Population Analysis and Protein Stability Assays Illustrate Xenobiotic Metabolizing Enzymes Have No Detectable Effect on Breast Cancer Development and Progression

Thomas Daniel Crowder
University of South Carolina - Columbia
POPULATION ANALYSIS AND PROTEIN STABILITY ASSAYS ILLUSTRATE XENOBIOTIC METABOLIZING ENZYMES HAVE NO DETECTABLE EFFECT ON BREAST CANCER DEVELOPMENT AND PROGRESSION

By
T. Daniel Crowder

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University of South Carolina
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Accepted By:
Bert Ely, Major Professor
John Grego, Committee Member
Hexin Chen, Committee Member
David Reisman, Committee Member
Johannes Stratmann, Committee Member
Lacey Ford, Vice Provost and Dean of Graduate Studies
Abstract

Xenobiotic Metabolizing Pathway works to detoxify the cell from numerous carcinogenic, mutagenic, and toxic hydrophobic compounds. As a member of the phase I enzymes (the first phase in the Xenobiotic Metabolizing Pathway) the Cytochrome P-450 Family 1 Sub-family B Protein 1 (Cyp1B1) works to attach an oxygen molecule to its hydrophobic substrate. In performing this reaction, Cyp1B1 often increases the reactivity of the xenobiotic compound. If these reactive Cyp1B1 products migrate into the nucleus and they can cause damage by reacting with DNA. However, the Glutathione S-Transferase Theta 1 (GSTT1) and Glutathione S-Transferase Mu 1 (GSTM1), members of the phase II xenobiotic metabolizing pathway, are able to inactive these reactive Cyp1B1 products through the addition of a glutathione molecule. Previous studies have shown that four single nucleotide polymorphisms, which lead to amino acid substitutions, in the cyp1B1 gene and gene deletions in gstt1 and gstm1 genes lead to differences in cancer susceptibility. However, our analysis genotyped 473 European Americans and 177 African Americans at each locus and found no correlation between genotype and any of 13 tumor characteristics and breast cancer risk factors that impact breast cancer progression or development. Due to high substrate overlap between xenobiotic metabolizing enzymes, we hypothesize the cell can compensate for differences in protein levels and enzymatic rates can by increased expression of highly related enzymes.
We have also investigated the role of four *cyp1B1* polymorphisms on protein stability using endogenous Cyp1B1 variant proteins in human cell lines. We determined that an Asparagine to Serine amino acid substitution at amino acid position 453 decreases stability by 20% compared to our ancestral control. This result was modest when compared to previously published data, which used African Green Monkey cells with overexpressed Cyp1B1 proteins. Furthermore, we showed that Arginine to Glycine substitutions at amino acid position 48 in conjunction with Alanine to Serine substitution at amino acid position 119 increased stability by 50%. We hypothesize that these differences in protein stability have little effect on the production of carcinogenic compounds and thus cancer development and progression.
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1.1 Breast Cancer and the Knudson hypothesis:

Breast cancer is the most common form of cancer in women, and while less common, it does occur in men. The National Cancer Institute estimates that almost a quarter of a million people were diagnosed with breast cancer in 2011 and more than 40,000 of those cases were fatal. The estimated lifetime risk of a woman living in the United States being diagnosed with breast cancer is 1 in 8 (1).

While we are gaining new insight into breast cancer treatments every day, the basic origins of cancer have been understood for sixty years. In 1953, a statistician by the name of Carl Nordling published a paper in the British Journal of Cancer describing the multi-mutational theory of cancer (50). His hypothesis stated that an accumulation of mutations leading to an increase in the ratio between cell division and cell loss could cause cancer. Nearly twenty years later, Dr. Alfred Knudson demonstrated statistically that a specific set of mutations leads to the development of retinoblastoma (51). Over the years, this theory of an accumulation of mutations has become known as the Knudson Hypothesis. During the 60 years since it was first conceived, this hypothesis has been well established. In fact, research into the development of colorectal cancer has yielded a model demonstrating a series of mutations that lead to the formation of malignant tumors (Figure 1.1) (61). In early stages, loss of function mutations in the adenomatous polyposis coli (APC) gene leads to an increase in β-catenin, a protein normally inhibited by the APC gene (62, 63). Deregulation of β-catenin leads to an increase in cell division. This allows for the formation of a small benign tumor known as
Figure 1.1. Molecular Progression of Colorectal Cancer. Cells leave the crypt of the intestines and differentiate to become normal epithelium. Through contact with mutagenic compounds the normal epithelial cells undergo a series of mutations that transform it to malignant metastatic tumor.

an adenoma. To grow past this stage, a number of other mutations must occur in the precancerous cells (61). Ras GTPase gain of function mutations, which are present in 50% of larger adenomas, allow for increased proliferation, increased invasion, and eventually metastasis (64). As the adenomas grow and develop towards cancer, parts of the long arm of chromosome 18 are lost (63, 64). These deletion events occur in approximately 50% of large adenomas and 75% of carcinomas (63). Loss of function in the cell adhesion gene (Deleted in Colorectal Cancer; DCC) and cell cycle suppression genes (SMAD2, SMAD4, and Transforming Growth Factor-β) also allow for increased growth of the adenoma (63, 64). In the last step of colorectal cancer development, the benign tumor must transform to a malignant tumor. The mutations needed to make this switch usually involve rendering the tumor suppressor p53 non-functional. Either through missense mutations, chromosomal breaks or gene deletions, loss of function mutations in p53 must occur in both copies to create a cancerous cell (64). Once p53 is inactivated, regulation of DNA repair, cell cycle, and apoptosis is lost, allowing the cells to divide unconditionally (64). This in turn leads to decreased chromosomal stability and the eventual formation of metastatic cancer (63, 64, 65).

Unlike colorectal cancer, mutations that lead to the development of breast cancer are less understood, and it is clear that there are multiple pathways that lead to the development of breast tumors. In 2010, Schnitt describes the molecular classification of breast cancer as four distinct subgroups based on hormone receptor expression, histological grade, molecular markers and invasiveness (71). However, in a more recent publishing by Curtis et al., who analyzed breast cancer based on somatic
mutation, germ line mutations, hormone receptor status, and other molecular markers, was able to delineate ten breast cancer subgroups (72). Based on the high heterogeneity of breast cancer, it is possible that as research increases, a more informed study may find an even greater diversity of breast cancer subtypes.

Cancer causing mutations are created through multiple pathways. Some of these mutations are caused by errors that occur during cellular activities (e.g. DNA synthesis error during replication), while others may be due to external factors (e.g. Benzopyrene entering the cell and intercalating into DNA, forming DNA adducts, and causing mutations or alternatively an exposure to ultraviolet light that causes Thymine dimers). Benzopyrene and many other environmental mutagenic compounds are hydrophobic, which allows them to easily cross the hydrophobic cellular and nuclear membranes. In order to reduce the effect of hydrophobic chemical mutagens and other potential carcinogens, organisms use the xenobiotic metabolic pathway to modify and eliminate these compounds.

1.2 The Xenobiotic Metabolic Pathway

As one of the main pathways of cellular detoxification, the xenobiotic metabolic pathway consists of hundreds of different enzymes. These enzymes alter the chemical structure and charge of many toxic hydrophobic compounds to facilitate their excretion from the body. This pathway is also very active in the metabolism of many pharmaceutical drugs and hormones. The xenobiotic metabolic pathway consists of three phases designated phase 1, 2, and 3 (Figure 1.2). Once a hydrophobic substrate has crossed the lipid bilayer and moved into the cytoplasm of the cell, it is met by a
Figure 1.2. Xenobiotic Metabolic Pathway: 1) After a xenobiotic compound (A) diffuses through the cell membrane, it is met by a phase I metabolic pathway enzyme (B). 2) In the modification step, a reactive group is added by the phase I metabolic pathway enzyme to the xenobiotic compound to produce a reactive xenobiotic compound (C). 3) The modified substrate (C) eventually is bound by a phase II xenobiotic compound (D). 4) The conjugation reaction of the phase II xenobiotic compound attaches a large polar group to the modified xenobiotic substrate to produce a hydrophilic molecule (E). 5) The newly created hydrophilic compound (E) is taken to the phase III protein known as the multidrug resistance channel, where it is eventually exported from the cell.
phase 1 enzyme that adds reactive groups (e.g. a hydroxyl group added to Estradiol) (Figure 1.3 and Figure 1.4). After phase 1 modification, the substrate is ready for a subsequent conjugation reaction in phase 2, in which charged molecules are added to the reactive phase 1 products. The addition of a charged moiety creates a polar hydrophilic molecule. In phase 3, a multidrug resistant protein channel spanning the lipid bilayer can then export this hydrophilic molecule into the extracellular space where its charged nature will allow it to enter the blood stream and be excreted from the body.

The xenobiotic metabolic enzymes have a high amount of substrate overlap. However, though the substrate may be the same, the reaction products often differ from enzyme to enzyme (Figure 1.3). This difference in product can cause an issue. As illustrated in Figure 1.3, the hydroxylation of estradiol by Cyp1A1 and Cyp1B1 can lead to 2-OHE$_2$ and 4-OHE$_2$, respectively. While 2-OHE$_2$ can eventually undergo methylation to become 2-MeOE$_2$ (a tumor inhibitor), 4-OHE$_2$ undergoes a conversion into a known carcinogen (10, 11). This phenomenon of mutagenic activation is not uncommon with phase 1 enzymes, and the hydroxylation of xenobiotics can often create reactive oxygen species, which lead to DNA adducts and genetic mutations (2).

