 T>C	rs2733739	Intronic	unknown
	IVS 16 -12 A>G	Novel	Intronic	unknown
	2739 C>T	Novel	No change	unknown
	IVS 18 -49 C>T	rs12977572	Intronic	unknown
ATP4B	5'US -147 A>C	ENSSNP10841904	Non-coding	unknown
	106 A>C	rs9285616	R36R; no change	unknown
	144 C>T	rs11164142	Y48Y; no change	unknown
	743 C>T	Novel	A248V	unknown
	801 G>A	rs17852631	A267A; no change	unknown
	3'UTR +376 T>C	Novel	Non-coding	unknown

Table 5: Site Specific Methylation of the *ATP4B* CpG Island 1 Shows a Statistical Correlation with Gene Expression of Both the β and α Subunits

CpG Site	ATP4A Gene Expression			ATP4B Gene Expression		
	Effect of additional unit of CpG (95% CI)	**P-value**	**Bonferroni adjusted p-value**	**Effect of additional unit of CpG (95% CI)**	**P-value**	**Bonferroni adjusted p-value**
1 D8	-2.16(-5.10, 0.78)	0.157	1	-1.98(-5.16, 1.19)	0.227	1
2,3	-2.20(-5.21, 0.81)	0.159	1	-0.84(-4.07, 2.39)	0.612	1
4	0.74(-1.96, 3.43)	0.595	1	-1.12(-4.58, 2.34)	0.531	1
5,6	-1.52(-4.18, 1.14)	0.268	1	-1.71(-4.49, 1.08)	0.236	1
7	-4.41(-7.39,-1.43)	0.006	0.17	-4.49(-7.63,-1.36)	0.007	0.21*
8 D1	-2.16(-5.10, 0.78)	0.157	1	-1.98(-5.16, 1.19)	0.227	1
10	-3.43(-6.05,-0.81)	0.014	0.39	-2.89(-5.72,-0.07)	0.052	1
11	-4.77(-7.59,-1.95)	0.002	0.05^	-4.72(-7.82,-1.62)	0.005	0.13*
12	-0.73(-2.57, 1.11)	0.441	1	0.35(-1.55, 2.25)	0.721	1
13 D14	-1.62(-4.65, 1.40)	0.298	1	-1.77(-5.02, 1.47)	0.29	1
14 D13	-1.62(-4.65, 1.40)	0.298	1	-1.77(-5.02, 1.47)	0.29	1
15	-1.80(-4.69, 1.09)	0.228	1	-0.84(-3.99, 2.31)	0.605	1
16	-2.10(-5.11, 0.91)	0.179	1	-2.57(-5.70, 0.57)	0.116	1
17	-1.29(-3.93, 1.34)	0.342	1	-0.68(-3.47, 2.12)	0.638	1
22	-2.28(-4.29,-0.26)	0.032	0.9	-2.47(-4.63,-0.31)	0.03	0.84
AVG	-4.40(-7.79,-1.01)	0.014		-4.26(-7.94,-0.57)	0.029	

*q-value = 0.03

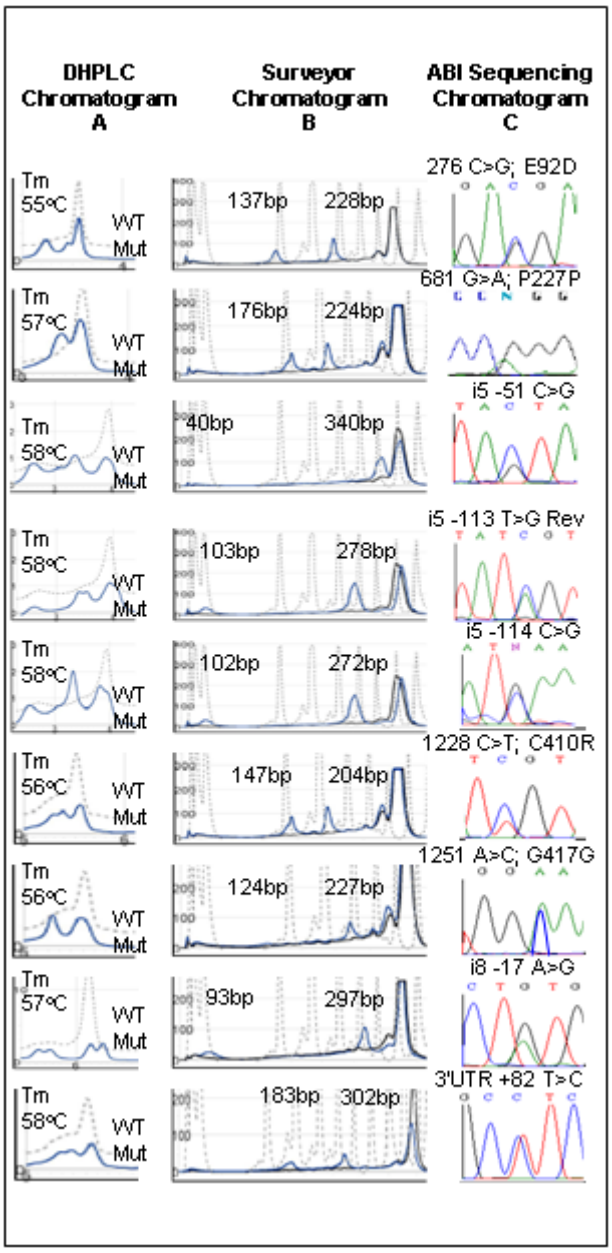
^q-value = 0.05

Table 6: Site Specific Methylation of the *ATP4B* CpG Island 1 Shows a Statistical Correlation with Decreased BAO

CpG Site	Basal Acid Output		
	Effect of additional unit of CpG (95% CI)	**P-value**	**Bonferroni adjusted p-value**
1 / 8	-1.23(-3.40, 0.94)	0.2746	1
2,3	-2.09(-4.32, 0.13)	0.0726	1
4	-3.31(-5.68,-0.94)	0.0097	0.27*
5,6	-1.77(-3.73, 0.19)	0.0842	1
7	-1.11(-3.52, 1.30)	0.371	1
10	-0.80(-2.90, 1.30)	0.4604	1
11	-1.04(-3.35, 1.28)	0.3848	1
12	-0.28(-1.69, 1.12)	0.6946	1
13 / 14	-2.77(-4.86,-0.69)	0.0124	0.35*
15	-2.16(-4.21,-0.10)	0.0455	1
16	-1.61(-3.87, 0.66)	0.1721	1
21	-0.61(-2.16, 0.93)	0.4407	1
AVG	-2.63(-5.16,-0.09)	0.0487	

