GENETIC POLYMORPHISMS IN AH RECEPTOR AND CYTOCHROME P450 DRUG-METABOLIZING ENZYMES IN RELATION TO ESTRADIOL METABOLISM AND BREAST CANCER SUSCEPTIBILITY

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ABSTRACT

Genetic Polymorphisms in *AH* Receptor and Cytochrome P450 Drug-metabolizing Enzymes in Relation to Estradiol Metabolism and Breast Cancer Susceptibility
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Bioactivation of environmental procarcinogens and prolonged exposure to 17β-estradiol and catechol estrogens have been implicated in the etiology of mammary carcinogenesis. Endogenous 17β-estradiol is oxidized to catechol estrogens by cytochrome P450 CYP1A2 and CYP1B1 enzymes which are transcriptionally regulated by the AH receptor. These enzymes are also involved in the bioactivation of numerous environmental procarcinogens such as benzo[*a*]pyrene. Consequently, those genetic polymorphisms in *CYP1A2*, *CYP1B1* and *AHR* that result in changes of enzyme or receptor function may affect 17β-estradiol clearance and metabolic activation of procarcinogens. I genotyped two populations for a total of 7 polymorphisms in the *AHR*, *CYP1A2* and *CYP1B1* genes to examine whether these gene polymorphisms modify estrogen metabolism (Project 1) and breast cancer susceptibility (Project 2). Data of our estrogen metabolism study (Project 1) suggest that the *AHR* 554 polymorphism is related to alterations in 17β-estradiol metabolism. Results of our Quebec French Canadian breast cancer case-control study (Project 2) indicate that the *AHR* 554, *CYP1A2*1F*, *CYP1B1*3* and *CYP1B1*4* polymorphisms may modify breast cancer susceptibility.

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bp	base pair
µg	micrograms
µl	microlitres
°C	degree Celsius
16αOH	16α-hydroxyestradiol
17β-HSD	17β-hydroxysteroid dehydrogenase
2OH	2-hydroxyestradiol
2OH:16αOH	2-hydroxyestradiol/16α-hydroxyestradiol metabolite ratio
4-ABP	4-aminobiphenyl
4OH	4-hydroxyestradiol
4OH:2OH	4-hydroxyestradiol/2-hydroxyestradiol metabolite ratio
95% CI	95% confidence interval
A	adenine
AHH	aryl hydrocarbon hydroxylase
AHR	aryl hydrocarbon receptor
AHREs	AHR-responsive elements
AIP	AHR-interacting protein
Ala	alanine
App	appendix
Arg	arginine
ARNT	aryl hydrocarbon receptor nuclear translocator
ASA	allele-specific amplification
Asn	asparagine
ASO	allele-specific oligonucleotide
BaP	benzo[*a*]pyrene
BMI	body mass index
C	cytosine
CFLP	cleavase fragment length polymorphism
COMT	catehol-O-methyltransferase
DGGE	denaturing gradient gel electrophoresis
DMBA	7,12-dimethylbenz[*a*]anthracene
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DREs	dioxin-responsive elements
E1	estrone
E2	17β-estradiol
EDTA	ethylenediaminetetraactetic Acid
EGFR	epidermal growth factor receptor
EROD	ethoxyresorufin-O-deethylase
ERs	estrogen receptors
f	allele frequency
G	guanine

HAHs	halogenated aromatic hydrocarbons
HAs	heterocyclic amines
Hsp90	90-kDa heat shock protein
Ile	isoleucine
IPTG	Isopropyl β-D-thiogalactopyranoside
IQ	2-amino-3methylimidazo[4,5]quinoline
kg	kilograms
Leu	leucine
ln	natural logarithm
Lys	lysine
m^2	metre square
n	sample population size
ng	nanograms
OLA	oligonucleotide ligation assay
OR	odds ratio
p	probability
PAHs	polynuclear aromatic hydrocarbons
PBS	phosphate buffered saline
PCB congener no. 153	2,2',4,4',5,5'-hexachlorobiphenyl
PCB congener no. 183	2,2'3,4,4',5,6'-heptachlorobiphenyl
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
PhIP	2-amino-1-methyl-6-phenylimidazole [4,5-*b*] pyridine
Pro	proline
PRs	progesterone receptors
RFLP	restriction fragment length polymorphism
r_s	Spearman correlation coefficient
RT-PCR	reverse transcription-polymerase chain reaction
Ser	serine
SERMs	selective estrogen-receptor modulators
SSCP	single-stranded conformation polymorphism
T	thymine
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N',N'-tetramethylethylenediamine
UTR	untranslated region
Val	valine
WT	wild-type
X-GAL	5-Bromo-4-chloro-3-indolyl β-D-galacto-pyranoside
XREs	xenobiotic-responsive elements
PAGE	polyacrylamide gel electrophoresis
K_m	Michaelis constant
V_m	maximum velocity

INTRODUCTION

OVERVIEW

The toxicities of aromatic hydrocarbons are mediated through the AH receptor, a transcriptional regulator of genes encoding drug-metabolizing enzymes including *CYP1A1*, *CYP1A2* and *CYP1B1*. The AH receptor also plays a role in the regulation of other genes involved in growth and development.

In humans, the cytochrome P450 species CYP1A1, CYP1A2, CYP1B1 and CYP3A4 are the major enzymes that metabolize 17β-estradiol. CYP1A1 and CYP1A2 metabolize 17β-estradiol to 2-hydroxyestradiol. CYP3A4 is involved in the formation of the estrogenic and genotoxic 16α-hydroxyestradiol while CYP1B1 catalyzes the formation of the genotoxic 4-hydroxyestradiol. Of these cytochrome P450 enzymes CYP1A1, CYP1A2 and CYP1B1 are also known to bioactivate carcinogenic polycyclic aromatic hydrocarbons, heterocyclic aromatic amines and mammary carcinogens in laboratory animals. In humans, exposures to these chemicals have been postulated as a risk factor for various types of cancer including breast cancer.

In humans the genes for *AH* receptor, *CYP1A1*, *CYP1A2*, *CYP1B1* and *CYP3A4* are polymorphic. In light of this, genetic variations in these genes may be related to alterations in estrogen metabolism as well as in activation of environmental procarcinogens, ultimately potentiating mammary carcinogenesis.

My goal was to determine the effects of eight polymorphisms in *AHR*, *CYP1A2* and *CYP1B1* on 17β-estradiol metabolism and the risk of breast cancer. This was achieved through designing, validating and implementing various genotyping assays on DNA samples from two independent populations. Both populations were phenotyped for estrogen metabolite ratio by collaborating laboratories of Dr. Bradlow and Dr. Dewailly. To study the effects on 17β-estradiol metabolism, we obtained DNA from 509 ethnically-mixed

Toronto healthy young women subjects from Dr. Steven Narod. A subset (n=168) of this population has been phenotyped for CYP1A2 activity by *in vivo* caffeine-phenotyping. To determine the association of these genetic polymorphisms and breast cancer risk, we embarked on a breast cancer case-control study of French Canadian women. This population comprises 300 cases and 437 controls; DNA was provided by Dr. Pierre Ayotte.

BREAST CANCER

Breast cancer is one of the leading causes of premature death in North American women. In Canada, breast cancer accounts for 30% of new cancer cases in women and it has been estimated that 1/9 women will develop this disease during their lifetime. Breast cancer incidence in North American women has increased during the past fifty years while the overall rates of mortality from breast cancer have decreased probably due to earlier detection and improved treatment. Despite the tremendous research ongoing for several decades, the etiology of breast cancer remains largely unknown.

Breast cancer is characterized by uncontrolled growth and spread of abnormal cells of the mammary tissue. Breast cancer risk is influenced by both external factors, such as chemicals, radiation and hormones, and internal factors such as hormones and inherited gene mutations. Causal factors may act together or in sequence to initiate or promote carcinogenesis.

The risk of breast cancer increases with age. Women with a personal or family history of breast cancer, biopsy-confirmed atypical hyperplasia, and a long menstrual history are at greater risk. Other risk factors include older age at first birth, use of oral contraceptives or postmenopausal estrogens (Table 1). Worldwide, breast cancer incidence rates appear to correlate with variations in diet, especially fat intake, although a causal role of dietary factors has not been firmly established. Additional factors that may be related to breast cancer risk and are currently being studied include exposures to pesticides and other chemicals, weight gain, induced abortion, physical inactivity and selective estrogen-receptor modulators (SERMs) such as tamoxifen and raloxifene. Research on BRCA1 and BRCA2 susceptibility genes for breast cancer is also in progress.

Table 1

Established risk factors and protective factors for breast cancer in humans.

Risk factors
Family history
Early menarche
Late menopause
Alcohol consumption
Post-menopausal obesity
Hormone replacement therapy

Protective factors
Young age at first full term pregnancy
Prolonged lactation
Exercise

Cancer risk

Overall cancer risk is dependent on the lifetime risk and the relative risk for an individual. Lifetime risk refers to the probability that an individual, over the course of a lifetime, will develop cancer or die from it. Relative risk is a measure of the strength of the relationship between risk factors and the particular cancer. It compares the risk of developing cancer in persons with a certain exposure or trait to the risk in persons who do not have this exposure or trait.

Chemical exposures

Known risk factors for breast cancer only account for less than 50% of breast cancer cases; inherited germ-line mutations occur in no more than 10% of all cases. Environmental factors such as polynuclear aromatic hydrocarbons (PAHs), heterocyclic amines (HAs) and organochlorines are receiving particular attention for their potential role in the etiology of breast cancer. The popular belief is that exposure to these chemicals may increase breast cancer risk.

Development and progression of breast cancer in women is dependent on diverse factors including genetic predisposition, exposure to exogenous and endogenous chemicals. These factors modulate initiation, promotion and progression of the disease. Several exogenous chemical compounds have been proposed as initiators of chemical-induced mammary carcinogenesis in humans. These compounds include aryl hydrocarbons and aromatic amines.

Polynuclear aromatic hydrocarbons

Endogenous and exogenous chemicals can potentially play important roles during the multistep development of mammary tumors. Several classes of environmental chemicals have been demonstrated to induce mammary cancer in rodent models; and these chemicals may act as initiators (*1, 2*). Polynuclear aromatic hydrocarbons, such as 7,12-dimethylbenz[*a*]anthracene (DMBA), benzo[*a*]pyrene (BaP) in cigarette smoke, and N-methylnitrosourea, induce

mammary cancer in rodent models. Their effectiveness is dependent on the timing of exposure (*2*). For instance, DMBA is most effective as a carcinogen when administered to 30-55 day-old female Sprague-Dawley rats (*3*). The carcinogenic efficacy of DMBA is abolished when administered outside of this critical window of exposure and the resulting tumor incidence is markedly decreased. Little is known about the timing of initiation or the detection of initiators in humans. Several studies suggested that the critical period in females may be between age of menarche and first full-term pregnancy (*3*). For example, exposure to ionizing radiation before 19 years of age increased the incidence of breast cancer in women; however, the incidence did not increase when women were exposed after pregnancy or lactation (*2, 3*).

Several studies reported that breast tissue samples from breast cancer patients contained significantly higher levels of aromatic DNA adducts than samples obtained from controls (*4, 5*). Adducts may serve as initiators of human mammary carcinogenesis. Another study suggested that benzo[*a*]pyrene inhibits BRCA-1 expression of human MCF-7 breast cancer cells (*6*). In addition, a recent study conducted by Health Canada showed that passive and active smoking are related to breast cancer with statistically significant dose-response relationships (*7*).