1.3 Polymorphic Variations and Cancer Risk

The Knudson Hypothesis not only describes the role of new mutations on cancer development, but also those that are inherited. Throughout the genomes of the general
Figure 1.3 The modification of Estradiol by Cyp1A1 and Cyp1B1: Estradiol (E$_2$), a common estrogen derivative undergoes different modifications through different reactions with Cyp1A1 and Cyp1B1. In the Cyp1A1 reaction E$_2$ is converted to 2-OHE$_2$, which can undergo further modifications to become a tumor inhibitor (2-MeOE$_2$). In its reaction with Cyp1B1, E$_2$ is converted to 4-OHE$_2$. After additional reactions, 4-OHE$_2$ is transformed into E$_2$-3,4-Q, a known carcinogen.

Figure 1.4 Phase I and Phase II Xenobiotic Metabolic Pathway Enzymes: Moving from left to right, the molecule (X) is passing through the xenobiotic metabolic pathway. First, it is modified by different phase I enzymes, and then it is passed on to phase II xenobiotic metabolic enzymes to undergo conjugation reactions. Through this process, the hydrophobic toxin is converted into a hydrophilic compound.
population there are a number of inherited mutations and polymorphisms that have been shown to increase or decrease the risk of cancer development (4). For example, inherited mutations in the DNA repair protein Breast Cancer 1 (BRCA1) and Breast Cancer 2 (BRCA 2) genes can lead to a significant increase in breast cancer risk (up to 80%)(5). Women who inherit these BRCA mutations also have a higher chance of developing ovarian, colon, pancreatic, and thyroid cancers (5). In similar fashion, single nucleotide polymorphisms and acquired missense mutations in the p53 tumor suppressor gene are found in 20% to 40% of breast cancers, and women who inherit these defective p53 genes more commonly develop breast cancer than any other cancer (68). Other genes that play a fundamental role in genome integrity have also been shown to have increased risk in breast cancer development. Chek2, a protein kinase responsible for cell cycle arrest due to DNA damage, has been shown to contain deletion mutations in some breast cancer patients. Studies have demonstrated that this deletion is associated with a greater risk of breast cancer development, reoccurrence, and mortality (73). Similarly, mutations in the PI3K-related protein kinase ATM that decrease its ability to repair DNA double strand breaks have been shown to increase breast cancer risk (74). A further example includes mutations and polymorphisms in the tumor suppressor Her2 tyrosine kinase, which promotes cell proliferation. These genetic variations have been shown to only moderately increase the risk of breast cancer development. However, these resulting changes in the Her2 protein levels and function are known to yield a poorer prognosis and higher risk of cancer recurrence (68). Like Her2, most polymorphisms do not yield such major changes in susceptibility, however it
is reasonable that multiple mutations that individually yield small increases in the risk of cancer development can have significant effects in combination. Previous studies have examined the effects of polymorphisms in the xenobiotic metabolism pathway on cancer development in different populations throughout the world, yielding variable results regarding the effects of each polymorphism (18-20, 23, 27-31, 36-43). By measuring differences in catalytic activity and protein stability of the polymorphic xenobiotic metabolizing pathway enzymes, several studies were able to provide potential mechanisms for differences in associated risk (6, 18-26, 32, 40). We hypothesized that by looking at single nucleotide polymorphisms (SNPs) and gene deletions in the xenobiotic metabolic pathway genes in breast cancer patient DNA, we would be able to determine which mutations contribute to an increased or decreased likelihood of developing certain types of breast cancer.

1.4 Polymorphisms in the Xenobiotic Metabolic Pathway and Their Enzymatic Effects

As indicated above, the xenobiotic metabolic pathway is composed of hundreds of enzymes; however based on their expression patterns in breast tissue and breast cancer cells and previously published data, 3 genes (cyp1B1, gstm1, and gstt1) with 6 mutations in total were chosen for this study (18, 19, 20, 27, 36-40). Cytochrome P450 family 1 subfamily B protein 1 (Cyp1B1) is considered a member of the phase I xenobiotic metabolic enzyme superfamily Cytochrome P-450, which includes 18 families and 43 subfamilies and totals 57 genes and 59 pseudogenes (12). Studies have shown that Cytochrome P-450 (Cyp) enzymes can play a role in both cancer treatment and
development (60). By acting as a cellular detoxifier, Cyp enzymes have been shown to deactivate and play a role in the expulsion of many cellular toxins. In addition, they also activate many chemotherapeutic drugs (11, 15-17). However, Cyp enzymes also have been shown to activate pro-carcinogens, which can lead to the formation of DNA-Adducts and the development of genomic mutations (2, 3, 7, 10, 11, 15-17).

**Cytochrome P-450 1B1 Polymorphisms**

While, less well known than the closely related Cytochrome P-450 family 1 subfamily A protein 1 (Cyp1A1), Cyp1B1 is produced at higher levels in breast tissue and can yield quantitatively more reactive products (2, 3, 13, 14) (Figure 1.5). In fact, cyp1B1 null mice have shown a significant reduction in the number of tumors caused by Benzo[a]pyrene and DB[a,l]P, two common products of Cyp1A1 and Cyp1B1 (3). Also, as described above, the conversion of Estradiol to the carcinogenic 4-OHE2 is specific to the Cyp1B1 enzyme. Steady state levels of Cyp1B1 are determined by the rate of synthesis and the rate of degradation. Previous studies have found that the rate of degradation for Cyp1B1 is dependent upon movement through the proteasome, however the exact mechanism is not fully understood (44). The cyp1B1 gene is found on chromosome 2 region 22 and contains 3 exons and 2 introns. As shown in figure 1.6, translation begins near the start of exon 2 and continues into exon 3. Thus far, 23 variants of the cyp1B1 gene have been discovered, 17 of which lead to differences in amino acid sequence. Several of these mutations have been thought to increase the risk of certain types of cancer (lung, endometrial, head and neck squamous, ovarian and breast cancer) along
Figure 1.5 Expression of Cyp1A1 and Cyp1B1 in normal and carcinogenic breast tissue:
Data was gathered using Genevestigator biomedical software. 1) Cyp1B1 expression in normal breast tissue. 2) Cyp1A1 expression in normal breast tissue. 3) Cyp1B1 expression in cancerous breast tissue. 4) Cyp1A1 expression in cancerous breast tissue.
Figure 1.6 Cyp1B1 from Gene to Protein: From top to bottom: Gene: a depiction of the exons and introns within the cyp1b1 gene. mRNA: The Cyp1B1 mRNA transcribed from cyp1b1 gene with introns removed. Protein: A depiction of the Cyp1B1 coding sequence, which begins in exon 2, from mRNA that is translated into a five hundred and forty three amino acid protein.
with congenital glaucoma (6-9). In particular, the four SNPs that have been examined in this study, which each yield an amino acid change in the protein sequence, 142 C>G, 355 G>T, 4326 C>G, and 4390 A>G (Figure 1.7) all have been shown to alter enzymatic efficiency or have been implicated in cancer development, either individually or in combination (6, 18-26, 32). For example, Shimada et al. reported an increase in the rate of metabolism of a number of benzopyrene derivatives with the Cyp1B1 355 G>T mutant enzyme (23). They also showed that the Cyp1B1 4326 C>G mutant enzyme and the Cyp1B1 355 G>T and 4326 C>G double mutant enzyme produced 4-OHE2 at an increased rate. In a different study with different Cyp1B1 haplotypes, Aklillu et al. demonstrated that the 4390 A>G mutation could lead to a decrease in the rate of production of benzopyrene derivatives, however they did not investigate the production of 4-OHE2 (22). These studies suggest that certain polymorphisms along the cyp1B1 gene could lead to a reduction in carcinogen production via the xenobiotic metabolizing pathway yielding a reduction in the overall risk of tumor formation. However, other polymorphisms may increase the rate of mutations and thus lead to an increase in risk of tumor formation (23). Despite clear data about the production of mutagenic compounds through Cyp1B1 enzymatic activity, the determination of the role each polymorphism plays in altering cancer risk has produced conflicting data. In 2010, the MARIE-GENICA consortium showed an increase in breast cancer risk with mutations at both 142 C>G and 355 G>T (20). Also, Miller et al. showed that in African American women, but not in Caucasian women, mutations at site 4326 could increase breast cancer susceptibility (19). However, Rylander-Rudqvist et al. showed that in a Swedish
**Figure 1.7 Polymorphisms in the Cyp1B1 gene:** Four polymorphisms found in the Cyp1B1 gene, which yield amino acid substitutions and their relative positions. 142 C>G causes a Arginine to Glycine change at amino acid (AA) number 48. 355 G>T causes an Alanine to Serine change at AA 119. 4326 C>G yields a Leucine to Valine change at AA 432. 4390 A>G causes an Asparagine to Serine change at AA 453.
population a 4326 C>G mutation increased the risk of breast cancer (27). Other studies have failed to show an association between cancer risk and Cyp1B1 polymorphisms (28-31). However, many of these differing results could be due to race or lifestyle factors, such as smoking, dietary habits, or local environmental conditions. Thus further investigations into the role of cyp1B1 polymorphisms in breast cancer development are warranted.