*q-value = 0.04

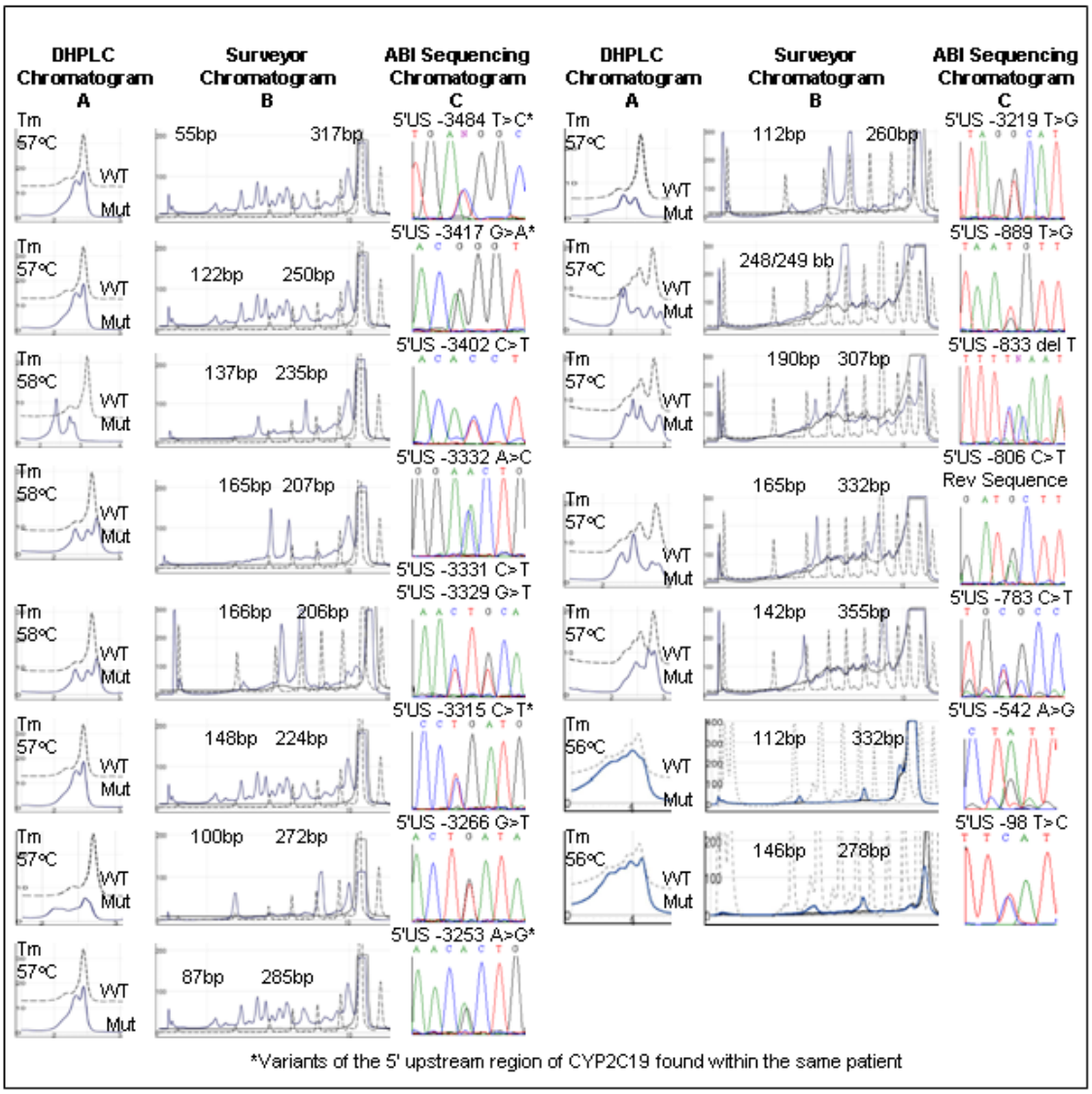
Supplementary Figure 1: Genetic Analysis of the *CYP2C19* Gene in 50 Gastric Acid Hypersecretors.



Sequence variants of the *CYP2C19* gene in studied patients were detected by DHPLC using two methods: temperature-modulated heteroduplex analysis (column A) and Surveyor mutation detection fluorescence analysis (column B) on the Transgenomic WAVE system. In column A, changes in DHPLC chromatogram peak patterns of

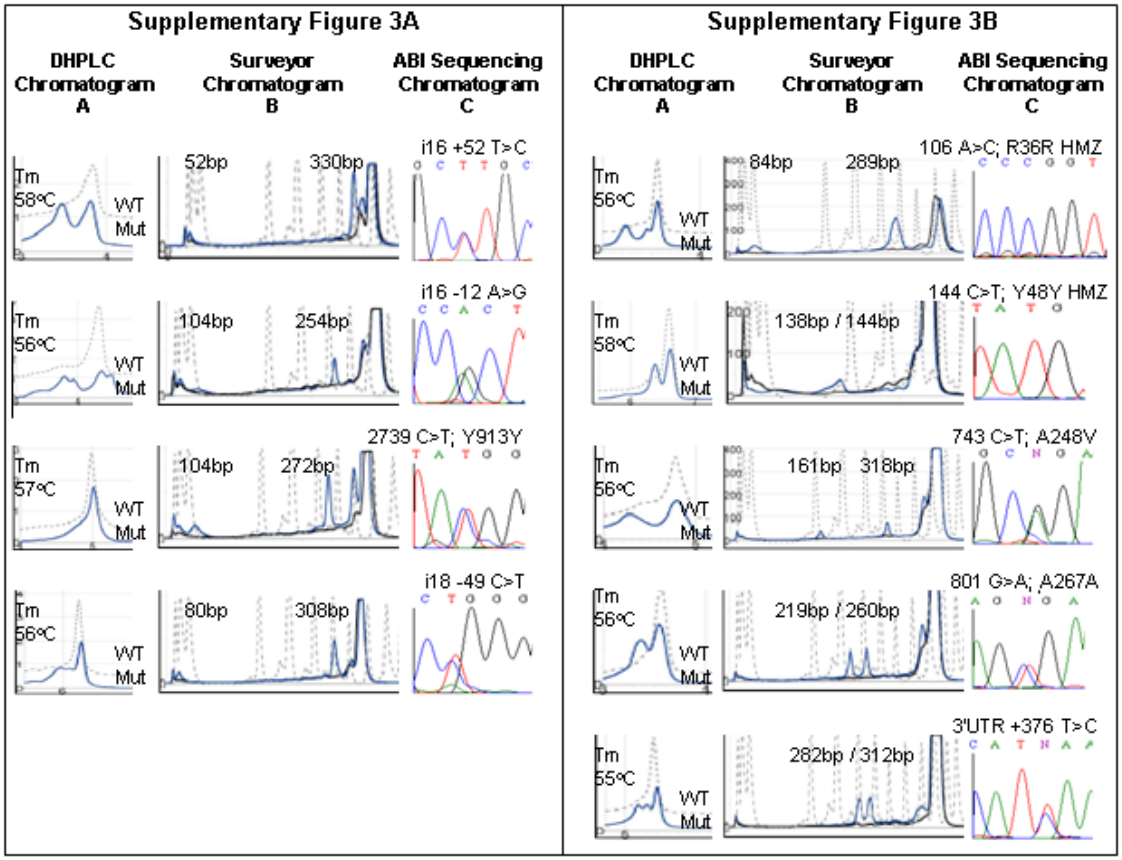
samples with unknown *CYP2C19* genotype (labeled Mut and shown as a solid blue line) from the wild type sample (labeled WT and shown as a dashed line) were detected at previously optimized screening temperatures specific for each *CYP2C19* fragment. PCR products were also digested with the Surveyor endonuclease and injected onto the DHPLC at 48^{o}C using a pUC18 sizing gradient. Peak patterns were compared to that of a digested, PCR-amplified known WT sample (solid black line) against a 50 bp sizing ladder (grey dashed line) (column B). PCR products of samples showing chromatogram differences from that of the WT pattern were then sequenced to determine the identity of the sequence variant (column C).