There are only limited reports on the role of industrial chemicals and environmental contaminants as risk factors for breast cancer in women. Lewis-Michl and coworkers (*8*) assessed the relationship between breast cancer incidence in women residing in Nassau and Suffolk Counties on Long Island and their residential proximity to industrial facilities. The incidence of breast cancer significantly increased in postmenopausal women in Nassau County who were potentially exposed to chemical industrial plants. Another study reported a significant relationship between premenopausal breast cancer and occupational exposure to benzene and PAHs (*9*).

Organochlorines

Exposure to organochlorines has also been suggested as a possible cause of breast cancer (*2, 3, 10-14*). The link between exposure to organochlorines and breast cancer has been the subject of intense research due to their lipophilicity and environmental persistence. This chemical group is characterized by its long half-life in the environment and *in vivo*. It consists of industrial compounds such as polychlorinated biphenyls (PCBs) as well as agricultural pesticides such as chlordane and DDT, which were extensively used in the past and can still be detected in human tissues. Upon consumption, these organochlorines accumulate in various fat tissues including adipose tissue (*15, 16*), blood lipids (*17*), breast milk (*18*) and breast adipose tissue (*15*). Case-control studies of organochlorine levels in serum or adipose tissue and breast cancer risk have yielded conflicting results regarding the connection between exposure to these chemicals and breast cancer. Early studies in humans generally supported the relationship between breast cancer risk and organochlorine exposure (*13, 19-21*), in particular with p,p-DDE, the main metabolite of DDT. However, recent studies involving larger sample sizes failed to detect such association (*22-26*). The more recent studies did not find any overall association between risk of breast cancer and exposure to DDT (*27*) or PCBs (*13, 27, 28*).

PCBs are broad-acting toxicants occurring in complex mixtures. Thus, accurate risk assessment may be hampered by incomplete residue data. Furthermore, exposure to more labile mixtures may contribute to adverse outcomes without leaving a residue record. Moreover, variability in methods used to assess exposure levels may also lead to inconsistent results between studies. Finally, depending on the specific PCB congener, its serum $T_{1/2}$ and lipophilicity, serum levels of PCBs may or may not be correlated with concentrations in the breast tissue. In fact, a study comparing 17 organochlorines and PCB residues in human breast adipose tissue and serum suggested that serum levels of most PCBs analyzed did not correlate with the concentration found in the breast adipose tissue (*29*). Interestingly, a recent

case-control study conducted in Ontario demonstrated a clear association between breast cancer risk and PCB concentrations in breast adipose tissue (*15*).

Heterocylic amines

The heterocylic amines are a family of procarcinogenic compounds formed during the pyrolysis of proteins found in the human diet. Most, but not all of the major heterocyclic amines have been shown to be carcinogens in animals (*30, 31*). In fact, mutations of the Ha-ras and p53 genes have been found in 2-amino-1-methyl-6-phenylimidazole[4,5,-*b*]pyridine- (PhIP) induced rat mammary carcinomas (*32*).

In humans, great emphasis has been given to heterocyclic amines to which humans are continuously exposed in an ordinary lifestyle. Primary cultures of human mammary epithelial cells have been shown to bioactivate heterocyclic amines and form heterocyclic amine-DNA adducts with substantial interindividual variation (*30, 33, 34*). Results of a case-control study in Uruguay suggested that meat intake and chemicals formed during the cooking process may be strong risk factors in human breast carcinogenesis (odds ratio=3.34) (*35*). Another study by Zheng *et al.* also showed a relation between breast cancer risk and intake of well-done meat+ (*36*).

Involvement of cytochrome P450 enzymes in chemical-induced breast cancer

Exogenous chemicals can affect different stages of multistep breast cancer development. Cytochrome P450 enzymes are phase I enzymes responsible for the metabolism of numerous environmental chemicals as well as the most potent physiologic estrogen, 17β-estradiol. P450 enzymes can bioactivate or deactivate environmental procarcinogens. For example, the heterocyclic amine 2-amino-3-methylimidazo[4,5]quinoline (IQ) is a potent mutagen and is a mammary carcinogen in rodents. In man, hepatic activation of

IQ is carried out by CYP1A2 (*37*). Williams and coworkers (*37*) have shown that CYP1A1 and CYP1B1 are likely the activating enzymes responsible for IQ-DNA adduct formation in human mammary epithelial cells. It has been proposed that individual variations in levels of CYP enzymes in the mammary duct epithelial cells will affect individual susceptibility and that N-hydroxy-IQ produced in the liver may be O-acetylated to adduct-forming products in breast epithelial cells (*37*).

Benzo[*a*]pyrene (BaP), an ubiquitous environmental, tobacco and dietary carcinogen, has been implicated in human cancer etiology (*38, 39*) Both CYP1B1 and CYP1A1 significantly metabolize BaP to the postulated ultimate 7,8-diol epoxide mutagenic species (*40*). Indeed, a recent study reported that reactive metabolites of PAHs (*41*) specifically target mutational hotspots of the p53 tumor suppressor gene. Such DNA damage can abrogate the p53-dependent pathways involved in important cellular functions.

It has been shown that in rats, chemical inducers or suppressors of drug-metabolizing enzymes, when coadministered with procarcinogens such as PAHs, can act as cocarcinogens or anticarcinogens depending on whether PAHs are metabolized to their ultimate carcinogens. Furthermore, adduct formation following DMBA exposure can also be modulated by inducers of various cytochrome P450 genes. This is exemplified by the aryl hydrocarbon (AH) receptor agonists such as benz[*a*]anthracene which induces *CYP1A1* and *CYP1B1* gene transcription and DMBA adduct formation through enhanced DMBA bioactivation (*3*).

Hormone exposure

Considerable epidemiological and experimental data implicate estrogens in the etiology of human breast cancer (*42, 43*). Studies in animal models repeatedly demonstrate that various estrogens (17β-estradiol and catechol estrogens) can induce and promote mammary tumorigenesis in rodents while removal of the animals' ovaries or administration of anti-

estrogens results in the opposite effect (*44*). In animal models, treatment with 4-hydroxyestradiol but not 2-hydroxyestradiol induces renal cancer in Syrian hamsters (*45, 46*). 4-hydroxyestradiol also is capable of inducing uterine adenocarcinoma, a hormonally related cancer in mice.

The well-established risk factors for breast cancer are shown in Table 1. Early menarche or late menopause prolongs estrogen exposure by maximizing the number of ovulatory cycles experienced over time. Alcohol consumption is associated with a linear increase in breast cancer incidence in women who consume more than 2 drinks per day. It has been suggested that alcohol consumption can increase plasma estrogen. Physical activity and prolonged lactation can reduce the number of ovulatory cycles and therefore, circulating ovarian hormone levels (*47-50*). In post-menopausal women, the major source of estrogen originates from the conversion of androstenedione to estrone in adipose tissue. Consequently, post-menopausal obesity increases breast cancer risk through increased production of estrogen. In this regard, there is a basis for the association between risk of breast cancer and serum androgens, as androgens provide a large pool of substrate for conversion to 17β-estradiol (E2) via the action of aromatase in breast tissue (*51*).

International studies comparing estrogen levels in populations at differing breast cancer risk have supported the role of estrogens, in particular estradiol, in the pathogenesis of breast cancer. Two recent studies characterized the relationship between serum estradiol and lower breast cancer risk in Asian compared to North American populations. In one study, Berstein and coworkers (*52*) found that premenopausal subjects residing in Los Angeles have 20% higher E2 levels compared to Shanghai pre-menopausal controls (*52*). Another study involving the comparison of post-menopausal women in Los Angeles to age-matched Japanese women showed that women in Los Angeles have 36% higher E2 levels than Japanese controls (*53*). The reasons behind such differences remains unknown but may be explained by genetic differences affecting 17β-estradiol biosynthesis. Collectively, these results show

a possible link between elevated E2 levels and breast cancer. Few studies have assessed prospectively the risk of breast cancer according to the levels of hormones in blood. The results from other studies have varied, perhaps because of the difficulty in measuring hormone levels in postmenopausal women. The data from prospective studies have recently been combined in a systematic review and meta-analysis by Thomas *et al.* (*54*). The authors reported that there was no substantial heterogeneity in results among the prospective studies. These authors observed a statistically significant higher risk of breast cancer among women with higher levels of serum estrogen. More specifically, post-menopausal women who subsequently develop breast cancer have a 15% higher mean serum estradiol concentration than unaffected women ($p= 0.0003$). Exposure to catechol estrogens has also been postulated as a factor for mammary cancer. For instance, examination of microsomal 17β-estradiol hydroxylation in human breast cancer showed significantly higher 4-hydroxylation activity in tumor tissues than in adjacent normal breast tissues (*55*).

Cytochrome P450-mediated estrogen metabolism

17β-estradiol (E2) is the most potent naturally occurring estrogen in mammals and is formed by various tissues including the ovary, placenta, testis, adrenal cortex and to a lesser extent, adipose tissue. CYP17 is involved in steroidogenesis and synthesis of 17β-estradiol by catalyzing two distinct steps in steroid hormone production.

In adipose tissue, androstenedione is aromatized to estrone (E1) by CYP19 aromatase and then converted to 17β-estradiol. The metabolic interconversion between estradiol and their respective estrone (E1) metabolites is catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD). Estrone has considerably less biological activity than the parent hormone. In the human body, 17β-estradiol and estrone primarily undergo oxidative metabolism