**Cytochrome P-450 1B1 Polymorphisms and Protein Stability**

Although the effects of the cyp1B1 polymorphisms described above have been characterized, the effects of these amino acid substitutions on Cyp1B1 protein stability need to be investigated. Consequently we used NetPhos 2.0 (49) to predict potential protein phosphorylation sites which might impact protein stability (Figure 1.8). When the Cyp1B1 enzyme had a serine substitution at position 119 or position 453 (the 355 G>T and 4390 A>G polymorphisms, respectively) potential new phosphorylation sites were formed. These newly created phosphorylation sites may cause the Cyp1B1 enzyme to be degraded more rapidly through the phosphodegron pathway of the proteasome. In this pathway, two or more phosphorylated amino acids in close proximity to each other on the protein of interest serve as recognition sites for the E3 ubiquitin ligase. The E3 ubiquitin ligase is then able to tag the protein for degradation by the attachment of a chain consisting of at least 4 ubiquitin molecules to a lysine on the protein of interest. This tag directs the ubiquitinated protein of interest to the proteasome. The proteasome is a large multi-subunit enzyme consisting of a barrel shaped core, a lid, and
Figure 1.8 Prediction of Potential Phosphorylation Sites on Cyp1B1: From top to bottom: Frame 1 depicts the ancestral Cyp1B1 protein displaying serine, threonine, and tyrosine phosphorylation sites. The phosphorylation threshold is depicted by a gray horizontal line. Frame 2 shows the Cyp1B1 enzyme with an alanine to serine substitution at site 119. As indicated by the black arrow, a phosphorylation site is created by this substitution. Frame 3 illustrates the potential phosphorylation sequence of Cyp1B1 with an asparagine to serine amino acid substitution at site 453. As marked by the black arrow, the presence of the 453 serine causes a neighboring serine at location 458 to be phosphorylated.

a base. Once recognized by the proteasome, the ubiquitin tags are removed and the protein of interest enters the proteasome to be degraded into amino acids. We hypothesize that the variant Cyp1B1 enzymes will be phosphorylated at the newly created phosphorylation sites in addition to the normal phosphorylation sites. The resulting increase in phosphorylation could cause the E3 ubiquitin ligase to recognize and bind Cyp1B1 more efficiently as a substrate. Once bound, E3 will transfer the ubiquitin tag to the phosphorylated Cyp1B1. After a sufficient number of ubiquitin tags have been transferred from E3 molecules to the Cyp1B1 protein, Cyp1B1 will be targeted by the proteasome for degradation (Figure 1.9). Experimental data produced by Bandiera et al. demonstrated that the asparagine to serine amino acid substitution at site 453 caused a reduction in the stability of this Cyp1B1 variant in monkey Cos-1 cells (44). Therefore, it is likely that a Cyp1B1 enzyme with an alanine to serine amino acid substitution at position 119 would have decreased stability as well. Since these serine-encoding alleles are relatively common in human populations, the decreased stability of the Cyp1B1 variant enzymes could have a direct impact in steady state levels of the protein. This reduction in Cyp1B1 enzyme stability could decrease the metabolism of hormones, pharmaceutical drugs and xenobiotic toxins and impact cancer development and treatment. Therefore, it is important to determine the half-life and steady state levels of the CYP1B1 variant enzymes in human cells.
Figure 1.9 Proteasome Degradation Pathway: Ubiquitination and degradation of a protein substrate by E1, E2 and E3 ubiquitin ligases and the 26S proteasome. Recognition of certain amino acid or phosphorylation sequences by the E3 ubiquitin ligase causes ubiquitin to be covalently attached to the protein substrate. Once the protein substrate has been tagged by a chain of 4 or more ubiquitin molecules it will be targeted by the proteasome for degradation.

Glutathion S-Transferase

Like the phase I enzymes discussed above, phase 2 enzymes also play an important role in cancer development and treatment. Composed mainly of transferases (an enzyme that transfers a functional group from one molecule to another), phase 2 enzymes detoxify many of the carcinogenic, mutagenic, and potentially toxic products of phase I reactions. Glutathione S-Transferases (GSTs), which make up approximately a sixth of the phase 2 enzymes and are divided into five classes (Alpha, Mu, Pi, Kappa, and Theta), form thioether conjugates between glutathione and the phase I xenobiotic product (Figure 1.10) (33). GSTs primarily metabolize reactive oxygen species and are often the downstream detoxifier of the phase I Cytochrome P450 reactions (34). Glutathione S-Transferase class Mu protein 1 (GSTM1) and Glutathione S-Transferase class Theta protein 1 (GSTT1) are two of the most studied GSTs in relation to cancer (35). Independent deletions that remove either the gstm1 or gstt1 gene regions (Figure 1.11) are present at frequencies of 62% (gstm1) and 15% (gstt1) in European Americans and 26% (gstm1) and 38% (gstt1) in African Americans and have been implicated in cancer development (18, 19, 36-40). For example, Miller et al. showed an increase in breast cancer risk in women who lack the gstt1 gene (19). Also, Helzlsouer et al. found an increase in breast cancer risk with both the gstt1 and the gstm1 null genotypes (40). However, other results have been contradictory with a number of reports finding no association with gstt1 or gstm1 null genotypes and breast cancer risk (41-43). As with cyp1B1, these differences could be due to racial or lifestyle disparities and further investigation is needed to clarify the role of these enzymes in cancer development.
Figure 1.10 Conjugation of Glutathione by GST:
Glutathione is conjugated to the phase 1 xenobiotic metabolic pathway product through a thioether bond. This reaction is catalyzed by members of the Glutathione S-Transferase family.

Figure 1.11 Homologous Recombination and the loss of GSTT1 and GSTM1: High homology sequences, which flank the gstt1 and gstm1 genes on chromosome 22 and chromosome 1, respectively, resulted in independent instances of homologous recombination and the loss of the gstt1 and gstm1 genes.

1.5 Research Goals

Chapter Two

Chapter two of this dissertation focuses on genotyping six SNPs in the phase 1 and phase 2 xenobiotic metabolic enzymes described above and determining their association with breast cancer risk factors and tumor characteristics. Since polymorphisms in the xenobiotic metabolic pathway have been shown to yield changes in mutagenic activity, these polymorphisms in phase 1 and phase 2 xenobiotic metabolic enzyme genes could alter the risk of cancer development or tumor progression and aggressiveness.

Cyp1b1 Population Study

Studies have shown that Cyp1B1 has an increased rate of mutagen production compared to its most closely related enzyme, Cyp1A1 (2). In addition, the amino acid substitutions in Cyp1B1 caused by the cyp1b1 genetic polymorphisms have shown an even higher rate of metabolism and mutagen production with certain substrates. Due to the fact that polymorphisms in the cyp1b1 gene yield an increased rate of mutagenic compounds, and because it is the main phase 1 xenobiotic metabolic compound in breast tissue, it is our hypothesis that the four SNPs in the cyp1b1 gene will impact the development of breast cancer.
GSTT1 and GSTM1 Population Study

Phase 2 xenobiotic metabolic enzymes detoxify the cell from the reactive and potentially mutagenic products produced from phase 1 reactions. Members of the Glutathione S-Transferase family neutralize these toxic substances by attaching a glutathione molecule to the reactive site on the mutagenic compound. As two of the most highly abundant phase 2 xenobiotic enzymes in breast tissue, GSTM1 and GSTT1 play a pivotal role in detoxification of the cell (52). The absence of the GSTM1 and GSTT1 enzymes caused by independent deletions in the gstm1 and gtt1 genes could potentially yield a buildup of phase 1 mutagenic products. As stated above, a number of these products have been shown to be carcinogenic (3, 10, 11). Because GSTM1 and GSTT1 reduce the levels of mutagenic and carcinogenic compounds in breast tissue by the addition of glutathione, we hypothesize that deletions in the gstm1 and gtt1 genes speed the development of breast cancer. However, previous studies examining the role of gtt1 and gstm1 gene deletion polymorphisms on breast cancer have found inconsistent associations (19, 40-43). To test this hypothesis, we determined the gtt1 and gstm1 genotypes in African American and European American breast cancer patients. The resulting patient genotypes were then analyzed using logistic regression to detect associations with breast cancer patient and tumor characteristics.

Chapter Three

Chapter three of this dissertation examines the effect of four amino acid substitutions on Cyp1B1 protein stability and steady state levels. As indicated above,
polymorphisms in the cyp1b1 gene (142 C>G, 355 G>T, 4326 C>G, and 4390 A>G) cause amino acid substitutions (R48G, A119S, L432V, and N453S, respectively) in the Cyp1b1 enzyme. Numerous studies have been performed analyzing the role of these substitutions on the enzymatic rate (6, 18-26, 32). However, only two studies, which assessed degradation through overexpression of Cyp1B1 variants in African Green Monkey cells, have examined how these substitutions affect protein stability and steady state levels (21, 44). Measuring the rate of endogenous Cyp1B1 protein degradation in human cells will allow us to more accurately assess the role that these amino acid substitutions play on Cyp1B1 stability.

Preliminary data gathered from NetPhos 2.0 predicted that amino acid substitutions at 119 and 453 both yield changes in the phosphorylation status (49). Therefore, we hypothesized that these substitutions at site 119 and 453 could cause a decrease in Cyp1B1 stability since the newly created phosphorylation sites could lead to the formation of a phosphodegron. This phosphodegron would increase the recognition and binding of E3 ubiquitin ligase and thus would increase the rate of Cyp1B1 degradation through the proteasome pathway. Therefore, we determined the cyp1b1 genotype of three breast cancer cell lines (BT20, MCF7, and T47D) and measured the steady state levels and stability of their Cyp1B1 proteins. To verify the role of the proteasome in Cyp1B1 degradation, we used the proteasome inhibitor MG132 with a cycloheximide chase to measure differences in Cyp1B1 degradation rates.
CHAPTER 2: POLYMORPHISMS IN THE PHASE I AND PHASE II XENOBIOTIC METABOLIC PATHWAY

AND THEIR ASSOCIATION WITH PATIENT AND TUMOR CHARACTERISTICS
Abstract

Xenobiotic metabolizing enzymes play an important role in detoxifying the cell from toxic and often carcinogenic hydrophobic molecules. While enzymes such as GSTM1 and GSTT1 work to create non-reactive polar molecules from these hydrophobic compounds, in the process of detoxification, the enzyme Cyp1B1 often produces mutagens. Four single nucleotide polymorphisms found in the cyp1b1 gene have been experimentally shown to increase this mutagen production. Furthermore, deletions of the gstm1 and gstt1 genes reduce the cells ability to safely remove these toxic compounds. As such, we hypothesized that polymorphisms in the cyp1b1, gstm1, and gstt1 genes would increase the mutagenic rate, and thus could be associated with breast cancer risk factors and tumor characteristics. To test this hypothesis, genotypes were determined at each locus for 601 European American and African American breast cancer patients. Logistic regression was used to compare patient genotypes with breast cancer tumor and patient characteristics. Our analysis found no significant association between any of the tested polymorphisms of the xenobiotic metabolic pathway and any of the breast cancer tumor and patient characteristics analyzed in this study.