Supplementary Figure 2: Genetic Analysis of the 5' Upstream Region of the *CYP2C19* Gene in 50 Gastric Acid Hypersecretors.



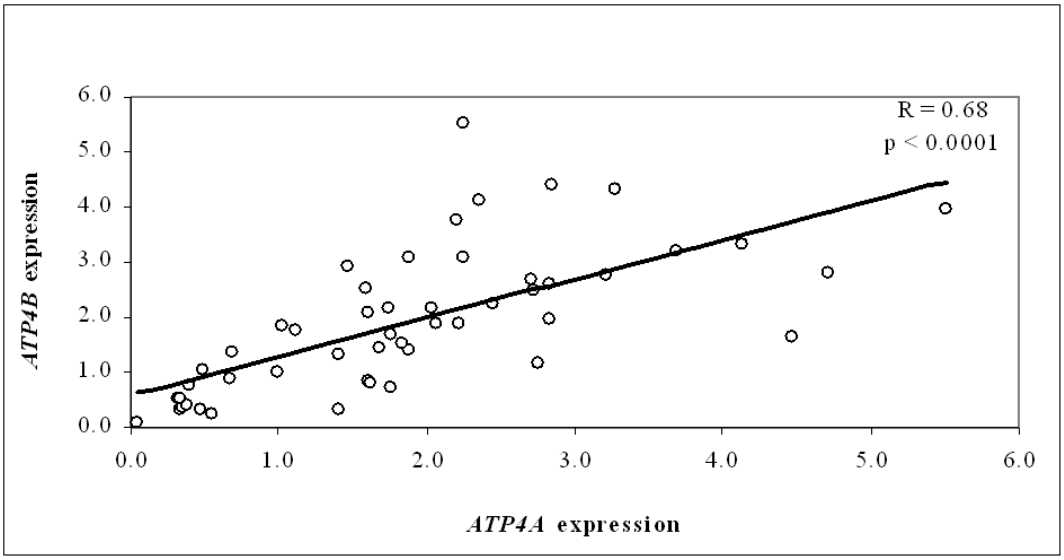
Sequence variants of the 5'US region of the *CYP2C19* gene in studied patients were detected by DHPLC using two methods: temperature-modulated heteroduplex analysis (column A) and Surveyor mutation detection fluorescence analysis (column B) on the Transgenomic WAVE system. PCR products of samples showing chromatogram differences from that of the WT pattern were then sequenced to determine the identity of the sequence variant (column C).

Supplementary Figure 3: Genetic Analysis of the *ATP4A* and *ATP4B* Genes in 50 Gastric Acid Hypersecretors.



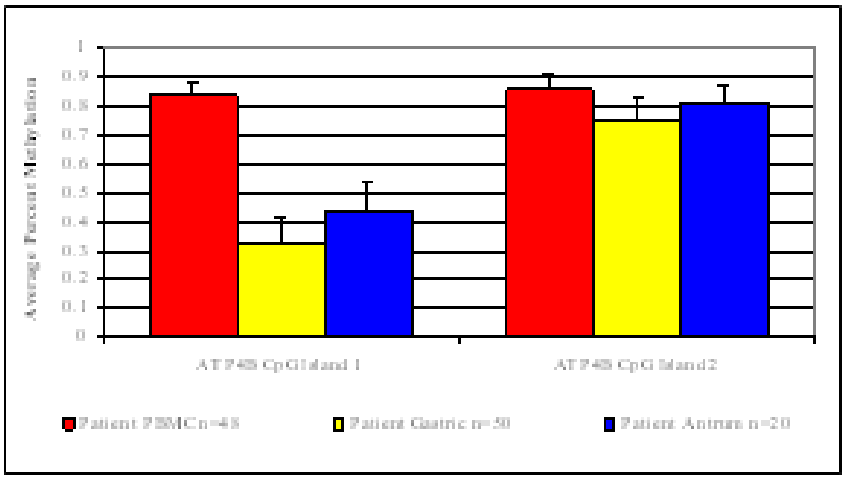
Sequence variants of the *ATP4A* (3A) and *ATP4B* (3B) genes in studied patients were detected by DHPLC using two methods: temperature-modulated heteroduplex analysis (column A) and Surveyor mutation detection fluorescence analysis (column B) on the Transgenomic WAVE system. PCR products of samples showing chromatogram differences from that of the WT pattern were then sequenced to determine the identity of the sequence variant (column C).

Supplementary Figure 4: *ATP4A* and *ATP4B* Gene Expression in Gastric Tissue of Gastric Acid Hypersecretors.



RNA from gastric biopsies obtained from 50 patients displayed a wide range of *ATP4A* and *ATP4B* relative gene expression (0.1 – 5.5; mean = 1.9) while PBMCs (n=48) and antrum biopsies (n=20) showed no expression. Statistical analysis also indicated a direct relationship between changes in gene expression of the α and β subunits (p value <0.0001).

Supplementary Figure 5: Differential Methylation Patterns of CpG Islands 1 and 2 of the *ATP4B* Gene.



CpG sites of *ATP4B* CpG island 1 showed significantly different levels of methylation between patients' gastric (average percent methylation = 33% $\pm$ 9) and antrum biopsies (average percent methylation = 44% $\pm$ 9) when compared to patients' PBMCs (average percent methylation = 89% + 4). Though antrum biopsies showed a relatively increased methylation percent in comparison to gastric biopsies, the difference was not significant. A substantial increase in methylation was observed in the CpG sites of *ATP4B* CpG island 2 for patients' gastric (average percent methylation = 75% $\pm$ 8) and antrum (average percent methylation = 81% $\pm$ 6) biopsies when compared to *ATP4B* CpG island 1 indicating that differential methylation was isolated to the first island of the *ATP4B* gene.