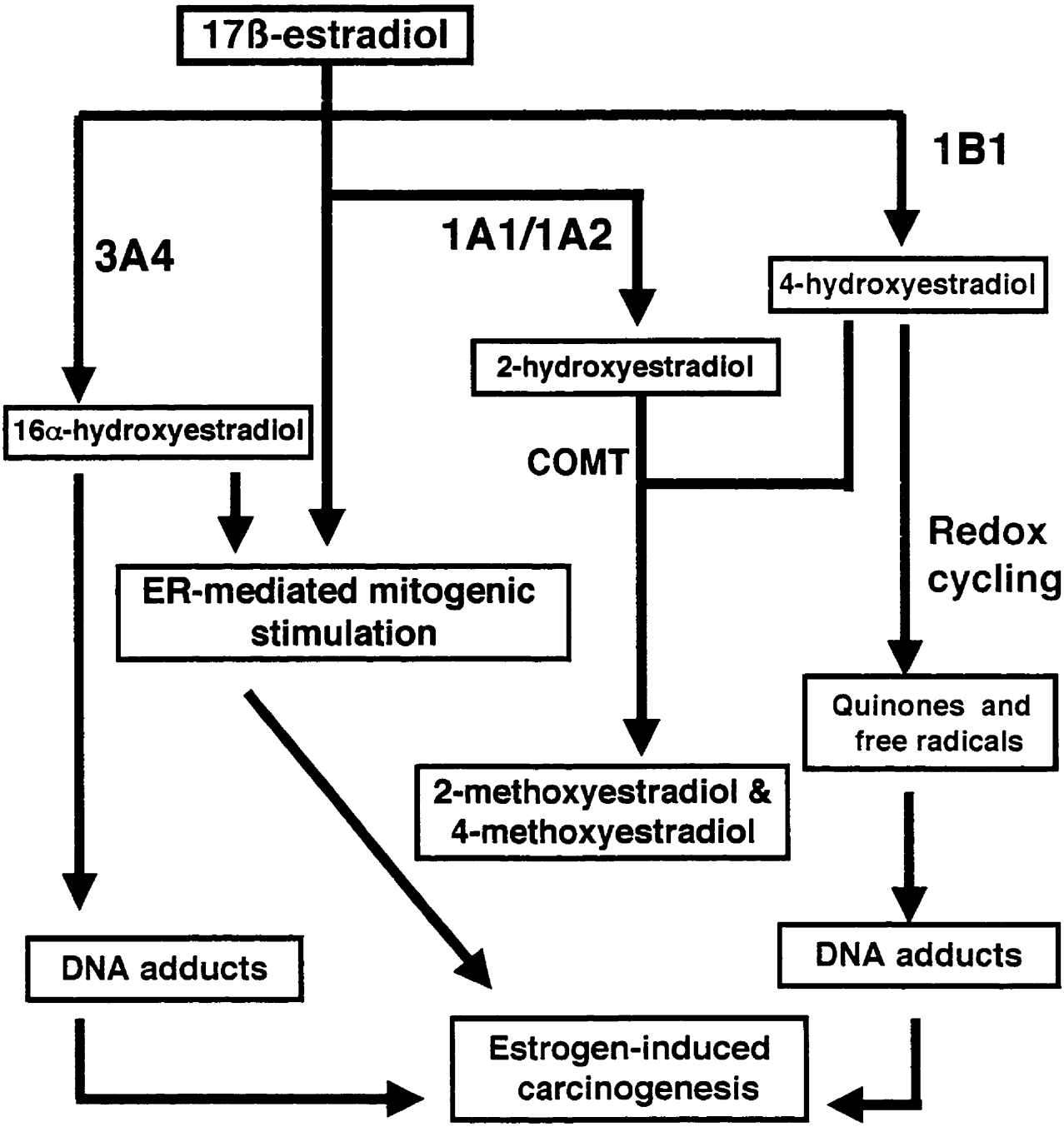
mediated by P450 enzymes. CYP1A1 and CYP1A2 preferentially metabolize 17β-estradiol to 2-hydroxyestradiol (*56-58*), a catechol metabolite devoid of estrogenic activity. Alternatively, 17β-estradiol and estrone can be oxidized by CYP1B1 to the 4-hydroxyestradiol catechol metabolite (Figure 1). This catechol estrogen retains significant estrogenic activity (*59-61*) and also is genotoxic (*55, 62*). Moreover, the 17β-estradiol parent hormone can be hydroxylated by CYP3A4 at the C16 position to yield 16α-hydroxyestradiol (*56*). 16α-hydroxyestradiol is a uniquely reactive estrogen also believed to be responsible for the carcinogenicity of estrogens in their target organs (*62*).

Catechol estrogens are known to be carcinogenic (*63*) via free radicals generated through redox cycling (*64, 65*). Fortunately, these catechol estrogens can be detoxified to 2- and 4-methoxyestradiol via catechol-O-methyltransferase (COMT). Defects in this methylation pathway favor shunting of these metabolites toward redox cycling thereby generating potentially mutagenic free radicals that can damage DNA and other cell structures (*60, 63*). In fact, an association was found between the allele coding for low COMT activity and breast cancer risk (*66*). Since 2-hydroxyestradiol is the preferred substrate of COMT, 4-hydroxyestradiol is accumulated in cells before detoxification by COMT. It is believed that accumulation of 4-hydroxyestradiol and its entrance into the redox cycling pathway confers its carcinogenic potential. In this regard, 4-hydroxyestradiol is a strong renal carcinogen in Syrian hamsters (*45*) whereas 2-hydroxyestradiol does not induce any tumors at similar concentrations. Furthermore, chronic administration of quercertin, a commonly ingested dietary polyphenol that is a potent inhibitor of COMT, significantly increased the severity of estradiol-induced kidney tumor formation in Syrian hamsters (*46, 61*).

Although 17β-estradiol 4-hydroxylation is a relatively minor pathway in the liver for the formation of catechol estrogens, significant estrogen 4-hydroxylase activity by CYP1B1 has been observed in several extrahepatic

Figure 1

Cytochrome P450-mediated estrogen metabolism.



estrogen-responsive tissues including the breast. It should be noted that CYP1B1-dependent activities can be induced via the AHR pathway and the activity of CYP1B1 is found to be higher in breast cancer than in adjacent normal tissue (*55, 67*). Interestingly, a recent epidemiologic study involving a population of Chinese women in Shanghai revealed an association between the *CYP1B1*3* polymorphism and breast cancer risk (*68*). In contrast, expression of CYP1A2 is abundant but confined to liver, making 2-hydroxylation the predominant metabolic pathway of 17β-estradiol. Taken together, these P450 enzymes are likely involved in the pathogenesis of breast cancer (Figure 1).

REGULATION OF P450 ENZYMES BY THE AHR PATHWAY

AHR (aryl hydrocarbon receptor) became of interest to the science community when the soluble AHR was observed to play key roles in the adaptive metabolic response to PAHs (*69*) and in the toxic mechanism of halogenated dioxins and dibenzofurans (*70, 71*). Many familiar environmental toxicants are agonists of the AHR; PAHs are byproducts from incomplete pyrolysis of various carbon sources typically found in cigarette smoke, charbroiled foods and diesel exhaust (*72, 73*). Halogenated aromatic hydrocarbons (HAHs) such as TCDD (2,3,7,8,-tetrachlorodibenzo-*p*-dioxin) are trace contaminants in industrial processes that involve chlorination in the presence of phenolic substrates. Both PAHs and HAHs have carcinogenic potential. HAHs have been more thoroughly studied due to their remarkable environmental persistence and acute toxicity. Antagonists of AHR have been identified. These chemicals include resveratrol and α-naphthoflavone.

The cytosolic form of unbound AHR associates with two molecules of 90-kDa heat shock protein (Hsp90) (*74*). Other factors have been shown to interact with the AHR-Hsp90 complex including the AHR-interacting protein (AIP) (*75, 76*) and c-Src protein tyrosine kinase (*77*).

TCDD is an ubiquitous environmental contaminant and a potent AHR agonist. In the laboratory, TCDD has been the prototype agonist for the study of AHR for many years since it is poorly metabolized and has high affinity for the AHR. Binding of TCDD or other agonists to AHR results in a receptor transformation process where changes in AHR conformation lead to subsequent translocation into the nucleus.

In the nucleus, release of Hsp90 is followed by interaction of AHR with its partner protein, the aryl hydrocarbon receptor nuclear translocator (ARNT) via the basic-helix-loop-helix (bHLH) motif found in both proteins. This ligand-AHR-ARNT complex recognizes and bind to specific dioxin-responsive elements (DREs; also known as AHREs or XREs) which regulate transcription of specific genes. The AHR-ARNT complex is thought to disrupt local chromatin structure, thus increasing the accessibility of transcription factors to bind to downstream promoter elements and initiate transcription (*78*) (Figure 2).

AHR-mediated transcriptional up-regulation remains the most clearly understood aspect of AHR biology and much of our understanding of this phenomenon comes from the extensive analysis of the regulatory regions that govern the expression of DRE-containing genes like *CYP1A1*, *CYP1A2* and *CYP1B1* xenobiotic-metabolizing enzymes (*79*). Of these genes, *CYP1A1*, which is predominantly responsible for inducible aryl hydrocarbon hydroxylase (AHH) activity, has been most extensively studied. In addition to the cytochrome P450 enzymes, other genes regulated by similar mechanisms are phase II conjugating enzymes such as UDP-glucuronosyl transferase 1*06 (*80, 81*) and glutathione S-transferase Ya subunit (*82*), as well as other metabolizing genes including NAD(P)H:quinone reductase 1 and 2 (*83*) and class 3 aldehyde dehydrogenase (*84*). All these genes are regulated in part by a functional DRE and encode enzymes with importance in the metabolism of foreign and endogenous chemicals. Compared to the wild-type *Ahr* +/+ mice, *Ahr* -null mice express very low levels of CYP1A1 in the skin and CYP1A1 is not detectable in liver. In contrast, expression of CYP1A2 was not observed in the skin but was constitutive even in the liver of *Ahr*-null mice (*85*). AHR-regulated P450

Figure 2: Aryl hydrocarbon receptor and its regulated genes.

enzymes appear to have metabolic activity toward PAHs. It is possible that the AHR pathway evolved partly to handle and reduce an organism's body-burden of chemicals with polycyclic aromatic structures with capacity to intercalate into nuclear and mitochondrial DNA and act as nonspecific inhibitors of enzymatic reactions (*84*).

Other genes that encode factors with a broad range of effects on cellular proliferation and differentiation also are targets of AHR regulation. Such genes include epidermal growth factor receptor (EGFR) (*86*), interleukin 1β (*87*), transforming growth factor α and β2 (*88*) and plasminogen activator inhibitor 2 (*89*). Although the relationship between TCDD toxicity and induction or suppression of these genes has not been well-established, disregulation of factors that play a role in generating molecular signals to regulate cell proliferation and differentiation may be responsible for many of the detrimental effects of TCDD. TCDD toxicity observed in animal models includes porphyria, tumor promotion, lymphoid involution, epithelial hyperplasia and metaplasia, teratogenesis and a severe wasting syndrome that ultimately leads to death (*71*). In humans, a wide variety of cellular processes have been shown to be affected by TCDD. In addition to acute toxic effects observed in animals, dioxin exposure also results in tumor promotion, immunosuppression, cardiac dysfunction and disregulation of lipid metabolism and chloracne (*71, 90*).

Cytochrome P450 gene expression and mammary chemical carcinogenesis

It is clear that members of the cytochrome P450 supergene family such as CYP1A1, CYP1A2 and CYP1B1 are responsible for bioactivation of numerous procarcinogens to ultimate carcinogens in the body. These procarcinogens include the dietary mutagens, organochlorines and PAHs. The P450 profile in a cell determines the capacity of the cell to form reactive metabolites. Furthermore, environmental factors influencing expression of P450 will alter the fate of procarcinogens in a cell.

The human liver plays a prominent role in metabolism of ingested and circulating environmental toxicants by virtue of various P450 enzymes found in the tissue. CYP1A1 is not constitutively expressed in any tissue; however, its expression is inducible by dioxins and PAHs in almost all tissues and cell lines examined including the breast. Human CYP1A2 is exclusively expressed in liver (*91*). It is constitutively expressed and can be further induced by environmental chemicals such as PCBs and PAHs. With its substrate specificity and high constitutive expression, CYP1A2 plays an important role in bioactivation of dietary mutagens (eg. heterocyclic amines).

Mouse carcinogen bioassays on *Cyp1a2*-null mice revealed that injection of the food mutagen, 4-aminobiphenyl (4-ABP), resulted in only marginal differences between *Cyp1a2*-null and wild-type mice that develop liver tumors (*92*). This indicated that CYP1A2 is not the only enzyme responsible for the activation of this carcinogen. This result parallels those in previous *in vitro* studies which suggested that human CYP1A1 and CYP1B1 also can catalyze N-hydroxylation of heterocyclic amines (*93, 94*). Absence of CYP1A2 expression in the *Cyp1a2*-null mice may enhance channeling of heterocylic amines through bioactivation pathways catalyzed by CYP1A1 and CYP1B1. Overall, reactive metabolites generated by hepatic CYP1A1 and CYP1A2, if not detoxified completely, may reach peripheral tissues and initiate carcinogenesis locally.