2.1 Introduction

Breast cancer is known to be one of the most commonly occurring and deadliest cancers in the world. For women in the United States, breast cancer ranks as the second highest diagnosed cancer and the number two cause of cancer related death. However, disparities in breast cancer type, aggressiveness, and age at diagnosis exist between African American and European American women. In a study performed in conjunction
with our laboratory, it was shown that African American women in South Carolina have a younger age of diagnosis than that of their European American peers (AA: 58.1 ± 14.8, EA: 62.5 ± 13.9) (66). In addition, Bauer et al. showed that African American women are also at higher risk to develop triple negative (Estrogen receptor negative, Progesterone receptor negative, Her2 negative) breast cancer, a prognosis associated with increased aggressiveness and poorer survival rates (67). Furthermore, it has been shown that African American breast cancer patients also have a higher incidence of developing the most aggressive subtype of breast cancer (Estrogen Receptor negative / Grade 3 tumor) compared to that of the European American breast cancer patients (AA: 33% of study population, EA: 16% of study population) (66). To understand the differences in aggressiveness, breast cancer type, and age at diagnosis between African American and European American women it is essential to examine genomic polymorphisms that could alter mutational rate.

In an effort to examine the impact of polymorphisms on the risk of developing aggressive forms of breast cancer, we began studying xenobiotic metabolic pathway genes. The xenobiotic metabolic pathway has been shown to participate in the metabolism of numerous hydrophobic toxins, drugs, and hormones. Studies have shown that enzymes in phase 1 of this pathway often increase the reactivity of many of these compounds which can lead to the formation of DNA adducts and eventually DNA mutations (2, 3, 7, 10, 11, 15-17). The phase 2 xenobiotic metabolic enzymes work to counteract this potential effect by attaching large polar groups to the reactive site on these active mutagenic compounds. The conjugation reaction performed by the phase 2
enzymes render the mutagens inert and allows them to be safely expelled from the body.

A common phase I enzyme in breast tissue is Cyp1B1. As one of the most abundant and reactive members of the Cytochrome P-450 superfamily in breast tissue, Cyp1B1 has been shown to produce much higher amounts of carcinogenic compounds when reacting with benzopyrene derivatives and estradiol (2, 3, 13, 14). Four cyp1b1 gene polymorphisms have found that alter the reaction rate of the Cyp1B1 enzyme (6, 18-26, 32). Each of the four polymorphisms (142 C>G, 355 G>T, 4326 C>G, and 4390 A>G) result in amino acid substitutions in the Cyp1B1 protein (R48G, A119S, L432V, and N453S). Due to their effect on reaction rates, these amino acid substitutions are thought to increase the risk of breast cancer in certain populations (19,20,27). However, other investigators found no association between these polymorphisms and cancer risk (28-31). The difference in results in each of these studies could be due to differences in race or lifestyle factors of these populations. However, the observed variability could be due to a small effect size that impacts only certain types of breast cancer. Therefore, additional research is needed to elucidate the role of cyp1b1 polymorphisms on breast cancer development. Unlike the previously cited studies, we used a case-case approach so that we could evaluate the association of the cyp1b1 polymorphisms with specific types of breast tumors or patient characteristics to determine if the polymorphisms impact traits such as breast cancer aggressiveness and progression.
We used a similar approach in the analysis of phase II xenobiotic metabolic enzymes. In the pathway of xenobiotic metabolism, phase II enzymes detoxify the cell through conjugation reactions. Conjugation reactions involve the attachment of large polar groups to the reactive compounds produced by the phase I enzymes. Deviations in the activity or amount of phase II enzymes could lead to an accumulation of mutagenic compounds and an increase in the rate of mutations within the genome. We focused on two phase II xenobiotic metabolic enzymes, GSTM1 and GSTT1. Both are members of the glutathione S-transferase family, are highly expressed in breast tissue, and deletions of each gene exist as common polymorphisms in our study population. Many studies have found an association between the gstm1 and gstt1 gene deletions and cancer risk (18, 19, 36-40). However, conflicting data have been published as well (41-43). As with cyp1b1, racial and lifestyle disparities could account for these differences, and additional investigations into the role of gstm1 and gstt1 polymorphisms in breast cancer development, progression, and aggressiveness are necessary. Therefore, we determined patient gstm1 and gstt1 genotypes and evaluated whether particular genotypes were associated with specific types of breast tumors or patient characteristics.

2.2 Materials and Methods

Polymerase Chain Reaction

Sequence information for exon 2 and exon 3 of the cyp1b1 gene were obtained from the National Center for Biotechnology Information (NCBI) SNP database. Small
sections of the sequence (18-24 bases) were selected as potential forward and reverse primers based on average GC content and unique nucleotide compositions. The potential primer sequences were analyzed for Tm, self-dimerization, heterodimerization, and hairpin formations using OligoAnalyzer software (Integrated DNA Technologies; Coralville, Iowa). Primers were ordered from Eurofins MWG Operon (Forsyth, Georgia).

In total, 601 patient DNA samples were obtained from the South Carolina Cancer Research Repository and amplified for sequencing of cyp1b1 exon 2 and exon 3. Since SNPs on exon 2 were 214 bases apart and SNPs on exon 3 were within 65 bases of each other, primers for each exon were designed to amplify both SNPs in each region (Table 2.1).

Each exon 2 amplification reaction contained 0.2 uM of each primer, 2.5 mM of each dNTP, 1U Taq DNA Polymerase (New England Biolabs; Ipswich, Massachusetts), 2.5 uL of ThermoPol buffer, 10 mg/mL BSA, 2.5 uL of 99% DMSO, and 5 to 50 ng of genomic DNA. The amplification was performed with an initial denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 20 sec, and 72 °C for 1 min. The final extension was at 72 °C for 7 min. The resulting amplification products were analyzed by agarose gel electrophoresis and successful amplification was indicated by the presence of a 914 bp band. Exon 3 reactions contained 0.2 uM of each primer, 2.5 mM of each dNTP, 1U Taq DNA Polymerase (New England Biolabs; Ipswich, Massachusetts), 2.5 uL of ThermoPol buffer, 10 mg/mL BSA, 25 mM MgCl₂, and 5 to 50 ng of genomic DNA. The
Table 2.1 Polymorphisms amplified and their corresponding primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>142 C&gt;G</td>
<td>Exon2-Forward</td>
<td>TGAGTGTCACGGCTTCTCCTCTCTCTCT</td>
<td>743 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon2-Reverse</td>
<td>AAACACACGGCACTCATGACGTGGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>355 G&gt;T</td>
<td>Exon2-Forward</td>
<td>TGAGTGTCACGGCTTCTCCTCTCTCTCT</td>
<td>743 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon2-Reverse</td>
<td>AAACACACGGCACTCATGACGTGGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyp1b1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4325 C&gt;G</td>
<td>Exon3-Forward</td>
<td>TCACTTGCTTTTCTCCTCTCC</td>
<td>650 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon3-Reverse</td>
<td>AATTTCACTCTTGCTCCTCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4390 A&gt;G</td>
<td>Exon3-Forward</td>
<td>TCACTTGCTTTTCTCCTCTCC</td>
<td>650 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exon3-Reverse</td>
<td>AATTTCACTCTTGCTCCTCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSTM1</td>
<td>Deletion</td>
<td>GAACCTCCCTGAAGGCTAAAGC</td>
<td>209 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GSTM1-Forward</td>
<td>GAACTCCCTGAAGGCTAAAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GSTM1-Reverse</td>
<td>GTTGGGCTCAAATATACGGTGGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSTT1</td>
<td>Deletion</td>
<td>TCCCCCTACTGGTCCTCACATCTCT</td>
<td>459 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GSTT1-Forward</td>
<td>TCCCCCTACTGGTCCTCACATCTCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GSTT1-Reverse</td>
<td>TCACCCGGATCATGGCCAGCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-globin</td>
<td>none</td>
<td>CAACCTTACACCTCCTACCC</td>
<td>268 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-globin-Forward</td>
<td>CAACCTTACACCTCCTACCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-globin Reverse</td>
<td>GAAGAGGCAAGGACAGGTAC</td>
<td></td>
</tr>
</tbody>
</table>
amplification was performed with an initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 20 sec, 54 °C for 20 sec, and 72 °C for 1 min with a final extension of 5 min at 72 °C. The resulting amplification products were analyzed by agarose gel electrophoresis and successful amplification was indicated by the presence of a 652 bp band. The amplified products for both regions were kept at 4 °C until shipped for sequencing.

$gstm1$, $gstt1$, & $\beta$-globin

The multiplex PCR was performed using the primers listed in Table 2.1 as described previously by Khan et al. (52).

Products from the resulting multiplex PCR were separated through electrophoresis on a 1.25% agarose gel and stained with ethidium bromide. Bands were visualized using ultraviolet light. Homozygous deletions of the $gstt1$ and $gstm1$ gene region resulted in the absence of the corresponding band ($gstt1$: 459 bp, $gstm1$: 209 bp) (Figure 2.1). The amplification of the $\beta$ globin gene was used as a positive control for the multiplex PCR reaction.

**Nucleotide Sequencing**

The $cyp1b1$ exon 2 and exon 3 PCR products were shipped to the High Throughput Genomics Center (Seattle, Washington) for nucleotide sequence identification. Genotypes
Figure 2.1 Genotype Determinations of GSTM1 and GSTT1 through Multiplex PCR: Using gstm1, gstt1, and β-globin specific primers, each gene was amplified via multiplex PCR. The resulting bands were separated by size through agarose gel electrophoresis. Genotypes were then determined by reviewing banding patterns. The absence of a band at 459 bp signified a deletion of the gstt1 gene (lane 5). The absence of a band at 209 bp indicated a deletion of gstm1 (lanes 3 and 4). The presence of a band at 459 bp or 209 bp signified the patient was either heterozygous or homozygous at the respective locus. β-globin was used as a positive control for the multiplex PCR reaction.
were determined through analysis of chromatograms using Sequencher 5.0 (Gene Codes Corporation; Ann Arbor, Michigan) (Figure 2.2).