Supplementary Table 1: Oligonucleotide primers for the amplification of the *ATP4A*, *ATP4B* and *CYP2C19* genes

Gene	Primer Name	5' - SEQUENCE - 3'	Size (bp)	Tm (^{o}C)	Product Size (bp)
ATP4B	ATP4B 5'UTR/E1 Fwd	CCCTGGTGTTTGGTGAGG	18	60	373
	ATP4B 5'UTR/E1 Rev	CTCCTGAGCTCACCTGGAG	19	59	
	ATP4B E2 Fwd	AGGTAGGGAAGATGGTCACG	20	59	282
	ATP4B E2 Rev	CAGCCTGGCCTGTCACAC	18	62	
	ATP4B E3 Fwd	GCATTCTGACTGTGCTGTCC	20	59	398
	ATP4B E3 Rev	CACCTGCTGCTTCACACCT	19	60	
	ATP4B E4 Fwd	CACAGCTTCGGCCTTTTCTA	20	61	373
	ATP4B E4 Rev	GCCCTGACTGCACTGTTGTA	20	60	
	ATP4B E5 Fwd	GCTGTGGCTCTGAATGATGA	20	60	367
	ATP4B E5 Rev	GGCTGACATTCCTGGAGAGA	20	60	
	ATP4B E6 Fwd	TTATGCAAGCACCTACACAGC	21	59	297
	ATP4B E6 Rev	GAAGGGGCTTATTGCTTTCC	20	60	
	ATP4B E7 Fwd	GGTCAGGGCCAGTCTTACAC	20	60	479
	ATP4B E7 Rev	ACTGATACTCGCGAGCAGGT	20	60	
	ATP4B 3'UTR Fwd	CTTACCTTGCCCGTCAGTTC	20	60	594
	ATP4B 3'UTR Rev	GGAGCTGTGAGCGTCCTTAG	20	60	
ATP4A	ATP4A 5'UTR/E1 Fwd	AGGAGGGAATGCCTGAGGT	19	61	226
	ATP4A 5'UTR/E1 Rev	ATTCCACTGCAACCCCTGT	19	60	
	ATP4A 5'UTR/E1+2 Fwd	CACCCCAAGGAGCCTGTAAT	20	61	460
	ATP4A 5'UTR/E1+2 Rev	TTGCTCTGGGCTCTCCTG	18	60	
	ATP4A E3 Fwd	AGGGTAGGCTGGGAACCA	18	61	286
	ATP4A E3 Rev	GCCTCCCTCCCTCTCTCTAT	20	59	
	ATP4A E4 Fwd	GCTGGAGAAGGGAAACTGAA	20	59	392
	ATP4A E4 Rev	ATCCTTCTCTGCCCCTCTTG	20	61	
	ATP4A E5 Fwd	GAGGGGGCTAAAGGCTACAG	20	60	378
	ATP4A E5 Rev	AGATGGAGGCCACAGATGAG	20	60	
	ATP4A E6 Fwd	TGTAGCCTCCTTCCATCAGG	20	60	486
	ATP4A E6 Rev	GAAGCCCCCTGTCCTAGAAG	20	60	

	ATP4A E7 Fwd	CAGTGACTGGCTGTCCTTCA	20	60	475
	ATP4A E7 Rev	TGGATGGGAGCTAAGTGGAC	20	60	
	ATP4A E8 Fwd	TGTTTGCAGAAGCACGTTG	19	60	400
	ATP4A E8 Rev	CCTCCCTATCCCTCTTCAGG	20	60	
	ATP4A E9 Fwd	GCGATATGAGCCTAGGAGGA	20	59	248
	ATP4A E9 Rev	CGAAGCCGGCTACACCAG	18	63	
	ATP4A E10 Fwd	CAGGATGCAGTGCCTGTG	18	60	359
	ATP4A E10 Rev	GTTTGGCTGCGGAGAGAAG	19	61	
	ATP4A E11 Fwd	CTCCATCTTCCACCTTCACC	20	60	473
	ATP4A E11 Rev	CTGTGGACTGCGACAAATCA	20	61	
	ATP4A E12 Fwd	TCCATAGGTGTTTGGTGCAA	20	60	392
	ATP4A E12 Rev	GCATTTTGGTTCAAGCCAGT	20	60	
	ATP4A E13 Fwd	GCTCCTCAAGGACAGGGATT	20	61	364
	ATP4A E13 Rev	CTCGGTCTGTGCCAGATGT	19	60	
	ATP4A E14 Fwd	AGCGAGACAGTGGAGGACAT	20	60	378
	ATP4A E14 Rev	GGTGGGTGGTCAGTGAGAG	19	59	
	ATP4A E15 Fwd	AGAAGCTGGTGATCGTGGAG	20	60	381
	ATP4A E15 Rev	CACCTCAGCCTCCTCACAG	19	60	
	ATP4A E16 Fwd	CAACTTTGCCTCCATTGTGA	20	60	382
	ATP4A E16 Rev	TCTGTGCCCAAGAGTGTCTG	20	60	
	ATP4A E17 Fwd	CTGCCTGCTAACCTCAGTCC	20	60	358
	ATP4A E17 Rev	GGACCAAATGCAGACACTGA	20	60	
	ATP4A E18 Fwd	CTGTCTCTCTTCCCGTGTCC	20	60	376
	ATP4A E18 Rev	CACACGTGGAGGAACAAGTG	20	60	
	ATP4A E19 Fwd	TCTGATGCAAGGCAGAGAGA	20	60	388
	ATP4A E19 Rev	TTTTGGCATGTCACCATCTG	20	61	
	ATP4A E20 Fwd	TAGAACACCTCCCTGCTGCT	20	60	296
	ATP4A E20 Rev	CTGAAGGCACATGGCAAG	18	59	
	ATP4A E21 Fwd	TCTTCAACTTCATGCCCATTC	21	60	290
	ATP4A E21 Rev	GGCTTAGAGGCCAAGTGTTG	20	60	
	ATP4A E22 Fwd	AAGCCCTTGACCCTCCATAG	20	61	343
	ATP4A E22 Rev	GCTGCTGCTCCAGGAGGT	18	62	