In contrast, human CYP1B1 expression is primarily extrahepatic and is constitutive in the human mammary tissue. CYP1B1 expression is inducible by PAHs and PCBs. CYP1B1 shares many properties with CYP1A1. It metabolically activates PAHs and is induced by TCDD in many tissues and cell lines. The protein can efficiently metabolize DMBA, a highly potent PAH procarcinogen. CYP1A1 and CYP1B1 are responsible for the metabolic activation of PAHs to their ultimate carcinogens. Analysis of the *Cyp1b1*-null mice revealed that they were completely resistant to DMBA-induced tumors while wild-type mice developed several malignant cancers when administered with high doses of DMBA (*92*).

In the breast, human mammary epithelium and breast tumor tissue highly express CYP1B1 while expression of CYP1A1 is generally low and may not be detectable in some individuals (*67, 95-98*). Nevertheless, expression of both genes can be induced by the PAHs and PCBs through the AH receptor pathway (*99*). Therefore, these P450 enzymes are critical in the generation of ultimate carcinogens locally thus promoting subsequent *in-situ* tumorigenesis.

P450 expression and hormone-related breast cancer

Circulating 17β-estradiol (E2) undergoes significant first-pass metabolism by hepatic CYP1A2 and CYP3A4. While CYP1A2 inactivates E2 to the "protective" 2-hydroxyestradiol, CYP3A4 metabolically activates E2 to the estrogenic and genotoxic 16α-hydroxyestradiol. By circulating in the blood stream, 16α-hydroxyestradiol can reach peripheral estrogen-responsive tissues such as the breast and exert its carcinogenic effects. Inducible hepatic CYP1A1 similarly metabolizes E2 to 2-hydroxyestradiol thus, in part contributing to hepatic clearance of circulating E2 and environmental contaminants. In response to PAHs, PCBs and other environmental chemical inducers, induction of human mammary CYP1A1 and CYP1B1 expression further augments local activation or deactivation of E2. Given the regioselectivity of CYP1A1 and CYP1B1 in E2-hydroxylation, opposing actions of these two enzymes and the net effect may either potentiate or decrease breast cancer development.

GENETIC POLYMORPHISM

Definition and implications

Genetic polymorphism is defined as the occurrence in the same population of multiple discrete allelic states of which at least two have frequency of 1% or more (*100*).

Over the years, genetic alterations from single point substitution to deletions and other chromosomal aberrations have been reported to be responsible for the development of various genetic disorders and diseases including cancer. Genetic factors that contribute to cancer susceptibility include rare but highly penetrant dominant mutations in addition to more common genetic polymorphisms that influence individual response to environmental exposures (*101*). The BRCA genes (BRCA1 and BRCA2) constitute an example where rare but highly penetrant mutations have profound effects on breast cancer risk. Genetic alterations in genes that control cell proliferation (H-*ras*) or regulate cellular adaptation to chemical-mediated damage (*p53*) have been implicated in a variety of tumors (*102, 103*). These traits may contribute to high overall lifetime cancer risks of an individual; however, they only account for a small fraction of cancer incidence (*104*).

The more common genetic polymorphisms that confer modest elevations in individual risk are also important determinants of individual susceptibility to cancer or other disorders since interaction of several polymorphic genes may synergistically act to facilitate the development of cancer. In particular, those polymorphisms that influence metabolic activation or detoxification of exogenous and endogenous carcinogenic chemicals will influence individual susceptibility to cancers caused by exposures to these chemicals. For instance, a Japanese case-control study demonstrated that certain genotypes of *CYP1A1* and *GST1* genes act synergistically to increase the relative risk of lung cancer (*105*). Furthermore, genetic polymorphisms in genes encoding drug-metabolizing enzymes will also affect individual ability to metabolize certain drugs and thereby create a predisposition to clinical adverse drug interactions and idiosyncratic reactions. Genetic polymorphisms of the *CYP2D6* locus account for adverse effects as a result of impaired biotransformation of the therapeutic agent debrisoquin. This has led to a whole field of research in pharmacogenetics, which is the study of unusual drug responses that have a hereditary basis.

METHODS FOR DETECTION OF GENETIC POLYMORPHISMS

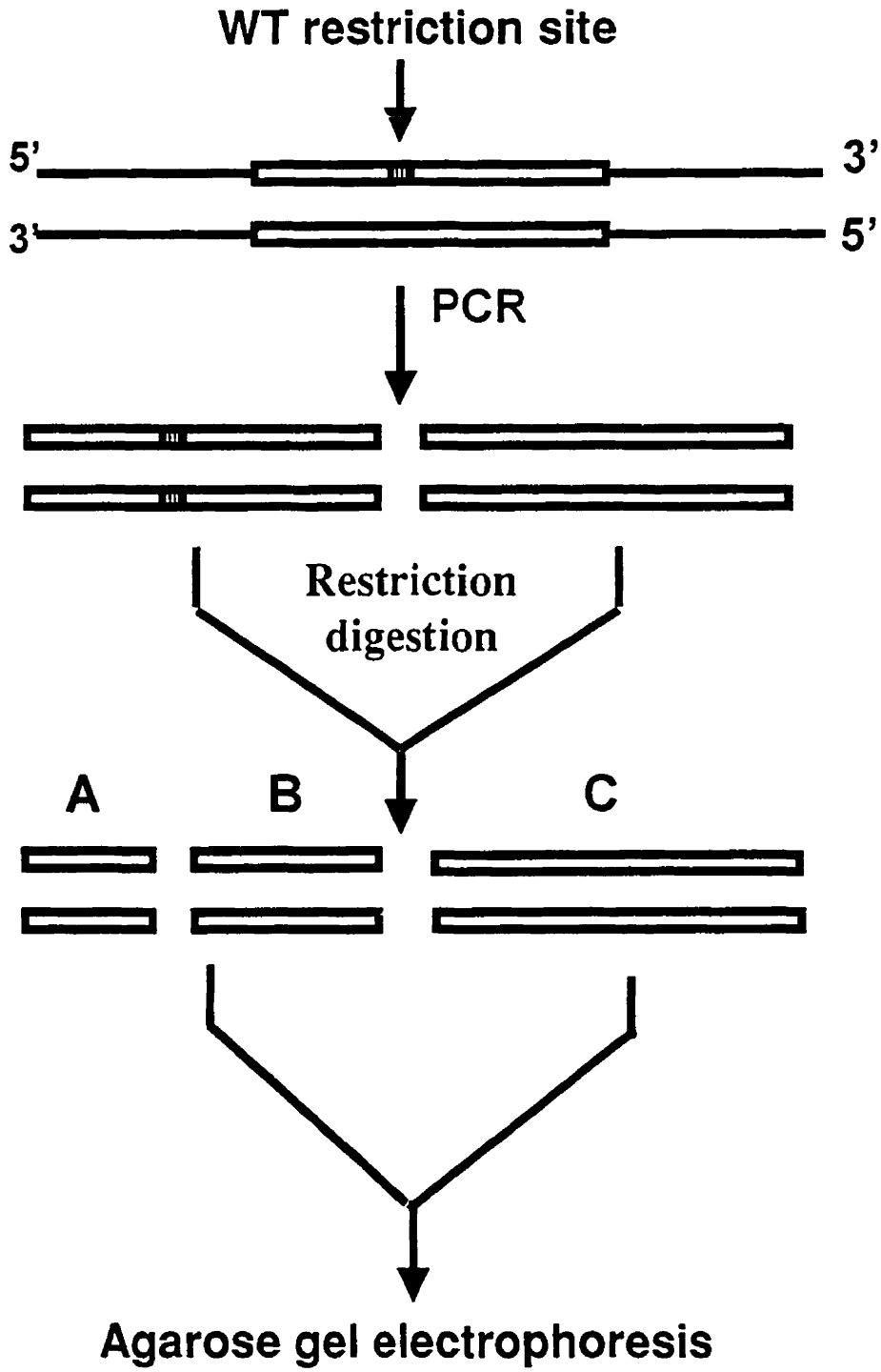
DNA sequencing is considered the gold standard and remains the definitive procedure for detection and identification of genetic polymorphisms. Nevertheless, there are a handful of screening methods that are more advantageous with relative ease of performance, enhanced detection rate and economically inexpensive. These methods are consequently more useful when high-throughput screening is required.

Restriction fragment length polymorphism (RFLP)

The principle of this method relies on the single base substitution that occurs within a restriction enzyme recognition site such that different allelic variations either retain or abolish the recognition and cleavage of this site by restriction enzymes. This in turn generates distinct patterns that define the corresponding genotypes (Figure 3). RFLP analysis of specific target DNA has been facilitated by the advent of polymerase chain reaction (PCR). The DNA fragment of interest potentially containing the polymorphism is first selectively amplified by PCR followed by restriction endonuclease digestion. The resulting pattern is most conveniently visualized by agarose gel electrophoresis. Alternatively, variable restriction sites can be monitored by Southern blot analysis. In this case, individual genomic DNA is digested by multiple restriction endonucleases followed by transfer onto a nitrocellulose membrane. This membrane is subsequently hybridized with a ^{32}P-radiolabled DNA probe that is complementary to the specific gene under analysis. RFLP analysis is relatively straightforward and efficient; however, RFLP is less effective in the identification of unknown polymorphisms. The usefulness of this technique is dependent on whether the unknown base substitutions introduce or eliminate a restriction site.

Figure 3

Restriction fragment length polymorphism (RFLP).



Denaturing gradient gel electrophoresis (DGGE)

Double-stranded DNA is electrophoresed through a gradient of increasing concentration of urea or formamide denaturing agents or of increasing temperature (*106*). Under these conditions regions of DNA dissociate according to their melting temperature; dissociation of strands results in a decrease of gel mobility. A 1-bp difference between two double-stranded DNA homoduplices can change the melting temperature by 1°C or more (*107*). This difference in melting temperature results in variable electrophoretic mobility once the fragments reach the portion of the gel where denaturation occurs at their respective denaturant concentration or melting temperature (Figure 4). For this reason, DGGE is sufficiently sensitive to detect single nucleotide polymorphisms between wild type and variant DNA fragments which differ by one base. To determine the location of base substitutions, DNA sequencing analysis must follow.

Single-stranded conformation polymorphism (SSCP)

SSCP, first described by Orita *et al.* (*108*), entails electrophoresis of denatured single-stranded DNA in a non-denaturing gel. SSCP relies on the principle that the mobility (and therefore migration pattern) of single-stranded DNA molecules is dependent on their structure and size by virtue of the secondary and tertiary structures they take on. These structures are formed by base pairing between nucleotides within each strand, and thus are determined by the DNA sequence of each strand. Discrimination of DNA molecules differing by a single nucleotide is made possible by this method (Figure 5). PCR-coupled SSCP is technically simple and effective in detecting polymorphisms in short DNA fragments of 100-300 bp. Similar to DGGE, once differences in the migration pattern are detected, DNA sequencing is required to ascertain the precise location of polymorphism. Modifications of SSCP have been developed to enhance detection of various polymorphisms. Gel temperature, glycerol and acrylamide concentration and inclusion of

Figure 4

Denaturing gradient gel electrophoresis (DGGE).