**Statistical Analysis**

Logistic regression analyses was performed using STATA/IC 10.1 statistical software (StataCorp LP; College Station, Texas). Associations between tumor or patient characteristics and polymorphisms were determined by calculating odds ratios (OR) and 95% confidence intervals (95% CI). Significance was determined by a P-value of <0.05.

**2.3 Results and Discussion**

In examining polymorphisms in the *cyp1b1*, *gstm1*, and *gstt1* genes, a total of 3,436 genotypes were determined from 473 European American and 177 African American breast cancer patients (Table 2.2 and Table 2.3). Allele frequencies for these genes were found to be in Hardy-Weinberg equilibrium.

**Cyp1B1**

Over the years, numerous polymorphisms have been discovered in the *cyp1b1* gene (54). Most of these have no effect on the enzymatic rate of the Cyp1B1 enzyme. However, four polymorphisms (142 C>G, 355 G>T, 4326 C>G, and 4390 A>G) have been shown to increase the xenobiotic metabolic rate of Cyp1B1, and thus increase its mutagenic potential (6, 18-26, 32). To measure the association of these polymorphisms with breast cancer patient and tumor characteristics, DNA samples were taken from African American and Caucasian breast cancer patients. Using these samples we were
Figure 2.2 Chromatogram Analysis of Cyp1B1 Single Nucleotide Polymorphisms:
As a result of Sanger sequencing, chromatograms were produced displaying levels of detection of the four bases at each site in the DNA sequence. The genotype of each SNP in cyp1b1 was determined by analyzing peak size, number, and color at each locus. 1) Cyp1b1 4390 A: Designated by the single large green peak. 2) Cyp1b1 4390 Heterozygote A/G: Indicated by the small double black and green peaks. 3) Cyp1b1 4390 G: Shown by the single large black peak.
Table 2.2 Genotype frequencies of the Cytochrome P-450 1B1 gene in Caucasian and African American breast cancer patients.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Caucasian</th>
<th>African American</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>142</td>
<td>CC</td>
<td>196</td>
<td>52.4</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>147</td>
<td>0.4</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>31</td>
<td>8.3</td>
<td>38</td>
</tr>
<tr>
<td>355</td>
<td>GG</td>
<td>194</td>
<td>51.9</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>146</td>
<td>39.0</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>34</td>
<td>9.1</td>
<td>31</td>
</tr>
<tr>
<td>4326</td>
<td>CC</td>
<td>67</td>
<td>30.6</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>106</td>
<td>48.4</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>42</td>
<td>21.0</td>
<td>65</td>
</tr>
<tr>
<td>4390</td>
<td>AA</td>
<td>156</td>
<td>71.2</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>57</td>
<td>26.0</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>6</td>
<td>2.8</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.3 Genotype frequencies of Glutathione S-Transferase Mu-1 and Glutathione S-Transferase Theta-1 in Caucasian and African American breast cancer patients

<table>
<thead>
<tr>
<th>Gene</th>
<th>Caucasian</th>
<th>African American</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>123</td>
<td>43.3</td>
<td>95</td>
</tr>
<tr>
<td>Absent</td>
<td>161</td>
<td>56.7</td>
<td>41</td>
</tr>
<tr>
<td>GSTT1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>247</td>
<td>87</td>
<td>108</td>
</tr>
<tr>
<td>Absent</td>
<td>37</td>
<td>13</td>
<td>28</td>
</tr>
</tbody>
</table>
able to determine the genotype of the aforementioned polymorphic sites and assemble the individual genotypes into exon-specific haplotypes of the patients in this study.

Haplotype determination was performed independently for the exon 2 (142 C>G and 355 G>T) and exon 3 (4326 C>G and 4390 A>G) loci. Chromosomes that contained the ancestral allele at both of the polymorphic sites of exon 2 were considered to have haplotype A. Therefore, individuals who were homozygous for the ancestral alleles at both polymorphic sites had an AA genotype. Similarly, if an individual was homozygous for the derived alleles at both loci, she would have a BB genotype. Alternatively, if an individual was heterozygous at the 142 C>G locus and homozygous for haplotype A at the 355 G>T locus, her genotype would be AC (Figure 2.3). This approach allowed us to determine the haplotypic genotypes of 62% of the women with allelic determinations at cyp1B1 exon 2. The remaining women were heterozygous at both loci. For these individuals, it is not obvious whether the two ancestral alleles are on the same chromosome or one is located on each chromosome. However, since only 1% of the identified haplotypes contained the ancestral allele at the 142 C>G locus and the derived allele at the 355 G>T locus, we assumed that this potential haplotype was rare enough to not affect statistical significance. Therefore, we were able to assign the haplotypes of the cyp1b1 gene in doubly heterozygous individuals by excluding the haplotype combinations that required the rare haplotype. A similar approach was successful for identifying the haplotypes present in exon 3 of the cyp1b1 gene. To measure statistical associations, logistic regression analysis was used to compare the cyp1b1 genotypes to the breast cancer patient and tumor characteristics found in Tables
Figure 2.3 Determination of *cyp1b1* haplotypes and genotypes in African American and European American breast cancer patients. Haplotypes were assigned first for patients who were determined to be at least homozygous at one of the polymorphic sites in exon 2 or exon 3. In each of the groups analyzed one potential haplotype was found to be absent or only present in 1% of the population. We were therefore able to determine the haplotype of individuals who were heterozygous at each locus in exon 2 or exon 3 by excluding the combinations that included a rare haplotype.
2.4, 2.5 and 2.6. No significant associations were discovered between any of the cyp1b1 genotypes and any of the 13 African American or Caucasian breast cancer patient or tumor characteristics. These results are consistent with previous studies, which have also failed to find an association between polymorphisms in the cyp1b1 gene and breast cancer susceptibility (28-31). As previously mentioned, Cyp1b1 has been shown to produce mutagenic metabolites and these four polymorphisms have experimentally demonstrated the ability to increase both the enzymatic rate and the mutagenic potential of the Cyp1b1 enzyme. Previous studies have shown that an increased mutation rate leads to increased breast cancer risk (50, 51, 55). However, it is plausible that an increase in phase I xenobiotic metabolic pathway products could induce phase II xenobiotic enzymes. This phase II induction would limit or nullify the effects of cyp1b1 polymorphisms. Alternatively, over the course of their lifetime, the patients in our study may have experienced different levels of xenobiotic compounds or hormones such that the presence of cyp1b1 polymorphisms does not correlate with mutation rates. Furthermore, it is possible that the cyp1b1 polymorphisms have only a small effect on breast cancer progression and aggressiveness. Thus, this study may lack the necessary sample size to detect those associations.

**GSTM1 and GSTT1**

As phase II xenobiotic metabolizing enzymes, the glutathione S-transferase family works to detoxify the cell by adding a large polar molecule to the toxic or carcinogenic hydrophobic substances produced from the phase I enzymes. It is believed that
Table 2.4 Distribution of African American and Caucasian Breast Cancer Patient Population Characteristics

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Caucasian</th>
<th>African American</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Menstruating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>118</td>
<td>28.20%</td>
<td>53</td>
</tr>
<tr>
<td>No</td>
<td>300</td>
<td>71.80%</td>
<td>107</td>
</tr>
<tr>
<td>Hormone Replacement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>165</td>
<td>39.01%</td>
<td>113</td>
</tr>
<tr>
<td>No</td>
<td>258</td>
<td>60.99%</td>
<td>48</td>
</tr>
<tr>
<td>Live Birth</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>364</td>
<td>85.80%</td>
<td>140</td>
</tr>
<tr>
<td>No</td>
<td>60</td>
<td>14.20%</td>
<td>22</td>
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<tr>
<td>Immediate Family History of Breast Cancer</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>105</td>
<td>24.70%</td>
<td>37</td>
</tr>
<tr>
<td>No</td>
<td>320</td>
<td>75.30%</td>
<td>125</td>
</tr>
<tr>
<td>Family History of Other Cancers</td>
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<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>426</td>
<td>83.40%</td>
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</tr>
<tr>
<td>No</td>
<td>85</td>
<td>16.60%</td>
<td>29</td>
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</table>
Table 2.5 Distribution of African American and Caucasian Breast Cancer Tumor Characteristics

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Caucasian</th>
<th></th>
<th>African American</th>
<th></th>
<th>Difference</th>
</tr>
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<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Laterality</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Left</td>
<td>235</td>
<td>52.80%</td>
<td>78</td>
<td>33.70%</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>Right</td>
<td>210</td>
<td>47.20%</td>
<td>153</td>
<td>66.20%</td>
<td></td>
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<tr>
<td>Estrogen Receptor Status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>303</td>
<td>78.90%</td>
<td>112</td>
<td>73.20%</td>
<td>P=.5</td>
</tr>
<tr>
<td>Negative</td>
<td>81</td>
<td>21.10%</td>
<td>41</td>
<td>26.80%</td>
<td></td>
</tr>
<tr>
<td>Progesterone Receptor Status</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>259</td>
<td>68.30%</td>
<td>98</td>
<td>64.10%</td>
<td>P=.25</td>
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<tr>
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<td>55</td>
<td>35.90%</td>
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<td>Her2 Receptor Status</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>73</td>
<td>24.80%</td>
<td>22</td>
<td>19.50%</td>
<td>P=.5</td>
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<tr>
<td>Negative</td>
<td>222</td>
<td>75.20%</td>
<td>91</td>
<td>80.50%</td>
<td></td>
</tr>
<tr>
<td>Lymph Node Involvement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>116</td>
<td>30.90%</td>
<td>42</td>
<td>30.00%</td>
<td>P=.975</td>
</tr>
<tr>
<td>No</td>
<td>259</td>
<td>69.10%</td>
<td>98</td>
<td>70.00%</td>
<td></td>
</tr>
<tr>
<td>Invasiveness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>377</td>
<td>85.70%</td>
<td>146</td>
<td>87.40%</td>
<td>P=.95</td>
</tr>
<tr>
<td>Noninvasive</td>
<td>63</td>
<td>14.30%</td>
<td>21</td>
<td>12.60%</td>
<td></td>
</tr>
<tr>
<td>Ductal vs. Lobular</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>388</td>
<td>92.20%</td>
<td>146</td>
<td>89.02%</td>
<td>P=.9</td>
</tr>
<tr>
<td>Lobular</td>
<td>33</td>
<td>7.80%</td>
<td>18</td>
<td>10.98%</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.6 Distribution of African American and Caucasian breast cancer patients age at time of diagnosis.