	ATP4A 3'UTR Fwd	CTCTGGACAGCCACCAAGAT	20	60	438
	ATP4A 3'UTR Rev	GGATAAACAGCCCCCATGTA	20	60	
CYP2C19	CYP2C19 5'UTR I Fwd	CTTCAGTGCTCTCAATTATGACG	23	59	386
	CYP2C19 5'UTR I Rev	TCCAGATTGAAAGGAGAAGCA	21	60	
	CYP2C19 5'UTR II Fwd	GGTCTCCCTTTTCCCATTTG	20	60	492
	CYP2C19 5'UTR II Rev	CCAGTGCCCACTCTGTACATT	21	60	
	CYP2C19 5'UTR/E1 Fwd	TCCATCAAAGAGGCACACAC	20	60	399
	CYP2C19 5'UTR/E1 Rev	AGGCAACAAAAGCCTTTCAA	20	56	
	CYP2C19 E2 Fwd	TTTGAGCCTGTGTGACTGAA	20	58	365
	CYP2C19 E2 Rev	TCTGCCCGAGAAGCTCTG	18	58	
	CYP2C19 E3 Fwd	TGCCTGTTTCAGCATCTGTC	20	60	387
	CYP2C19 E3 Rev	CACCCCTGAAATGTTTCCAA	20	58	
	CYP2C19 E4 Fwd	ATGCATGCCAAACTCTTTTT	20	54	400
	CYP2C19 E4 Rev	TGTACTTCAGGGCTTGGTCA	20	60	
	CYP2C19 E5 Fwd	CAATAAAAATTTCCCCATCAAGAT	24	62	400
	CYP2C19 E5 Rev	TTAAACATCCGTAGTAAACACAAAA	25	64	
	CYP2C19 E6 Fwd	CCCTCTCTCACCGCTCCTAT	20	64	381
	CYP2C19 E6 Rev	TCTAAGAGAAACAGAAAGGAGAACA	25	68	
	CYP2C19 E7 Fwd	TGATGTTTGGATACCTTCATCAT	23	62	389
	CYP2C19 E7 Rev	TGCACTTCTCTCACCCAGTG	20	62	
	CYP2C19 E8 Fwd	CTGCTCTTCTTTGGAATGGTG	21	62	351
	CYP2C19 E8 Rev	TGCAGAGAAGGCACATGTAAG	21	62	
	CYP2C19 E9 Fwd	TCACCGAACAGTTCTTGCAT	20	58	390
	CYP2C19 E9 Rev	GATGACGGGTCAGAAGAAGC	20	62	
	CYP2C19 3'UTR Fwd	TGAAATCTCTGATTGACCCAAA	22	60	485
	CYP2C19 3'UTR Rev	GCATTATGCACAAAGGAAGGA	21	60	

Supplementary Table 2: Oligonucleotide Primers for the PCR Amplification of the Two CpG Islands of the *ATP4B* Gene

Primer Name	**5' - SEQUENCE - 3'**	**Size (bp)**	**Tm (oC)**	**Product Size (bp)**	**CpG Sites**
ATP4B meth1 Fwd	aggaagagagGGGTTGTATTTAGTTTTTGGTGTTTGG	37	62	396	22
ATP4B meth1 Rev	cagtaatacgactcactatagggagaaggctACTCCCCTCCTCCTAAACTCACCTA	56	68		
ATP4B meth2 Fwd	aggaagagagGGGTTTTATTTGTTTTTTAGGGA	33	60	449	20
ATP4B meth2 Rev	cagtaatacgactcactatagggagaaggctTAAAAACCCAAACATCTACTTTCCT	56	66		

Lower case bases indicate the 5’-tags necessary for the MALDITOF MassArray technique.

Supplementary Table 3: CpG Sites of the *ATP4B* CpG Island 2 Show No Correlation with Changes in *ATP4B* or *ATP4A* Gene Expression.

CpG Site	ATP4A Gene Expression			ATP4B Gene Expression		
	Effect of additional unit of CpG (95% CI)	**P-value**	**Bonferroni adjusted p-value**	**Effect of additional unit of CpG (95% CI)**	**P-value**	**Bonferroni adjusted p-value**
2,3	1.65(-0.58, 3.88)	0.156	1	1.97(-0.49, 4.44)	0.125	1
4	0.19(-1.09, 1.47)	0.774	1	-0.19(-1.52, 1.14)	0.781	1
5	-2.19(-5.00, 0.62)	0.134	1	-2.38(-5.34, 0.57)	0.121	1
6	-0.94(-2.47, 0.59)	0.244	1	-0.42(-1.99, 1.14)	0.602	1
7	0.35(-2.75, 3.44)	0.828	1	1.78(-1.63, 5.19)	0.314	1
9	0.84(-4.12, 5.79)	0.743	1	-0.19(-5.27, 4.89)	0.942	1
10,11	-0.38(-1.40, 0.64)	0.471	1	-0.61(-1.66, 0.44)	0.261	1
12	-0.07(-1.10, 0.97)	0.901	1	-0.39(-1.46, 0.68)	0.483	1
13,14	-0.07(-1.10, 0.97)	0.901	1	-0.39(-1.46, 0.68)	0.483	1
15	0.35(-2.75, 3.44)	0.828	1	1.78(-1.63, 5.19)	0.314	1
16,17	3.51(-5.21,12.23)	0.434	1	-1.33(-10.3, 7.64)	0.773	1
18	-0.03(-2.26, 2.21)	0.982	1	-1.53(-3.86, 0.80)	0.205	1
19,20	1.59(-0.49, 3.66)	0.142	1	0.97(-1.36, 3.31)	0.42	1
AVG	-0.48(-1.88,0.91)	0.503		-0.91(-2.31,0.49)	0.22	

Supplementary Table 4: Site Specific Methylation of the *ATP4B* CpG Island 2 Shows No Statistical Correlation with Changes in BAO

CpG Site	Basal Acid Output		
	Effect of additional unit of CpG (95% CI)	**P-value**	**Bonferroni adjusted p-value**
2,3	-0.84(-2.88, 1.21)	0.4282	1
4	0.18(-0.79, 1.16)	0.7116	1
5	0.30(-1.95, 2.54)	0.7972	1
6	-0.84(-1.89, 0.21)	0.1348	1
7	0.64(-2.21, 3.48)	0.6639	1
9	-2.00(-5.85, 1.85)	0.3149	1
10,11	0.07(-0.73, 0.87)	0.863	1
12	0.12(-0.67, 0.92)	0.7599	1
13,14	0.12(-0.67, 0.92)	0.7599	1
15	0.64(-2.21, 3.48)	0.6639	1
16,17	-0.98(-7.84, 5.88)	0.7813	1
18	0.89(-1.12, 2.89)	0.3909	1
19,20	-0.22(-3.26, 2.81)	0.8871	1
AVG	0.21(-0.88,1.30)	0.707	

Supplementary Table 5: Site Specific Methylation of the *ATP4B* CpG Islands 1 and 2 Shows No Statistical Correlation with Total Daily Lansoprazole Dose