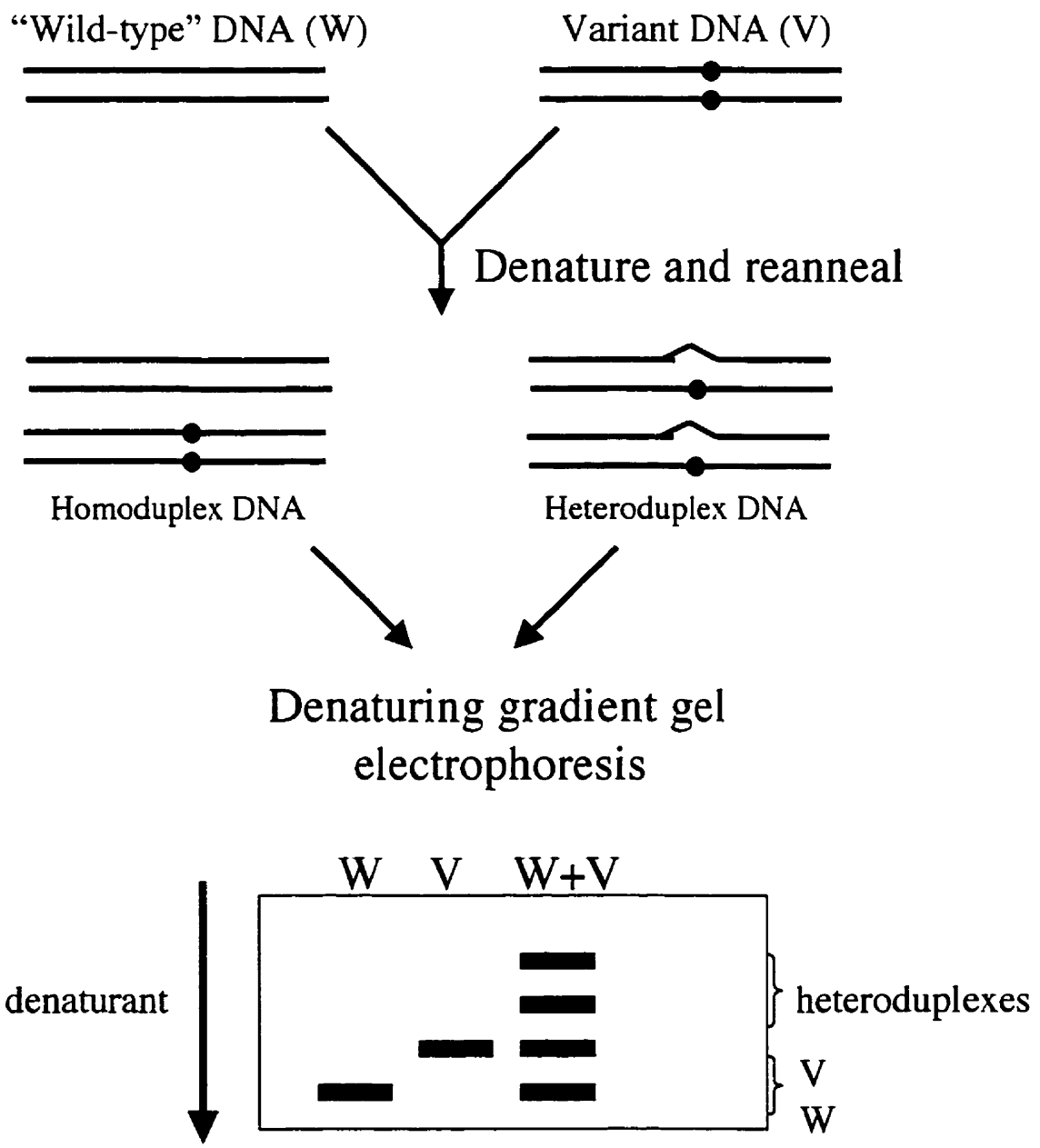
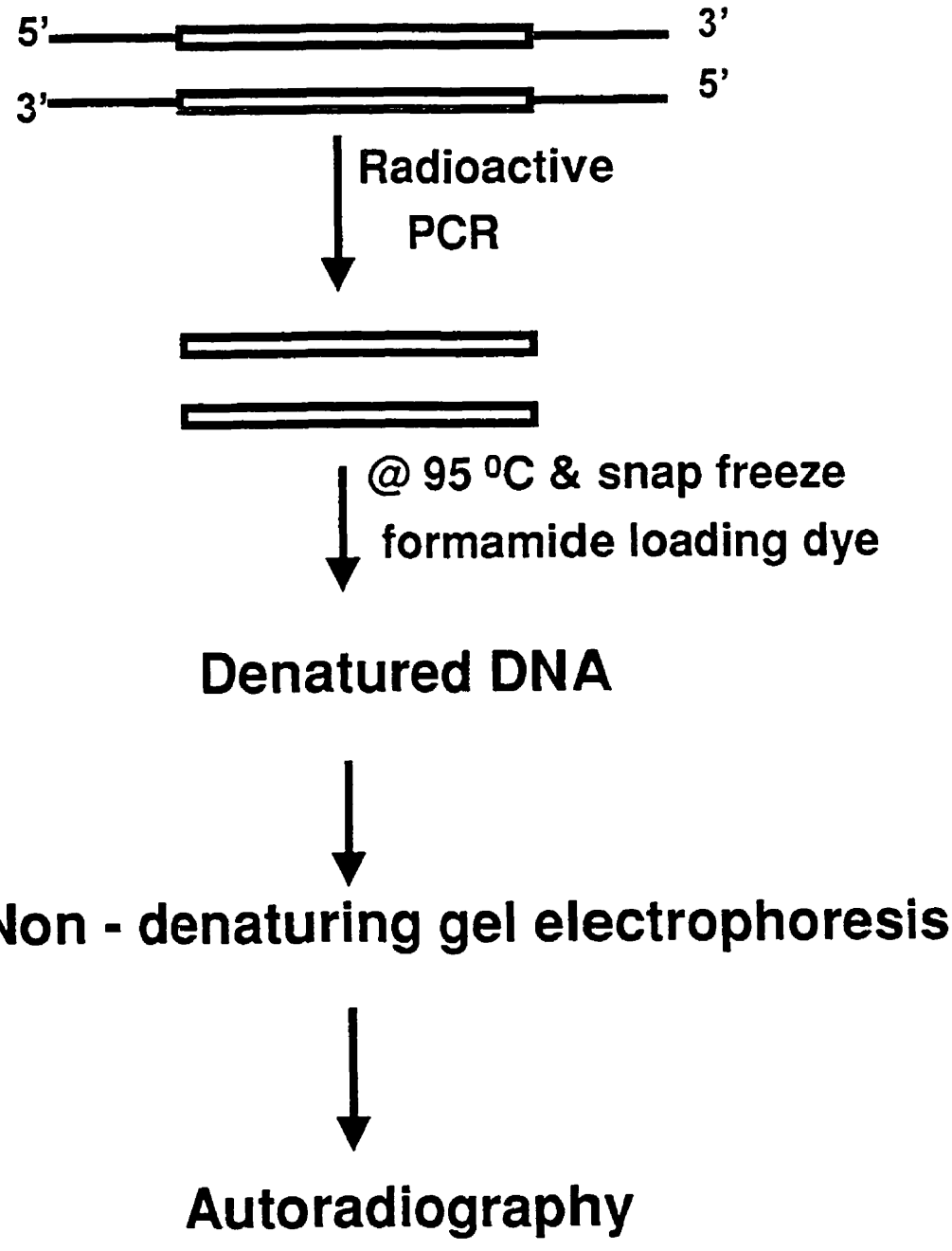


Figure 5

Single-stranded conformation polymorphism (SSCP).



denaturants are among the variables that can be changed to obtain different conditions which affect the separation patterns of the single-stranded DNA. Each polymorphism is optimally detected under specific conditions, and thus requires optimization.

Other methods for polymorphism detection

In recent years, there have been major advances in elucidating the relationship between genetic factors and human disease outcomes. This would not have been possible without the development of a vast array of genotyping approaches allowing the determination of individual DNA sequence differences for a particular trait. This has been valuable for risk assessment. High-throughput screening methods for single nucleotide polymorphisms, small deletions or insertions suited for diagnostic applications or analysis of allelic differences in hereditary disease are of importance in the understanding of environment-susceptibility interactions, particularly in the context of human cancers.

Cleavase fragment length polymorphism (CFLP) analysis is a relatively recent method (*109*). Upon refolding and renaturation of single strands of DNA, sequence-dependent secondary structures are formed. These structures consist of hairpin-like conformations which can be cleaved by cleavase I endonuclease that specifically cleaves at the junction between single-stranded and double-stranded DNA. This in turn generates a collection of fragments unique to that strand of DNA. In this way, different changes in the sequence will alter the secondary structures formed and the distinct CFLP pattern can be electrophoretically detected.

More recently, with the advance of DNA chips, the principle of allele-specific oligonucleotide (ASO) hybridization has been extended to a screening method for polymorphisms (*110*). Oligonucleotides of known sequences are immobilized on appropriate surfaces. Given a consensus sequence, a set of four probes can be defined for each nucleotide in the target. Labeled target sequences are hybridized to the immobilized oligonucleotides. Because of

high resolution, fluorescent dyes have been used for detection. Many commercial systems have been developed based on this principle.

Another method for rapid screening is allele-specific amplification (ASA). PCR is performed in two parallel reactions. In the first reaction, the 5' primer is complementary to the wild-type sequence. Conversely, the second reaction employs a 5' primer that is complementary to the mutant or polymorphic sequence. Based on the assumption that elongation occurs only when primer and target sequences match completely, only one allele of either mutant or wild-type DNA is amplified per round of PCR. The mismatch and consequent mispairing is either located at the far 3'-end of the primer or within the primer preventing primer annealing.

The principle of single nucleotide primer extension is similar to that of ASA. This method is based on the extension of the 3'-end of the primer by a single labeled nucleotide (*111, 112*). Extension by the differentially labeled nucleotides will help identify the nucleotide located at the polymorphic site.

Finally, oligonucleotide ligation assay (OLA) is another method commonly used. In this approach, two primers are hybridized to complementary stretches of DNA at sites of possible polymorphisms or mutations. Primers are designed such that the 3'-end of the first primer is located immediately adjacent to the 5'-end of a second primer. When the 3'-end of the first primer matches perfectly with the target DNA, both primers can be ligated by DNA ligases (*113, 114*). Otherwise, no ligation will be obtained when a mismatch occurs at the 3'-end of the first primer.

At present, RFLP and its modified approaches are technically simpler and sufficiently sensitive for the detection of low-abundance alleles in great excess of predominant alleles.

AHR AND CYTOCHROME P450 POLYMORPHISMS IN RELATION TO CANCER

From the point of view of cancer biology, an intriguing finding by Shimizu and coworkers provides direct evidence for the involvement of AHR in carcinogenesis (*85*). *Ahr*-knockout mice were totally protected from tumor formation induced by subcutaneous injection of BaP. Interestingly, all *Ahr*-positive mice, with either both or one *Ahr* allele, developed tumors under the same treatment. This clearly establishes that normal AHR function is critical in determining the carcinogenicity of BaP.

As a consequence of remarkably broad substrate specificity, cytochrome P450 enzymes play key metabolic roles in several aspects of cancer. Importantly, the majority of endogenous and exogenous chemical carcinogens require biotransformation to activated forms to be carcinogenic. The cytochrome P450 enzymes are preeminent in catalyzing such bioactivations of procarcinogens as well as detoxification of carcinogens. This superfamily of enzymes is also responsible for the biotransformation of therapeutic drugs, thereby either enhancing or diminishing the efficacy of the drugs.