<table>
<thead>
<tr>
<th>Age at Diagnosis</th>
<th>Caucasian</th>
<th>African American</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=452</td>
<td>n=168</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>56.2 ± 11.5</td>
<td>51.7 ± 11.67</td>
</tr>
<tr>
<td>Median; Range</td>
<td>54; 25-82</td>
<td>52; 26-84</td>
</tr>
</tbody>
</table>
decreased levels of phase II enzymes would increase the level of carcinogenic material in the cell and lead to an increase in mutations, thus potentially leading to the development of cancer. Therefore, we looked at two gene deletion polymorphisms that have previously been associated with the development of different cancers (18, 19, 36-40). By utilizing multiplex PCR with primers complementary to DNA sequences within the *gstt1* and *gstm1* genes, we were able to amplify the respective genes in the genomes that contained at least one copy. However, because this technique only detects the presence or absence of the glutathione s-transferase genes we are unable to detect the genomes that are heterozygous for either or both genes (figure 2.4). Our data indicated that the two glutathione S-transferase genes, *gstm1* and *gstt1*, are absent in 57% and 13% in the European American population 30% and 21% in the African American population, respectively. Homozygous deletions in both the *gstm1* and *gstt1* genes were present in only 6.3% of European Americans and 5.8% of African Americans.

To assess possible associations between the *gstm1* and *gstt1* gene deletions with breast cancer patient and tumor characteristics (Tables 2.4 and 2.5), we used logistic regression. No significant associations were found between *gstt1*, *gstm1*, or the *gstm1-gstt1* double deletion genotypes and any of the African American or Caucasian breast cancer patient or tumor characteristics. Products from phase I xenobiotic metabolism can lead to the development of cancer, and enzymes in phase II of the xenobiotic metabolic pathway are able to deactivate many of these carcinogens (2, 3, 7, 10, 11, 15-17). However, with high substrate overlap within classes of glutathione S-transferase family, it is possible that the effects of *gstm1* or *gstt1* deletions are nullified or greatly
Figure 2.4 Representative results for GSTT1 and GSTM1 multiplex PCR: Figure shows a representative gel for the amplification of β-globin, *gstt1* and *gstm1* through multiplex PCR. GSTT1 and GSTM1 genotypes are signified by a “+” for presence and a “−” for absence. No results were gathered from lanes 8 and 9 as positive control Beta Globin did not amplify.
reduced by increased expression or activity of other mu or theta class genes. Thus, further investigation into the expression levels of glutathione S-transferase mu class and glutathione S-transferase theta class genes in gstm1 and gstt1 null patients is warranted. Alternatively, as with cyp1b1, lifetime exposure of xenobiotics may impact the effect of the gstm1 or gstt1 null genotype of breast cancer. Also, the impact of polymorphisms in the gstm1 or gstt1 genes on breast cancer patient and tumor characteristics may be too small for our analysis to accurately detect. Previous studies have reported that the aforementioned cyp1b1 polymorphisms lead to increased carcinogen production and mutation rate. The effects of these polymorphisms on breast cancer may be exacerbated in patients with deletions in gstm1 and gstt1 genes, as they have reduced phase II xenobiotic metabolic enzymatic capacity. Once again, our study lacked sufficient power to analyze this hypothesis since these patients would represent only a small percentage of the population.

In summation, while this study failed to show any significant associations between polymorphisms in the cyp1b1, gstm1, or gstt1 genes and breast cancer patient or tumor characteristics, we cannot rule out a small effect on breast cancer development or progression. An increase in sample size and comparison of phase I and phase II polymorphisms with factors such as xenobiotic exposure could help to identify previously undetected associations. Furthermore, studies into phase II xenobiotic metabolic gene expression will help to elucidate how the cell compensates for varying Cyp1b1, GSTM1, and GSTT1 enzymatic rates.
CHAPTER 3: AMINO ACID SUBSTITUTIONS CAUSE MODEST EFFECT ON CYP1B1 PROTEIN STABILITY
Abstract

Cytochrome P450 Family 1 Subfamily B Protein 1 (CYP1B1) plays a fundamental role in the metabolism of numerous mutagenic and toxic xenobiotics. In reacting with these xenobiotic substrates, Cyp1B1 often increases their mutagenic and carcinogenic potential. We examined the effect of four single nucleotide polymorphisms in the Cyp1b1 gene, which lead to amino acid substitutions, on Cyp1B1 protein stability. Stability of the endogenous Cyp1B1 in human cell lines was measured utilizing cycloheximide chase and western blotting techniques. Our data indicated a significant 20% decrease (p: 0.042) in the half-life of Cyp1B1*4 (Asparagine to Serine substitution at amino acid position 453) compared to Cyp1B1*3 (Leucine to Valine substitution at amino acid position 432). We also noticed a significant 50% increase (p:0.03) in the half-life of Cyp1B1*2 (Arginine to Glycine substitution at amino acid position 48 and Alanine to Serine substitution at amino acid position 119) compared to Cyp1B1*3. Utilizing the proteasome inhibitor MG132 in conjunction with the cycloheximide chase techniques, we verified that Cyp1B1 is degraded through the proteasomal pathway. Compared to previous findings, our results show conservative changes in degradation rates, and as such we hypothesize that differences in half-life for the Cyp1B1 variants have little to no effect on the production of carcinogenic compounds and thus cancer development and progression.
3.1 Introduction

Xenobiotic metabolizing enzymes play an important role in purifying the cell from potential toxic and carcinogenic materials. To perform this function, enzymes of the phase I and phase II xenobiotic metabolic pathway must convert hydrophobic substrates into polar hydrophilic products. Often during the phase I modifications these toxic substrates are converted to highly mutagenic compounds. Thus mutations in the phase I metabolizing genes, which lead to amino acid substitutions, can cause an alteration in the functionality of these proteins and variation in carcinogenic production.

As a member of the largest family of proteins in the xenobiotic metabolizing pathway, and as one of the highest expressed xenobiotic metabolizing pathway phase I proteins, we chose to study Cyp1B1. Previous studies have examined the effects of four mutations in exon 2 and exon 3 of the cyp1b1 gene, which lead to amino acid substitutions (figure 1.6) (3, 6, 18-27, 32). Shimada et al. reported an increase in the rate of metabolism of benzopyrene derivatives in mutants that contained a Serine residue at position 119 instead of the ancestral Alanine (23). In contrast, Aklillu et al. found the formation of benzopyrene derivatives to be decreased in Cyp1b1 proteins with an Asparagine to Serine amino acid substitution at 453 (22). In addition, Shimada et al. also showed that amino acid substitutions at positions 432 and 432+119 yielded increased production of 4-hydroxyestradiol, a known carcinogen, and Aklillu et al. showed that a Serine substitution at position 453 of Cyp1B1 lead to a decrease in 4-hydroxyestradiol production (23, 32). In contrast, Hanna et al. found a 2.4 fold increase 4-
hydroxyestradiol production in Cyp1B1 Serine 453 variants. It was also shown that Serine 119 variants had a 3.4 fold increase in 4-hydroxyestradiol. Other Cyp1B1 variants proved to have little effect on $K_m$ or $V_{max}$ in relation to benzopyrene or estrogen catalysis (3, 6, 21-23, 32). It is clear that changes in the amino acid sequence of Cyp1B1 have varying effects on the catalysis of specific substrates; however few studies have been performed to analyze the effect of amino acid sequence changes on Cyp1B1 protein stability.

Previously, Ingleman-Sundberg et al. and Bandiera et al. examined the stability of the Cyp1b1*2 (Glycine 48, Serine 119) and Cyp1b1*4 (Serine 453) variant proteins through overexpression in COS-1 African Green Monkey cells (21, 44). In these experiments, it was shown that Cyp1b1*4 was degraded more than three times faster than the ancestral protein (5 hrs. Cyp1b1*4, 17hrs Cyp1b1*1), whereas Cyp1b1*2 displayed no significant difference in stability from that of the ancestral protein. However, these results may not reflect the rates of degradation that occur in human cells with endogenous rates of protein expression.

Through the use of predictive software (NetPhos 2.0 and GPS 2.1), we found that Serine substitutions at positions 119 and 453 generate potential phosphorylation sites at 119 and 458, respectively (figure 1.7). These additional phosphorylation sites may result in the formation of a phosphodegron, a pattern of phosphorylated amino acids used to identify proteins for ubiquitination and eventually degradation. In this pathway, two or more phosphorylated amino acids in close proximity to each other serve as
recognition sites for the E3 ubiquitin ligase. It is our hypothesis that the Cyp1B1 protein will be phosphorylated at the 119 and 458 phosphorylation sites in addition to the normal phosphorylation sites when the additional serine residues are present (figure 1.7). The proximity of these phosphate groups to each other would cause E3 ubiquitin ligase to recognize and bind Cyp1B1 more efficiently as a substrate. Once bound, E3 will transfer ubiquitin tags to the phosphorylated Cyp1B1 (Figure 1.8), which will direct the protein to the proteasome for degradation. To study the effects of these mutations on the degradation rate of Cyp1B1, we analyzed the endogenous Cyp1B1 protein in three different human cell lines and found that the presence of the serine residues had only a small impact on Cyp1B1 degradation rates (Table 3.1). Thus, overexpression of the Cyp1B1*4 protein in African Green Monkey cells apparently led to exaggerated rates of degradation.