Gene Fragment	CpG Site	Total Daily Dose		
		Effect of additional unit of CpG (95% CI)	P-value	adjusted p-value
ATP4B CpG island 1	1 / 8	-1.12(-3.19, 0.94)	0.2932	1
	2,3	-0.43(-2.65, 1.79)	0.7078	1
	4	-0.25(-2.84, 2.33)	0.8497	1
	5,6	-1.55(-3.40, 0.30)	0.1083	1
	7	-0.64(-2.93, 1.64)	0.5843	1
	10	-0.58(-2.54, 1.38)	0.5657	1
	11	-1.20(-3.36, 0.96)	0.2831	1
	12	-0.13(-1.43, 1.18)	0.8514	1
	13 / 14	-1.24(-3.39, 0.90)	0.2625	1
	15	-1.32(-3.32, 0.68)	0.2031	1
	16	-1.56(-3.70, 0.59)	0.1622	1
	21	0.34(-1.15, 1.83)	0.6544	1
	AVG	-1.67(-4.16,0.81)	0.1941	
ATP4B CpG island 2	2,3	-0.21(-2.06, 1.65)	0.8279	1
	4	0.20(-0.70, 1.10)	0.6688	1
	5	-0.02(-2.06, 2.03)	0.9885	1
	6	-0.72(-1.89, 0.45)	0.2452	1
	7	2.09(-0.41, 4.60)	0.1107	1
	9	0.65(-2.79, 4.08)	0.7147	1
	10,11	-0.01(-0.74, 0.72)	0.9703	1
	12	0.11(-0.64, 0.86)	0.7802	1
	13,14	0.11(-0.64, 0.86)	0.7802	1
	15	2.09(-0.41, 4.60)	0.1107	1
	16,17	4.69(-1.47,10.85)	0.1432	1
	18	0.82(-0.90, 2.55)	0.3558	1
	19,20	0.29(-1.44, 2.02)	0.743	1
	AVG	-0.21(-1.20,0.77)	0.6728	

GENERAL DISCUSSION

Interindividual variability in drug efficacy and toxicity is a consistently and commonly observed occurrence where administration of a standard drug dose to a population of patients produces a range of responses, from no effect to lethal events. These adverse drug reactions, particularly in the heterogeneity of patient response to chemotherapeutic agents, drastically interfere with health care and are estimated to increase significantly the overall hospital and drug costs [143]. Though numerous clinical variables have been associated with drug response, evidence increasingly shows that genetics is a major determinant of the observed variability in response to therapy. Due to advances in whole genome sequencing technology and high-throughput techniques, pharmacogenetics and pharmacogenomics are becoming an integral translational component in almost all clinical trials investigating new therapeutic agents and have the potential to revolutionize the way clinicians determine a patient's optimal treatment regimen through the prediction of an individual's responsiveness to therapy.

Pharmacogenetic and pharmacogenomic approaches may significantly enhance drug efficacy and limit toxicity by leading to an individualized approach to drug therapy that theoretically has the potential to allow for the selection of the optimum drug or drug combination and the optimum dosage to maximally benefit a specific population or a specific patient. However, clinical use of pharmacogenetics knowledge is restricted by several limitations. A vast majority of pharmacogenetic studies have mainly involved hypothesis-driven, focused investigations aimed at understanding the genetic basis of this variability through determining the effects of specific polymorphic genes on drug pharmacodynamics and pharmacokinetics and disease outcome. The influence of specific

polymorphic genes on drug response is variable, but no single polymorphic change can adequately explain all variations in therapeutic efficacy and safety. Other gene regulatory mechanisms, such as the epigenetic mechanism of DNA methylation, may offer an alternate explanation. To determine the impact genetic and epigenetic mechanisms, either alone or in concert, two test drugs, 5-FU and Lansoprazole, were utilized in this thesis.

In the case of 5-FU, variability in therapeutic response has often been attributed to the rate-limiting catabolic enzyme DPD. Previous studies in the Diasio lab involving the phenotypic characterization of DPD enzyme activity within a population of healthy volunteers and patients identified a number of cases where *DPYD* genotype provided no explanation for the measured DPD deficiency. The lack of genetic explanation for the phenotypically characterized DPD deficiency, coupled with previous studies indicating that promoter methylation could down-regulate DPD expression in cell lines, led to investigations of the *DPYD* promoter methylation in human samples.

To test for DPYD promoter methylation, a DHPLC method was developed which circumvents the cloning and sequencing steps required for methylation detection. It also correctly differentiates between methylated and unmethylated alleles with high sensitivity and rapidity (6.6 minutes) and permits a semiquantitative assessment of the methylation status, allowing approximation of the number of methylated CpG sites relative to a 100% methylated control reference sample. Additionally, this method permits the detection of single nucleotide polymorphisms within fragments screened for methylation.

In a cohort of samples phenotypically characterized for DPD activity by enzyme assay and breath test, aberrant methylation of the *DPYD* promoter was detected in all six DPD-deficient individuals without inactivating mutations in their DPYD gene. Out of

four DPD-deficient individuals genetically characterized as heterozygous for either *DPYD**2A or *DPYD**13, two samples showed varying *DPYD* promoter methylation. Taken collectively, our results indicate that methylation of the *DPYD* promoter is associated with decreased DPD activity in clinical samples and may act separately or in concert with genetic variants to down-regulate DPD activity.

In the case of the second test drug, Lansoprazole, variability in therapeutic response has often been attributed to the CYP2C19 enzyme activity which has been well correlated in previous studies with specific CYP2C19 genotypes. Utilizing a specific cohort of patients requiring a wide Lansoprazole dose range for therapeutic acid suppression, including a subset of patients who were unable to obtain successful acid inhibition, genetic analysis was performed not only on *CYP2C19* genetics, but also on other candidate genes such as the *ATP4A* and *ATP4B* genes encoding the gastric H^+/K^+ ATPase proton pump.

Though genetic analysis of the entire *ATP4A* and *ATP4B* genes failed to identify any significant associations with patients' BAO or total daily Lansoprazole, our comprehensive analysis of the *CYP2C19* gene indicated that patients homozygous for the *CYP2C19*17* allele were unable to achieve acid suppression at high Lansoprazole doses (n=2, BAO >5 mmol/h, lansoprazole doses 300-450 mg per day), while heterozygous patients achieved moderate to complete acid suppression at elevated lansoprazole doses (n=10, BAO < 5 mmol/h, Lansoprazole doses >90 mg per day). Though the *CYP2C19*17* allele showed no association with altered BAO, individuals homozygous for the *CYP2C19*17* allele may not be able to be achieve therapeutic acid suppression through PPI therapy due to the CYP2C19 URM phenotype even on high Lansoprazole

doses. Patients identified as homozygous for the *CYP2C19*17* allele are prime candidates for either the addition of a selective CYP2C19 inhibitor or alternate gastric acid suppression therapies that are metabolized through different pathways.