Many P450 genes are polymorphic generating variant forms of each enzyme. Polymorphisms associated with absent or low activity lead to consequent phenotypic alterations in susceptibilities to carcinogens. Genetic variations in noncoding regions that affect gene expression may also predispose an individual to altered cancer susceptibility. Variable expression of CYP1B1 between different types of tumor and their respective normal tissue has been documented (*67*). Moreover, epidemiologic studies suggest an elevation in risk of colon cancer and bladder cancer in subjects with increased CYP1A2 activity and/or inducibility (*115*). Furthermore, CYP1A1 Val462 (*CYP1A1*2C*) homozygote was reported to occur two to three fold more frequently in the lymphocytes of Japanese lung cancer patients versus healthy controls (*105*).

AHR AND CYTOCHROME *P450* POLYMORPHISMS

AH receptor

Human *AHR* appears remarkably conserved in that very few polymorphisms have been found. Recently, interindividual differences in AHR and ARNT mRNA levels have been examined using reverse transcription - polymerase chain reaction (RT-PCR) (*116*); in this study, CYP1A1 mRNA levels correlated with that of AHR and ARNT. This quantitative difference in AHR expression may be attributed to genetic variation in its gene locus. Nevertheless, previous studies in our laboratory revealed that *AHR* is well-conserved with very few genetic variations in the gene (Figure 6). Multiple polymorphism detection assays were employed to screen over 150 ethnically-distinct DNA samples. No sequence variation was detected in the ligand binding domain of the AHR. In the transactivation domain of exon 10, Wong *et al.* found three polymorphisms (Table 2 & Figure 6). Of these three sequence variations, the codon 554 polymorphism previously has been described in a Japanese population (*117*). This genetic variation occurs at nucleotide 1661, where guanine is replaced by alanine with corresponding change of amino acid arginine by lysine. The second polymorphism locates to a guanine nucleotide at position 1708 which is replaced by adenine (*118*); this base substitution similarly results in an amino acid change at codon 570 from valine to isoleucine (Table 2). The third base change at nucleotide 1549 is a novel polymorphism which had not been previously reported; this leads to replacement of cytosine by thymine and substitution of a proline residue at codon 517 by serine (*119*).

Wong *et al.* found that the codon 570 variant is in linkage-disequilibrium with the codon 554 variant. In fact, haplotype analysis revealed a strong association for codons 517, 554, 570 occurrence on the same DNA strand. However, this remains tentative due to the small number of samples tested (*119*).

Transactivating function of the AH receptors bearing these polymorphisms has been evaluated by Wong *et al.* by recombinant cDNA

Table 2

Human *AHR* polymorphisms.

Gene	Variation	Nucleotide change	Amino acid change	CYP1A1 induction	Ref. no.
AHR	codon 517	C1549T	Pro-->Ser	unknown	*(119)*
AHR	codon 554	G1661A	Arg-->Lys	increased	*(117)*
AHR	codon 570	G1708A	Val-->Ile	abolished	*(118)*

Figure 6

Organization of human *AHR* gene and location of known variations.

G1661A (554 variant): Arg->Lys

C1549T (517 variant): Pro->Ser

G1708A (570 variant): Val->Ile

expression studies (*119*). AHR with the codon 554-lys variant alone had no significant effect on CYP1A1 inducibility by TCDD. Conversely, AHR harboring both codon 554 and 570 variants displayed a time-dependent decrease in CYP1A1 induction. In contrast, Smart and Daly (*118*) recently showed a correlation between increased ethoxyresorufin-O-deethylase activity (EROD) in lymphocytes of individuals homozygous or heterozygous for the lysine variant allele (p=0.0001). Functional consequence of the *AHR* 517 variant remains elusive.

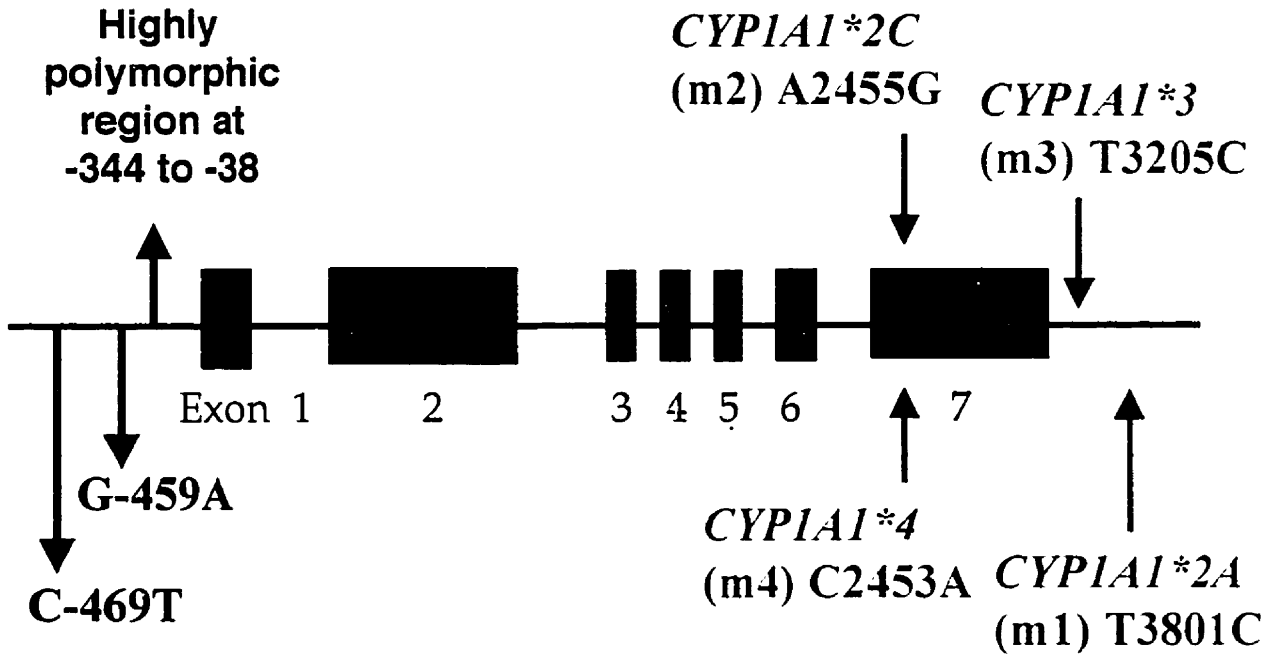
Overall, the AHR transactivation domain appears to be the most variable region of the human AHR. The AHR Ile570 variant form has diminished *CYP1A1* transactivation activity while the *AHR* 554-lys variant correlates with increased CYP1A1 EROD activity. The AHR also regulates transcription of others genes but the effects of these *AHR* variants on *CYP1A2* and *CYP1B1* transcriptional activation remains to be elucidated.

CYP1A1

Human CYP1A1 is expressed primarily in extrahepatic tissues and is polymorphic (Figure 7). Various comparative population-based genotyping studies have demonstrated racially distinct patterns of *CYP1A1* genotypes. Notably, RFLP analyses revealed significantly higher frequencies of a *CYP1A1*2A* variant in European-Americans, while African-American *CYP1A1*2A* genotypic frequencies more closely resemble those of Asians. This *CYP1A1*2A* polymorphism is in the 3'-non-coding region of *CYP1A1* and is closely linked in Asians (but less so in Caucasians) with a second *CYP1A1*2C* polymorphism. The *CYP1A1*2A* variant allele is associated with higher CYP1A1 inducibility (*105, 120*). In addition, this linkage between *CYP1A1*2A* and *CYP1A1*2C* alleles is absent in African-American population. The *CYP1A1*2C* polymorphism occurs in exon 7, in a region of the gene that codes for part of the catalytic site of the enzyme (*120*). The *CYP1A1*2C* allele results from an adenine to guanine substitution leading to isoleucine replacement by valine. Functionally, this variant confers a significant 3-fold increase in CYP1A1

Figure 7

Organization of the human *CYP1A1* gene and location of genetic polymorphisms.



*CYP1A1*1B:* C-469T
*CYP1A1*1C:* G-459A
*CYP1A1*2A* (m1): 3'-UTR (T3801C)
*CYP1A1*2C* (m2): codon 462 Ile->Val (A2455G)
*CYP1A1*3* (m3): intron 7 (T3205C)
*CYP1A1*4* (m4): codon 461 Thr->Asn (C2453A)

catalytic activity in lymphocytes of genotyped individuals (*120*); however, another study showed that cDNA-expressed CYP1A1 enzyme activity *in vitro* was not different from the wild type (*121*).

An African-specific *CYP1A1*3* polymorphism, locates to intron 7 upstream of the polyadenlyation site (*122*). This leads to a thymine to cytosine transition at position 3205. Finally, Cascorbi *et al.* (*122*) also reported another *CYP1A1*4* polymorphism which situates 2 bases upstream of the *CYP1A1*2C* polymorphic site with replacement of cytosine BY adenine and amino acid change from threonine to asparagine at residue 461 of the enzyme (Table 3). More recently, Smart and Daly reported two polymorphisms in the 5' regulatory sequence of *CYP1A1*. *CYP1A1*1B* results in the replacement of cytosine by thymine at position -469 while *CYP1A1*1C* leads to the substitution of guanine BY adenine at base -459. Functions of each sequence variation are currently unknown. Furthermore, a series of at least 10 single nucleotide polymorphisms (SNPs) at sites between -344 and -38 were found. With the complexity of this region, Daly and co-workers have termed this region "highly polymorphic" since it was not possible to correlate CYP1A1 activity with particular genotypes (*118*). Nomenclature of these new polymorphisms has not yet been ascribed.

CYP1A2

Cytochrome P4501A2 (CYP1A2) is a key enzyme in the metabolic activation of numerous chemical carcinogens including various heterocyclic amines and aromatic amines, aflatoxin B1 and other nitroaromatic compounds. Estrogens also are substrates of CYP1A2. In addition, CYP1A2 contributes to the metabolism of several common drugs and dietary constituents. Individual differences in CYP1A2 activity may therefore influence individual susceptibility to cancer risk and the therapeutic efficacy of some drugs. In humans, CYP1A2 is detected only in liver (*123*) where it is regulated by at least two mechanisms, one controlling constitutive expression while the other modulates inducible

Table 3

Selected human *CYP1A1* polymorphisms.

Gene allele	Trivial name	Variation	Base change	Codon change	Amino acid change	Function	Ref. no.
*CYP1A1*2A*	m1	3' UTR	T3801C	none	none	Higher inducibility	*(105)* *(120)*
*CYP1A1*2C*	m2	exon 7	A2455G	462	Ile->Val	3X higher activity	*(120)*
*CYP1A1*3*	m3	intron 7	T3205C	none	none	unknown	
*CYP1A1*4*	m4	exon 7	C2453A	461	Thr->Asn	unknown	

expression. Substantial interindividual variability in CYP1A2 activity has been reported. Currently, caffeine is the best *in vivo* probe drug for epidemiological studies where CYP1A2 activity is reflected by caffeine urinary metabolites (*124-126*). Several population-based studies have reported either bi- or tri-modal distributions in CYP1A2 activity (*126, 127*). Results of these *in vivo* phenotyping studies suggest a genetic basis for the large interindividual differences in CYP1A2 activity. Indeed two distinct functional genetic polymorphisms have just been identified in the non-coding regions of the gene that may partly explain the variability of CYP1A2 activity in the population (Table 4).