3.2 Materials and Methods

Cell Line and Reagents

BT20, T47D, and MCF7 cell lines were donated by the Pittman Laboratory (South Carolina College of Pharmacy). All cell lines were maintained at 37.5°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) (Corning Cellgro, Manassas, VA) supplemented with 10% new-born calf serum, 1% penicillin/streptomycin (Thermo Scientific, Waltham, MA), and 1% glutamate. This medium was designated primary growth medium.
Table 3.1 Cyp1b1 Variants Present in BT20, MCF7, and T47D Breast Cancer Cell Lines:
The T47D cell line has amino acid substitutions at locations 48 and 119. BT20 is used as
the ancestral equivalent with an amino acid substitution at location 432. It was
previously determined that a mutation at location 432 had no effect on degradation
rates (44). MCF7 has a Serine substitution at location 453.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Cyp1b1-Exon 2: R48G</th>
<th>Cyp1b1-Exon 2: A119S</th>
<th>CYP1b1-Exon 3: L432V</th>
<th>Cyp1b1-Exon 3: N453S</th>
<th>Cyp1b1 Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D</td>
<td>Mutant</td>
<td>Mutant</td>
<td>WT</td>
<td>WT</td>
<td>Cyp1B1*2</td>
</tr>
<tr>
<td>BT20</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
<td>WT</td>
<td>Cyp1B1*3</td>
</tr>
<tr>
<td>MCF7</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>Mutant</td>
<td>Cyp1B1*4</td>
</tr>
</tbody>
</table>
Genotyping Cell Lines

Sequence information for exon 2 and exon 3 of the cyp1b1 gene was obtained from the National Center for Biotechnology Information (NCBI) SNP database. Since SNPs on exon 2 were 214 bases apart and SNPs on exon 3 were within 65 bases of each other, primers for each exon were designed to amplify both SNPs in each region (Table 3.2) Small sections of the sequence (18-24 bases) were selected as potential forward and reverse primers based on average GC content and unique nucleotide compositions. The potential primer sequences were analyzed for Tm, self-dimerization, heterodimerization, and hairpin formations using OligoAnalyzer software (Integrated DNA Technologies; Coralville, Iowa). Primers were ordered from Eurofins MWG Operon (Forsyth, Georgia).

Each exon 2 amplification reaction contained 0.2 uM of each primer, 2.5 mM of each dNTP, 1U Taq DNA Polymerase (New England Biolabs; Ipswich, Massachusetts), 2.5 uL of ThermoPol buffer, 10 mg/mL BSA, 2.5 uL of 99% DMSO, and 5 to 50 ng of genomic DNA. The amplification was performed with an initial denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 20 sec, and 72 °C for 1 min. The final extension was at 72 °C for 7 min. The resulting amplification products were analyzed by agarose gel electrophoresis and successful amplification was indicated by the presence of a 914 bp band.

Exon 3 reactions contained 0.2 uM of each primer, 2.5 mM of each dNTP, 1U Taq DNA Polymerase (New England Biolabs; Ipswich, Massachusetts), 2.5 uL of ThermoPol
Table 3.2 Primer sequences used for amplification and sequencing of Cyp1B1 SNPs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>142 C&gt;G</td>
<td>Exon2-Forward</td>
<td>TGAGTGTACGCCTTCTCCTCTCT</td>
<td>743 bp</td>
</tr>
<tr>
<td></td>
<td>355 G&gt;T</td>
<td>Exon2-Reverse</td>
<td>AAAACACACGGCACTGACGTTG</td>
<td></td>
</tr>
<tr>
<td>Cyp1b1</td>
<td>4326 C&gt;G</td>
<td>Exon3-Forward</td>
<td>TCACTTGGCTTTCTCTCTCC</td>
<td>650 bp</td>
</tr>
<tr>
<td></td>
<td>4390 A&gt;G</td>
<td>Exon3-Reverse</td>
<td>AATTTCAGCCTGCTCCTG</td>
<td></td>
</tr>
</tbody>
</table>
buffer, 10 mg/mL BSA, 25 mM MgCl₂, and 5 to 50 ng of genomic DNA. The amplification was performed with an initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 20 sec, 54 °C for 20 sec, and 72 °C for 1 min with a final extension of 5 min at 72 °C. The resulting amplification products were analyzed by agarose gel electrophoresis and successful amplification was indicated by the presence of a 652 bp band. The amplified products for both regions were kept at 4 °C until they were shipped to High Throughput Genomics Center (Seattle, WA) for nucleotide sequence identification.

Genotypes were determined through analysis of chromatograms using Sequencher 5.0 (Gene Codes Corporation; Ann Arbor, Michigan).

**Determination of Cyp1B1 stability**

Once cells were grown to confluence in primary growth medium, 200 µg/mL of the protein synthesis inhibitor cycloheximide (CHX) was added to stop protein synthesis. Cells were harvested from the plates and lysed using cold lysis buffer at 0, 4, and 8 hours after CHX addition. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes using an electroblot process (Bio-Rad, Hercules, CA). The resulting blots were stained with Ponceau S (Sigma, St. Louis, MO) as described by Romero-Calvo et al. (58). After staining, the blots were incubated with 50 mg/ml of BSA for 1 hour (AMRESCO, Solon, OH), and the BSA-coated blots were incubated at 4°C overnight using the appropriate primary antibody (Cyp1B1: ab33586, Cyp1A1: ab124295, Abcam, Cambridge, MA). The
following day, the blots were washed for 15 minutes and incubated with secondary antibody for 1 hr (Anti-rabbit IgG: A2556, Sigma, St. Louis, MO). Following secondary antibody incubation, Lumi-Phos chemiluminescent substrate (Thermo-Scientific, Waltham, MA) was used with HyBlot autoradiography film to detect protein bands. Mean band intensity was measured to quantify degradation of Cyp1B1 using Adobe Photoshop.

To confirm that the proteasome was responsible for Cyp1B1 degradation, cells were grown to confluence in primary growth medium and then 200 µg/mL of the protein synthesis inhibitor cycloheximide (CHX) and 20µM of proteasome inhibitor MG132 was added. Cells were harvested and analyzed as described above.

**Statistical Analysis**

Experiments were performed in triplicate, and Z-score analysis was used to assess statistical significance of Cyp1B1 half-life.

**3.3 Results and Discussion**

Over the past two decades, numerous studies have examined the effect of four specific Cyp1B1 polymorphisms and their relation to xenobiotic catalysis and cancer (Figure 3.1) (3, 6, 18-27, 32). In more recent years, two studies investigated the role of these polymorphisms on protein stability (21, 44). However, as the these Cyp1B1 protein stability studies were conducted using overexpression techniques in African Green Monkey cells, we thought that it was important to determine degradation rates
**Figure 3.1 Polymorphisms in the Cyp1B1 gene and protein:** The relative positions of four polymorphisms found in the Cyp1B1 gene, which yield amino acid substitutions. **A)** 142 C>G causes an Arginine to Glycine change at amino acid number 48. 355 G>T causes an Alanine to Serine change at amino acid number 119. 4326 C>G causes a Leucine to Valine change at amino acid number 432. 4390 A>G causes an Asparagine to Serine change at amino acid number 453.
in human cell lines utilizing the endogenous Cyp1B1 proteins to obtain a more accurate estimation of the impact of *Cyp1b1* polymorphisms on Cyp1b1 protein stability. To determine Cyp1B1 protein stability, cell lines were selected based on Cyp1B1 genotype homozygosity and their relation to breast cancer. Nucleotide sequencing of cell line genomic DNA determined that the BT20 cell line had a polymorphism at position 4326 corresponding to an amino acid substitution (valine →leucine) at position 432. It has been shown previously that this mutation has no effect on degradation (44), and predictive data indicated no mechanism for altered degradation rate. Similarly, the T47D cell line was shown to contain a polymorphism at positions 142 and 355, which leads to amino acid substitutions at positions 48 (arginine →glycine) and 119 (alanine →serine). Genotyping of the MCF7 cell line revealed a polymorphism at site 4390 yielding an amino acid substitution at 453 (asparagine →serine). Predictive software (NetPhos 2.0 and GPS 2.1) indicated amino acid substitutions at position 119 and position 453 lead to increased phosphorylation (Figure 1.7). To determine if these phosphorylation sites lead to a change in rates of Cyp1B1 degradation, we grew T47D, BT20, and MCF7 cells (Cyp1B1*2, Cyp1B1*3, and Cyp1B1*, respectively) in the presence of cycloheximide to stop protein synthesis and measured the loss of Cyp1b1 protein over the course of eight hours using western blotting techniques (Table 3.1). Since Cyp1A1 is homologous with Cyp1B1, Cyp1A1 was used as a control to detect possible cell line differences affecting protein degradation. Using Cyp1B1*3 as reference and normalizing with Cyp1A1 degradation levels, we found significant variation among the half-lives of Cyp1B1*2, Cyp1B1*3, and Cyp1B1*4 (Figure 3.2). As found previously by Bandiera et al. (44), we
**Figure 3.2 Cyp1B1 degradation** Relative degradation rates of Cyp1B1 variants using cycloheximide chase from 0 to 8 hours. Cyp1B1*3 was used as the ancestral version of the gene. Cyp1B1*4 showed an increased rate of degradation and a half-life of 13 hrs. Cyp1B1*2 demonstrated a decreased rate of degradation and a half-life of 24 hrs. Cyp1B1*3 was determined to have a half-life of 16 hrs. # signifies significance.
found that Cyp1B1*4 had a decreased half-life compared to that of Cyp1B1*3. However, our data depicts Cyp1B1*4 degrading at a rate that is more than 2.5 times slower than that detected by Bandiera et al. (44). This difference in Cyp1B1*4 degradation rates may be due to differences between human and monkey cells or the fact that the human Cyp1B1*4 is a foreign protein in the monkey cells. Furthermore, overexpression of Cyp1B1*4 leads to a non-natural state, which could potentially alter the detectable rates of degradation.