Due to the importance of the β subunit in acid secretion and proton pump structure and the presence of two CpG islands within the *ATP4B* gene, we further investigated whether differential methylation patterns of the CpG islands of the *ATP4B* gene may alter expression of the gastric proton pump, potentially affecting gastric acid secretion and PPI efficacy. Analysis of site-specific methylation of the *ATP4B* promoter in gastric biopsies of the studied patients using MALDITOF MS technology indicated statistical associations between increased CpG site-specific methylation, decreased ATP4A and ATP4B gene expression, and decreased BAO. Associations observed between methylation, gene expression, and BAO need to be further validated in larger populations to determine whether methylation status of the *ATP4B* gene can be utilized as a potential marker for active proton pumps and variability of acid output in response to PPI therapy.

Taken collectively, these studies further expand our knowledge of genetics and epigenetics in drug response variability, and show that both genetics and epigenetics can act separately or in concert to provide a better understanding of the molecular mechanisms in drug response.

SUGGESTIONS FOR FUTURE STUDIES

High-Throughput Techniques for the Detection of DPYD Methylation

Examination of *DPYD* promoter methylation in this study was performed using the developed DHPLC technique which allows for the rapid differentiation of methylated and unmethylated alleles with high sensitivity. Additionally, this method permits the simultaneous detection of single nucleotide polymorphisms within fragments screened for methylation. Though reliable, the DHPLC methylation detection method permits only a semiquantitative assessment of the methylation status. It remains to be examined whether suppression of DPD expression by methylation is site-specific or pattern-specific. Due to the presence of two regulatory regions within the CpG island of the *DPYD* promoter region, a sensitive and reliable site-specific methylation detection method would be beneficial.

Recently, our lab has utilized a MALDI-TOF mass spectrometry based method in which analysis of methylation is based on the mass difference between methylated and unmethylated fragments generated from reverse transcribed and RNase A cleaved products. This methodology permits not only the quantitation (percentage) of 5-MeC within the analyzed fragment but also permits determination of specifically which cytosines are methylated. Though this methodology has proven useful in other genes, the RNase A cleavage of the *DPYD* promoter results in numerous fragments with masses that are too large to be accurately detected by mass spectrometry. Other high-throughput methodologies for the CpG site specific methylation analysis of the DPYD promoter, such as pyrosequencing, are currently under investigation in a larger cohort of samples.

Other Predictors of 5-FU Response

It is has been reported that partial to complete deficiency of the DPD enzyme is responsible for the side effects in approximately 43% of those patients suffering from 5-FU associated toxicity. Toxicity after the administration of 5-FU and its prodrugs (e.g., capecitabine), however, has also been observed in a subset of cancer patients with normal DPD enzyme activity, suggesting possible deficiencies in the two enzymes downstream of DPD in the uracil catabolic pathway: dihydropyrimidinase (DHP), encoded by the *DPYS* gene, or b-ureidopropionase (BUP-1), encoded by the *UPB1* gene. Studies performed in our lab by Dr. Holly Reed Thomas identified three non-conservative *DPYS* mutations in samples with deficient uracil catabolism as indicated by the UraBT and normal DPD enzyme activity, as well as two inactivating *UPB1* non-conservative mutations [144, 145]. All three enzymes of the catabolic pathway should, therefore, be considered as predictive markers for 5-FU response.

While approximately 85% of the administered 5-FU dose is catabolized, 15% of the administered dose is anabolized by a number of enzymes including orotate phosphoribosyl transferase (*OPRT*), uridine phosphorylase (*UPP1*), thymidine phosphorylase (*TP*), and thymidine kinase (*TK*). A deficiency in the enzymes of the catabolic pathway may explain the observed 5-FU toxicity, but the enzymes of the anabolic pathway should be considered for future studies. Examining both the genetic and epigenetic regulation of each of these enzymes could provide a more complete picture of the entire 5-FU metabolic pathway.

CYP2C19 Genetics and PPI Efficacy

The influence of *CYP2C19* genetic variants on the clinical outcome of PPIs has been extensively investigated. Though over 20 alleles have been identified to affect CYP2C19 activity, one of the two most common alleles associated with a poor metabolizer phenotype, *CYP2C19*2A*, showed no statistical association with decreased total daily Lansoprazole dose in the 50 studied samples. This lack of statistical association with decreased total daily Lansoprazole dose may in part be due to the lack of homozygous variant alleles linked to the CYP2C19 PM phenotype.

Genetic analysis also identified four novel variants of the *CYP2C19* gene's 5' upstream region (-3484 T>C, -3417 G>A, -3315 C>T, and -3253 A>G), found within one single patient taking 240 mg per day and achieving acid suppression of 0.7 mmol/h BAO. Three other novel variants within the same region (-3331 C>T, -3329 G>T, and -3219 T>G) were also found together within the same 3 patients. The identified novel variants within the 5' upstream region if the *CYP2C19* gene should be further analyzed to determine whether these variants affect potential transcription factor binding sites that may alter *CPY2C19* expression.

Most significantly, genetic analysis identified the *CYP2C19*17* allele, known to be associated with an URM phenotype. In the homozygous state, the *CYP2C19*17* allele has been shown to significantly reduce systemic exposure to PPIs, thus leading to decreased efficacy. As indicated in the current study, individuals homozygous for the *CYP2C19*17* allele may not be able to be achieve therapeutic acid suppression through PPI therapy due to the CYP2C19 URM phenotype even on high Lansoprazole doses. Patients identified as homozygous for the *CYP2C19*17* allele are prime candidates for

either the addition of a selective CYP2C19 inhibitor or alternate gastric acid suppression therapies that are metabolized through different pathways.

Though the *CYP2C19*17* allele showed significant correlation with increased total daily Lansoprazole dose required for therapeutic acid suppression, further studies are needed to determine the significance of the two mutations (-3402 C>T and-806 C>T) which comprise the variant allele. Contrary to previous reports, genetic analysis of the studied samples indicated that the two mutations did not always appear together and, in a small number of cases, were identified in patients heterozygous for *CYP2C19*2A* or **2B*. Further analysis is needed to determine the impact of two variants associated with both poor and rapid metabolism on PPI pharmacokinetics.

Methylation of the *ATP4B* Gene

Expression of the gastric H^+/K^+ ATPase is a characteristic feature of parietal cells and is regulated at the transcriptional level. Previous reports have related the well-established tissue specific expression of the α and β subunits to the presence of gastric-specific GATA DNA-binding nuclear proteins which recognize a specific sequence motif in the 5'-upstream regions of both the *ATP4A* and *ATP4B* genes. Methylation analysis of different tissues obtained from the same individual indicated the presence of differential CpG site-specific methylation of CpG island 1 of the *ATP4B* gene which may offer an alternate regulatory mechanism. Abolished *ATP4B* gene expression was detected in PBMCs which displayed an average percent methylation of 84.0% $\pm$ 5.0 for CpG island 1 of the *ATP4B* promoter. In gastric tissue, the average percent methylation was 33.0% $\pm$ 9.0 which correlated with a variable range of *ATP4B* gene expression. Abolished *ATP4B*

gene expression was detected in antrum tissues displaying an average percent methylation of 44.0% $\pm$ 9.0.