A study by MacLeod *et al.* reported a novel single base change in intron 1 (*128*) of *CYP1A2* at position 734 downstream of the first transcribed nucleotide (position 2640 of the gene) (Figure 8). This *CYP1A2*1F* polymorphic allele was detected by DNA sequencing with cytosine replaced by adenine (*115, 128*). Interestingly, in the large Caucasian population studied by Sachse *et al.*, the adenine variant was more frequent than cytosine at this position (*115*). Functional significance of this polymorphism was evaluated by an *in vivo* caffeine-phenotyping and genotyping correlation study. Data from this study indicate that, in smokers, a significantly higher (1.6 fold) CYP1A2 metabolic activity is found in homozygous individuals with two adenine alleles (*128*). Such a difference is not detectable in non-smoker populations and it appears that this difference in activity is only restricted to the homozygous genotype for adenine at this position (*115*).

More recently, Nakajima *et al.* (*129*) reported another polymorphism found in a Japanese population; the allele is denoted as *CYP1A2*1C* (Table 4). This sequence variant is situated in the 5'-flanking region of *CYP1A2* at position -2964 with substitution of guanine by adenine (Figure 8). Its effect on the transcriptional activation of the *CYP1A2* gene was investigated by measuring the rate of caffeine 3-demethylation in Japanese smokers. This *in vivo* study suggests that cigarette smokers with the *CYP1A2*1C* adenine allele have a significant decrease in CYP1A2 inducibility, and therefore *in vivo* activity. More

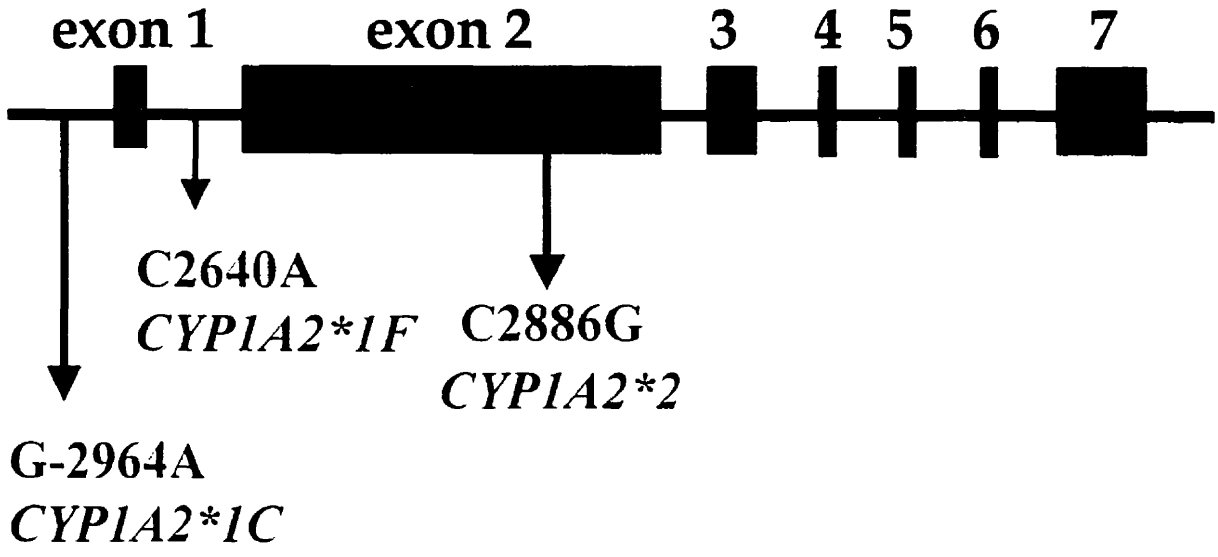
Table 4

Selected human *CYP1A2* sequence variations.

Gene Allele	Variation	Nucleotide change	Amino acid change	Enzyme/ receptor activity	Ref. no.
*CYP1A2*1C*	5' flanking sequence	G-2964A	none	decreased inducibility	*(129)*
*CYP1A2*1F*	intron 1	C2640A	none	higher inducibility	*(128)*
*CYP1A2*2*	exon 2	C2866G	Phe -> Leu	unknown	

Figure 8

Organization of human *CYP1A2* gene and location of variations.



***CYP1A2*1C*: 5' flanking sequence**
***CYP1A2*1F*: intron 1**
***CYP1A2*2*: codon 21 Phe→Leu**

specifically, a 1.6 fold decrease in CYP1A2 activity was detected in homozygotes with both adenine alleles while heterozygous genotype showed an intermediate 1.25 fold decrease in CYP1A2 inducibility (*129*).

PCR amplification and sequence analysis of DNA from eight Chinese subjects revealed a third polymorphism in exon 2 of the gene, known as *CYP1A2*2*. This base substitution involves a change of cytosine to guanine at nucleotide 2866 leading to replacement of phenylalanine by leucine. The functional consequence of this polymorphism, if any, is presently unknown (*130*). To date, all other sequence variations in *CYP1A2* have no functional impact on the expressed protein.

CYP1B1

In contrast to *CYP1A2*, *CYP1B1* appears to be genetically more variable. Bailey *et al.* reported two polymorphisms (*131*) in exon 3 which encodes the catalytically-important heme-binding domain of the enzyme (Figure 9). The first *CYP1B1*3* polymorphism occurs at nucleotide 1294. Guanine is substituted by cytosine at this position; the resulting valine at amino acid 432 is changed to leucine. The second polymorphism, *CYP1B1*4*, involves the substitution of adenine by guanine at position 1358. This leads to changes in the polypeptide sequence with codon 453 asparagine altered to serine (Table 5). Functional implications of these two polymorphisms were largely unknown until a recent study by Shimada *et al.* (*132*) which evaluated the metabolic activity of CYP1B1 Val432 and Leu432 variant enzymes. Activities for activation of nineteen PAHs and HAHs procarcinogens by both enzyme forms were essentially similar. However, kinetic studies on 17β-estradiol metabolism showed higher (1.45 fold) 4-hydroxyestradiol to 2-hydroxyestradiol metabolite ratio for Val432 compared to Leu432 (*132*). Another study reported that the Leu432 variant enzyme exhibits at least a 3-fold increase in its K_m for 17β-estradiol 4-hydroxylation with no effect on its V_{max} (*133*). However, a study by Hanna *et al.* (*134*) reported the opposite where the Leu432 variant displayed

Table 5

Selected human *CYP1B1* sequence genetic polymorphisms.

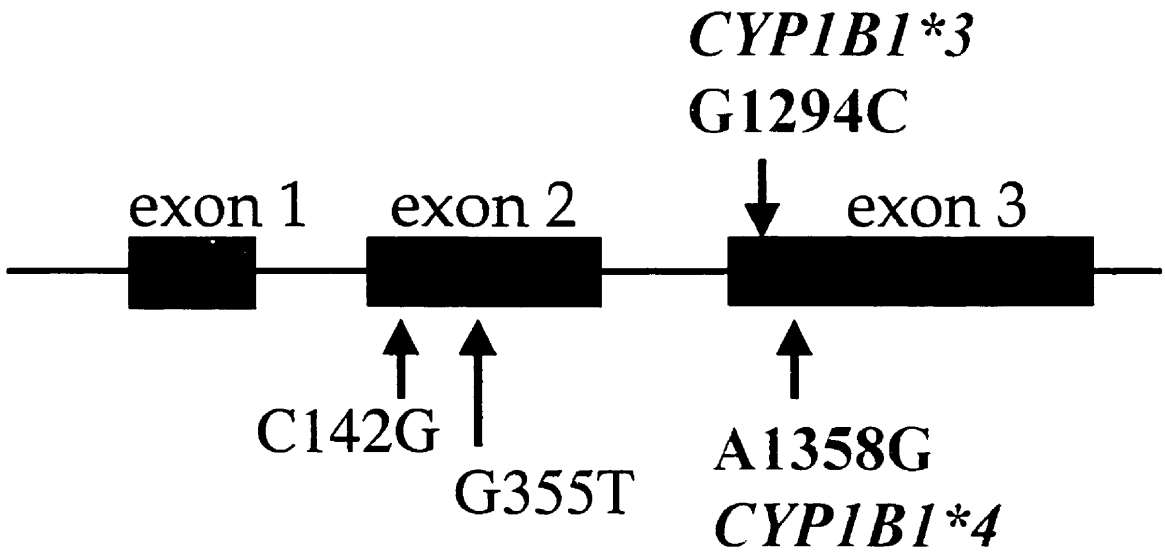
Gene allele	Variation	Nucleotide change	Amino acid change	Enzyme/ receptor activity	Ref. no.
*CYP1B1*3*	codon 432	G1294C	Val-->Leu	decreased 4OH:2OH	*(132)*
				increased K_m	*(133)*
				increased 4OH : 2OH	*(134)*
*CYP1B1*4*	codon 453	A1358G	Asn--> Ser	elevated 4OH:2OH	

higher 17β-estradiol 4-hydroxylation activity (3 fold). In the same study, Ser453 variant enzyme was associated with a 2.4 fold increase in 17β-estradiol 4-hydroxylation activity. A case-control study of Caucasian and African-American women with primary invasive breast cancer failed to show any significant association with these two exon-3 polymorphisms but the *CYP1B1*3* variant may be functionally important for steroid receptor expression in breast cancer of Caucasian women (*131*). A more recent study showed a positive correlation between breast cancer risk and the *CYP1B1*3* Leu/Leu genotype in a Chinese population in Shanghai (*68*).

Earlier sequence analysis of the *CYP1B1* translated regions in 22 primary congenital glaucoma families resulted in the identification of 16 mutations. These mutations were found to segregate with the disease phenotype in these families (*135, 136*). In the same study, these mutations were shown to occur only in affected patients but not in normal unrelated individuals. Of these normal individuals, DNA from 50 Turkish and 50 British subjects were sequenced which led to the identification of two additional polymorphic sites associated with amino acid substitution. The first polymorphism involves the arginine residue at codon 48 being replaced by glycine corresponding to a cytosine base at position 142 mutated to guanine. The second polymorphism occurs at nucleotide 355 from guanine to thymine and change of alanine to serine at amino acid 119. Positions of these two polymorphic residues (Figure 9) were characterized by a high degree of variability among humans and rodents and mapped outside of the conserved core structure (*136*).

Figure 9

Organization of human *CYP1B1* gene and location of genetic polymorphisms.



*CYP1B1*3*-m1: codon 432 Val → Leu (G1294C)
*CYP1B1*4*-m2: codon 453 Asn → Ser (A1358G)
*CYP1B1*2*: codon 48 Arg → Gly (C142G)
codon 119 Ala → Ser (G355T)

DESCRIPTION OF RESEARCH PROBLEM

Overview

Chemical exposure and lifetime exposure to17β-estradiol (E2) as well as catechol estrogens have been implicated in human mammary tumor formation. The cytochrome P450 supergene family is involved in the metabolism of numerous foreign and endogenous chemicals. Members of the *CYP1* genefamily; *CYP1A1*, *CYP1A2* and *CYP1B1* are principally involved in the metabolism of E2 and metabolic activation of environmental and dietary procarcinogenic chemicals into their ultimate carcinogens. E2 is also metabolized by hepatic CYP3A4.

CYP1A1, CYP1A2 and CYP1B1 activities are inducible by the AHR which is a ligand-activated transcriptional regulator. Following exposure to the AHR ligands (eg. PAHs and organochlorines), the AHR signaling pathway is triggered and induction of CYP1A1, CYP1A2 and CYP1B1 ensues. Enhanced metabolic activation of chemical procarcinogens results in the generation of reactive chemical species. In the absence of efficient detoxification or elimination by other conjugating enzymes, reactive metabolites can damage DNA and other cellular macromolecules leading to toxicity. Simultaneously, E2 metabolism is also affected by increased metabolic activities of these enzymes as well as competition with exogenous chemical substrates for the enzyme active sites.