The increased rate of Cyp1B1*4 degradation is probably due to creation of a new phosphorylation site in the Cyp1B1*4 variant protein by the Asparagine to Serine amino acid substitution at amino acid number 453. We hypothesize that this phosphorylation leads to the formation of a phosphodegron which would target the Cyp1B1*4 protein to the proteasome for degradation and increase the rate of Cyp1B1*4 turnover.

In addition to the Cyp1B1*4 data, we also found a significant difference in Cyp1B1*2 (24 hours) degradation compared to that of Cyp1B1*3 (16 hours) (Table 3.3). A previous study by Ingelman-Sundberg et al. (21), found a slower but non-significant difference in the rate of degradation between Cyp1B1*1 (ancestral) and Cyp1B1*2, which would be consistent with our results. The difference in our results could be due to the fact that Ingelman-Sundberg et al. (21) also compared Cyp1B1*2 to Cyp1B1*1 while we compared Cyp1B1*2 to Cyp1B1*3. The slower rate of degradation of Cyp1B1*2 could be due to an arginine to glycine substitution at position 48 in that could enhance the strength of the PPGP domain. The PPGP domain is thought to be integral in proper
Degradation rates of variant Cyp1B1 proteins were measured and used to calculate the protein half-life. Cyp1B1*3 was used as the ancestral version of the protein with half-life of 16 h. Each value is the average of three replicate experiments. # signifies significance at $\alpha = 0.05$.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cyp1B1</th>
<th>Half-life (h)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT20</td>
<td>Cyp1B1*3</td>
<td>16.3</td>
<td></td>
</tr>
<tr>
<td>T47D</td>
<td>Cyp1B1*2</td>
<td>24.3</td>
<td>0.03#</td>
</tr>
<tr>
<td>MCF7</td>
<td>Cyp1B1*4</td>
<td>13.1</td>
<td>0.042#</td>
</tr>
</tbody>
</table>
folding and stability of Cytochrome P450 family 1 and 2 proteins (21, 59). Thus amino acid substitutions that stabilize the folding of this region could increase the half-life of the protein. Bandiera et al. (44) showed that Cyp1B1 was degraded through the proteasome. To verify this result, we repeated the cycloheximide chase experiment as described above, but we blocked proteasomal degradation with the proteasome inhibitor MG132. For each of the three Cyp1B1 variant, we found a two-fold increase in the half-life of the Cyp1B1 protein in the presence of MG132 (Figure 3.3). These data demonstrate that the proteasome plays a key role in the degradation of Cyp1B1 in human cells confirming the observations of Bandera et al. (44) with Cyp1B1 degradation in monkey cells.

In conclusion, we were able to assess the effects of endogenous amino acid substitutions on Cyp1B1 protein half-life by measuring the stability of the variant proteins in human cell lines. For example we observed a half-life of 13.1 h for the Cyp1B1*4 variant protein indicating that it degrades about 25% faster than the rate of the Cyp1B1*3 protein (Table 3.3). In contrast Bandiera et al. (44) found that Cyp1B1*4 had a 5 hour half-life. We suspect that the 5 hour half-life reflects the fact that the degradation rate was measured in an African green monkey cell line where the human Cyp1B1*4 was over-expressed. It is our hypothesis that the examination of endogenous proteins in human cell lines would be closer to the actual degradation rates in human tissues. Since we observed a much smaller yet significant difference in half-life between Cyp1B1*4 and Cyp1B1*3, it is reasonable to assume that any resulting change in
Figure 3.3 Cyp1B1*2 degradation in the presence of cycloheximide alone and cycloheximide plus MG132. Cells were exposed to MG132 over for 8 hours. At 0, 4, and 8 hours, cells were harvested and Cyp1B1 levels were measured using western blotting techniques. The half-life of Cyp1B1*2 increased by 188% when MG132 was used to block proteasomal degradation. Cyp1B1*3 and Cyp1B1*4 increased by 220% when MG132 was added. All time points are the average of three replicate experiments.
xenobiotic metabolism in breast tissue would be very minor, and would have little to no
effect on breast cancer development or progression.
CHAPTER 4: SUMMARY
4.1 Polymorphisms in Xenobiotic Metabolizing Genes

As a major pathway of detoxification for the human cell, xenobiotic metabolizing enzymes (XME) work to reduce the effects of certain toxic and mutagenic compounds that diffuse into the cell. Our study set out to examine any link between polymorphisms in the phase I and phase II xenobiotic metabolizing pathways and breast cancer in African American and European American populations. For phase I XME, we measured the association between four common single nucleotide polymorphisms in Cytochrome P450 Family 1 Subfamily B Protein 1 (cyp1B1) gene and 13 patient and tumor characteristics. Using logistic regression analysis, we found no significant association between polymorphisms in the cyp1B1 gene and breast cancer risk factors. It is plausible that increased production of phase I products might increase the expression of phase II enzymes. This would help to reduce the amount of mutagenic compounds produced by Cyp1B1. Alternatively, since the production of mutagenic materials produced by Cyp1B1 protein is dependent upon substrate concentration, it is possible that a certain substrate concentration threshold would have to be reached before any effect could be seen.

In addition to our work with cyp1B1, we also assessed possible associations between polymorphisms in phase II XMEs. A major gene family in the detoxification of xenobiotics, Glutathione S-Transferase (GST) works by adding glutathione to reactive metabolites, which converts them to large polar non-reactive molecules. Many European Americans and African Americans have gene deletions in GST Family Theta Protein 1 (gstt1) and/or the GST Family Mu Protein 1 (gstm1) genes. It is thought that these loss of function polymorphisms could lead to development of certain cancers (18,
however we found no significant association between the absence of the *gstt1* or *gstm1* genes and breast cancer risk factors. We hypothesize that the overlap of substrate specificity with other GST enzymes may compensate for the loss of these genes.

In summary, the analysis of *cyp1B1*, *gstt1*, and *gstm1* polymorphisms in 473 European American and 177 African American breast cancer patients failed to show any significant association between polymorphic variance and breast cancer. However, it is possible that these polymorphic genes play a minor role in breast cancer progression that our moderate sample size renders undetectable. Furthermore, studies into the expression patterns of other phase II XMEs could be performed to assess how the cell compensates for varying protein levels and catalytic activity of Cyp1B1, GSTT1, and GSTM1.

### 4.2 Cyp1B1 Polymorphisms and Protein Stability

As the largest XME families in humans, Cytochrome P450 enzymes work to detoxify the cell from various toxins, carcinogens, and therapeutic drugs. In breast tissue, Cyp1B1 is the most highly expressed of any Cytochrome P450 enzymes (13). Cyp1B1 often converts xenobiotic compounds to highly reactive carcinogens, and previous studies have shown that common *Cyp1B1* single nucleotide polymorphisms that lead to amino acid substitutions can affect the rate of carcinogen production (3, 6, 18-27, 32, 44). To determine whether these polymorphisms affect Cyp1B1 stability, we examined three endogenous Cyp1B1 variant proteins in human breast cancer cells.
Through the use of cycloheximide chase and western blotting techniques, we were able to measure the half-life of Cyp1B1*2 (Arginine to Glycine substitution at amino acid position 48 and Alanine to Serine substitution at amino acid position 119), Cyp1B1*3 (Leucine to Valine substitution at amino acid position 432), and Cyp1B1*4 (Asparagine to Serine substitution at amino acid position 453). We detected a 20% increase in the rate degradation for Cyp1B1*4 compared to Cyp1B1*3, and a 50% decrease in the rate of degradation for Cyp1B1*2 compared to Cyp1B1*3. Both of these findings are much more modest compared to the three-fold differences reported in the previously published data (44). We suspect that the overexpression of the Cyp1B1 proteins in monkey cells might be responsible for the much greater differences in Cyp1B1 variant protein stabilities.

Previously, Bandiera et al. showed that Cyp1B1 was degraded through the proteasomal pathway in African Green Monkey cells (44). To verify this pathway in human cell lines, we used the proteasomal inhibitor MG132 along with cycloheximide chase and western blotting to measure Cyp1B1 degradation over 8 hours. In all three variants, we measured a two-fold increase in half-life when the proteasome was inhibited. This result verifies that the proteasome plays a key role in the degradation of Cyp1B1.

To further expand on this study, we plan to measure the half-life of Cyp1B1*1, Cyp1B1*5, and Cyp1B1*6. In addition, we plan to determine if amino acid positions 119 and 453 are phosphorylated when a serine substitution is present. If phosphorylation is
present, studies measuring enzymatic efficiency and substrate specificity can be used to examine the regulation of Cyp1B1*2 and Cyp1B1*4. The resulting data illustrate potential molecular mechanisms for previously published differences in breast cancer risk (19,20,27).

4.3 Cyp1B1 stability and Breast Cancer Risk

As one of the most common forms of cancer in women in the United States, it is imperative to determine the underlying mechanisms for breast cancer susceptibility. Numerous studies have examined genetic polymorphisms within the human genome to determine what effect they might have on breast cancer development or progression. Through our population study with cyp1B1, gstt1, and gstm1 genes, we were unable to measure any significant correlation between breast cancer tumor characteristics with genetic variance. These results are consistent with the modest differences in half-life we observed between the Cyp1B1 variants. These modest differences in Cyp1B1 protein stability are likely to have a minimal impact on the production of carcinogenic compounds and thus an even more minor effect on breast cancer risk. This is verified by the numerous case control studies examining the impact cyp1b1 mutations have on breast cancer (19,20,27-31) This conclusion argues against the previously published hypothesis which proposes a link between cancer risk and Cyp1B1 half-life (44).
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