While the average total methylation between the gastric and antrum biopsies show no significant differences, expression of the gastric H^+/K^+ ATPase α and β subunits were undetected in antrum biopsies. This suggests the presence of alternative molecular mechanisms that could down regulate gene expression, e.g., histone deacetylation (HDAC); the absence of the previously identified gastric-specific GATA DNA-binding nuclear proteins; and/or the absence of unidentified antrum-specific transcription factors required to activate the *ATP4A* and *ATP4B* expression. Further molecular studies are required in order to determine how these different regulatory mechanisms work in order to silence *ATP4B* expression in non-parietal cells.

Site-specific methylation analysis of the *ATP4B* gene promoter also identified specific CpG sites with differential methylation levels which seem to correlate with variations in gene expression of both the *ATP4B* and *ATP4A* genes, and in BAO of the studied patients. Further studies are necessary in order to understand the mechanisms underlying the association between the methylation status of the *ATP4B* promoter and *ATP4A* gene expression; however, their association may contribute to the importance of the β subunit in acid secretion. Previous reports have indicated that in the absence of the β-subunit, the α-subunit is retained within the rough endoplasmic reticulum and is relatively unstable without the β-subunit. Associations observed between methylation, gene expression, and BAO need to be further validated in larger populations to determine whether methylation status of the *ATP4B* gene can be utilized as a potential marker for active proton pumps and variability of acid output in response to PPI therapy.

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APPENDIX

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE APPROVAL FORMS

OMB No. 0925-0418
Approved for use through 01/31/2001

Protection of Human Subjects
Assurance Identification/Certification/Declaration
(Common Federal Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the common rule. See section 101(b) the common rule for exemptions. Institutions submitting applications or proposals for support must submit certification or appropriate institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the common rule. Institutions with an assurance of compliance that covers the research to be conducted on file with the Department, Agency, or the Department of Health and Human Services (HHS) should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency. Institutions which do not have such an assurance must submit an assurance and certification of IRB review and approval within 30 days of a written request from the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
☐ ORIGINAL ☐ FOLLOWUP ☐ EXEMPTION	☐ GRANT ☐ CONTRACT ☐ FELLOWSHIP ☐ COOPERATIVE AGREEMENT. ☐ OTHER:	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
Genetic Polymorphism of Dihydropyrimidine Dehydrogenase (DPD): Identification of DPD Deficiency	DIASIO, ROBERT B

6. Assurance Status of this Project *(Respond to one of the following)*

☐ This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification no. M- 1149 IRB identification no. 02

☐ This Assurance, on file with *(agency/dept)*_____, covers this activity.
Assurance Identification no._____ IRB identification no._____ *(if applicable)*

☐ No assurance has been filed for this project. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

☐ Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph _____.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

☒ This activity has been reviewed and approved by the IRB in accordance with the common rule and any other governing regulations or subparts on *(date)* 06-19-02 by: ☒ Full IRB Review or ☐ Expedited Review

☐ This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the common rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments	Title
Please note: UAB IRB Protocol Number is F020610007 Protocol subject to Annual continuing review.	Genetic Polymorphism of Dihydropyrimidine Dehydrogenase (DPD): Identification of DPD Deficiency

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed and certification will be provided.	10. Name and Address of Institution
	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
11. Phone No. *(with area code)* (205) 934-3789	12. Fax No. *(with area code)* (205) 934-1301
13. Name of Official Ferdinand Urthaler, M.D.	14. Title Chairman, IRB
15. Signature Ferdinand Urthaler M.D.	16. Date 06-26-02

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Public reporting burden for this collection of information is estimated to average less than an hour per response. An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: NIH, Project Clearance Office, 6701 Rockledge Drive, MSC 7730, Bethesda, Md. 20892-7730, ATTN: PRA 0925-0418. *Do not return the completed form to this address.*

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

July 24, 2009

MEMORANDUM

TO: UAB Graduate School

FROM: Marilyn Doss
Vice Chair, UAB IRB

RE: F020610007
Screening for Dihydropyrimidine Dehydrogenase (DPD) Deficiency Using an Oral Dose of 2-13C-Uracil, 2-13C-dihydrouracil or 2-13C-5-Fluorouracil

The Office of the IRB (OIRB) is in receipt of letter dated July 22, 2009, from Robert B. Diasio, M.D., regarding the above research. Dr. Diasio was the Principal Investigator of the above referenced protocol during the time that it was an active protocol at UAB.

In Dr. Diasio's letter, he verifies that Adam M. Lee worked on the above protocol as part of his work on his thesis entitled "Impact of Genetic and Epigenetic Variability in Response to Two Test Drugs 5-Fluorouracil and Lansoprazole"

The IRB was not notified that Mr. Lee would be working on the protocol prior to the work beginning. The UAB IRB cannot retrospectively approve research. However, had the IRB been notified, the research would have been approved as part of protocol F020610007.

cc: UAB Graduate School
file

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and expires on October 26, 2010. The Assurance number is FWA00005960.

Principal Investigator:	HIRSCHOWITZ, BASIL I
Co-Investigator(s):	
Protocol Number:	**X040830003**
Protocol Title:	*Molecular Basis for Individual Differences in Responses to Medications for Acid Suppression*

The IRB reviewed and approved the above named project on 5-2-08. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 5-2-08

Date IRB Approval Issued: 5-2-08

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

July 28, 2009

MEMORANDUM

TO: UAB Graduate School

FROM: Marilyn Doss
Vice Chair, UAB IRB

RE: X040830003
Molecular Basis for Individual Differences in Responses to Medications for Acid Suppression

The Office of the IRB (OIRB) is in receipt of letter dated July 22, 2009, from C. Mel Wilcox, M.D., regarding the above research. Dr. Wilcox was a Co-Investigator of the above referenced protocol during the time that Mr. Adam Lee worked on the protocol, and is the current UAB PI. The PI at the time Mr. Lee worked on the protocol, Dr. Basil Hirschowitz, is retired from UAB.

In Dr. Wilcox's letter, he verifies that Adam M. Lee worked on the above protocol as part of his work on his thesis entitled "Impact of Genetic and Epigenetic Variability in Response to Two Test Drugs 5-Fluorouracil and Lansoprazole"

The IRB was not notified that Mr. Lee would be working on the protocol prior to the work beginning. The UAB IRB cannot retrospectively approve research. However, had the IRB been notified, the research would have been approved as part of protocol X040830003.

cc: UAB Graduate School
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