The complex interplay among these metabolic pathways and processes is further complicated by the tissue-specific expression patterns of these genes. While CYP3A4 and CYP1A2 are abundant in human liver, CYP1A1 is not constitutively expressed in any tissue but can be induced in breast tissues by AHR-ligands. CYP1B1 is predominantly extrahepatic and is highly expressed in the mammary tissue.

Multiple genetic polymorphisms have been identified in each of these genes. It is conceivable that existence of genetic polymorphisms in *AHR, CYP1A1, CYP1A2, CYP1B1 and CYP3A4* affecting gene function will modulate

individual susceptibility to breast cancer by either disrupting E2 metabolism and/or affecting bioactivation of other dietary and environmental procarcinogens.

We focused on eight polymorphisms in *AHR*, *CYP1A2* and *CYP1B1*. *AHR* polymorphisms may alter the receptor's transactivating function therefore, induction of CYP1A1-, CYP1A2- and CYP1B1-dependent metabolism. Hepatic CYP1A2 is pivotal in inactivating circulating E2 as well as bioactivating dietary heterocyclic amines and other compounds. Consequently, polymorphisms in *CYP1A2* may potentially influence endogenous E2 clearance and the production of dietary mutagens. Finally, polymorphisms in *CYP1B1* may affect mammary CYP1B1 function hence, local metabolism of PAHs and E2. *CYP1A1* and *CYP3A4* are being investigated by collaborating laboratories.

HYPOTHESES

Project I : DNA polymorphisms in *AHR*, *CYP1A2*, and *CYP1B1* influence endogenous 17β-estradiol metabolism.

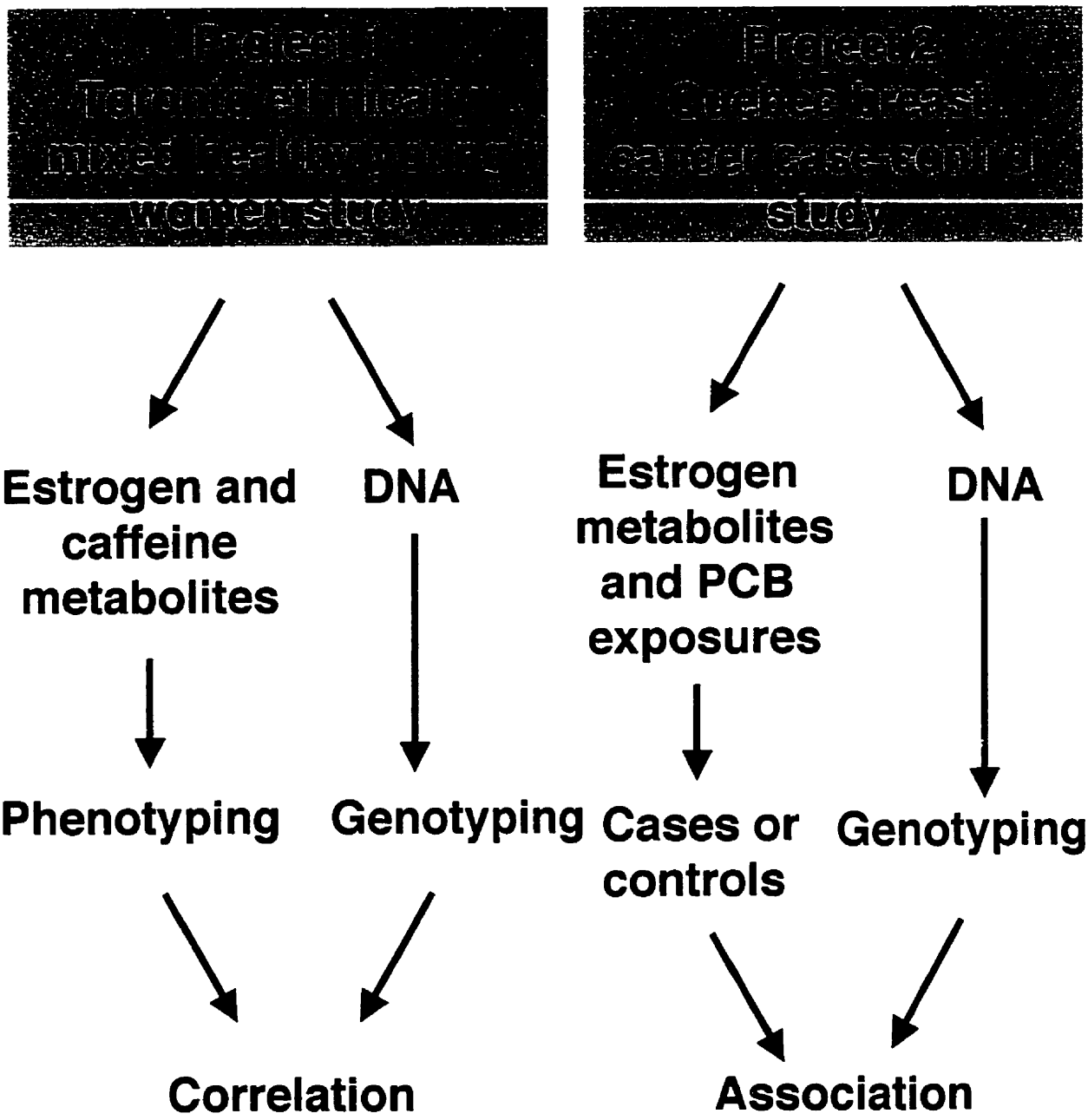
Project II : DNA polymorphisms in *AHR*, *CYP1A2* and *CYP1B1* are associated with breast cancer risk.

I first investigated whether variant alleles in *AHR*, *CYP1A2* and *CYP1B1* alter endogenous estrogen metabolism in an ethnically-mixed healthy Toronto population. This was achieved by Project I which involves the correlation of genotypes and the 2-hydroxyestradiol/16-hydroxyestradiol metabolite ratio.

I also assessed whether genetic polymorphisms in these genes influence breast cancer susceptibility. This was addressed in Project II, a case-control study of French Canadian women with breast cancer. Genotype frequencies were compared between cases and controls to detect any association between the genetic variations and breast cancer susceptibility (Figure 10).

Figure 10

Outline of research Projects 1 and 2.



DESCRIPTION OF COLLABORATIVE STUDIES

Project I: Toronto healthy young women study

Toronto healthy subjects were recruited by Dr. Steven Narod and Dr. Helena Jernstrom at the Center for Research in Women's Health, Toronto. Blood lymophocyte DNA and urine samples were collected by Dr. Steven Narod and coworkers. Caffeine-phenotyping was performed by Dr. Bing K. Tang. Estrogen metabolite ratios were assessed by Dr. Leon Bradlow and coworkers. Statistical analyses were carried out by Helena Jernstrom. I contributed the genotyping part of the study to allow genotype-phenotype correlations.

Project II: Quebec French Canadian case-control study

Recruitment of subjects and collection of DNA, serum PCBs and other variables were performed by Drs. Pierre Ayotte and Eric Dewailly at the Unite de Recherche en Santé Publique, Quebec. Statistical analyses were performed by Dr. Alain Demers. My component of this study was to genotype for the polymorphisms of interest. My genotyping results were integrated for the assessment of breast cancer susceptibility in relation to polymorphisms of *AHR* and P450 genes.

APPROACH

To screen for known polymorphisms, my first stage in the projects was to validate and optimize pre-existing genotyping assays before using them to screen DNA from subjects of these two studies. Next, I designed new assays suitable for detecting the polymorphisms of interest in the large populations (n=509 and n=737). The criteria for such techniques include the ability to detect the polymorphisms as well as being both time-efficient and technically feasible. In this regard, I chose PCR-based RFLP and SSCP (Figures 3 & 5).

OBJECTIVES

Validation and optimization of pre-established genotyping assays: Available genotyping assays were validated and optimized by following the procedures described in the literature.

Development of novel genotyping assays: Some of the genotyping assays to screen for the chosen polymorphisms were not available in the literature while other reported assays either were not reproducible or were of poor quality. In light of this, I developed my own genotype detection methods by choosing optimal primers enabling the amplification of DNA segments harboring the polymorphisms of interest. Restriction enzymes that could cleave DNA sequences generated by the presence or absence of polymorphic nucleotides were determined. Only enzymes capable of catalyzing complete digestion were used. Control DNA representing wild-type and homozygous variants or heterozygotes is necessary for each PCR-RFLP assay. This helps to demonstrate that the RFLP patterns obtained correspond to each of the genotypes. Thus, control DNAs for all 8 assays were developed. In addition to external controls (as mentioned), internal controls were incorporated into some assays to ensure completion of individual restriction enzyme reactions.

Assessment of allele frequencies: Allele frequencies of each polymorphism were detected by applying my novel and validated pre-existing assays to our two population studies. My control DNAs for each assay were included to generate typical patterns of different genotypes.

MATERIALS AND METHODS

SOURCES OF CHEMICALS AND REAGENTS

General chemicals such as ethidium bromide, acrylamide, N,N'-bisacrylamide, bromophenol blue, xylene cyanole FF, sodium dodecyl sulphate, phenol:chloroform:isoamyl alcohol, ampicillin, TEMED (N,N,N'N'-tetramethylethylenediamine) and glycerol were purchased from Sigma. Boric acid, glacial acetic acid, EDTA, isopropanol, formamide, sodium chloride and ammonium persulfate were from BDH. Agarose was from BioRad. Bacto tryptone, bacto yeast extract and bacto agar were supplied by Difco. Ethanol was from Commercial Alcohol Inc. Other chemicals including Xgal and IPTG were purchased from Canadian Life Technologies. Sodium hydroxide was from Fisher. Hydrochloric acid was from VWR. Acrylease was from Stratagene.

KITS AND PLASMIDS

The TA cloning kit was purchased from InVitrogen. Gel purification kit and plasmid maxiprep kit were obtained from Qiagen Inc. Plasmid vector pCRII and competent bacteria were also from InVitrogen.

OLIGONUCLEOTIDES AND CHEMICAL ISOTOPES

Oligonucleotide primers were synthesized by ACGT Corp. All oligonucleotides were cartridge-purified by ACGT Corp. [α-^{32}P]dCTP was supplied by Amersham.

CELL LINE AND TISSUE CULTURE REAGENTS

The human hepatoma cell line HepG2 was a gift from Dr. D. Grant. α-minimal essential culture medium and phosphate buffered saline (PBS) were purchased from the University of Toronto Media Center. Trypsin and fetal bovine (calf) serum were from Canadian Life Technologies. All sterile plastic pipettes and culture plates were from Falcon and Nunc respectively.

ENZYMES AND OTHER REAGENTS

Mwo I, *Msl* I, *Dde* I, *Hinc* II, *Bsu* 6I and *Mbo* II restriction enzymes were purchased from New England Biolabs. *Apa* I was obtained from Gibco BRL. Platinum *Taq* DNA polymerase was from Gibco BRL. Deoxynucleotide (dNTP) set was purchased from Amersham. 100 bp DNA ladder was from Gibco BRL or Amersham.

SOURCES OF HUMAN DNA SAMPLES

Project I : Toronto healthy young women population (n=509)

Blood lymphocyte DNA samples were collected in Toronto by Dr. Steven Narod and coworkers at the Center for Research in Women's Health, Toronto. An ethnically-mixed Toronto healthy young women (n=509) population consisting of 349 Caucasians and Jewish (White), 14 Blacks, 77 non-Indian Asians, 27 Indian and 42 women with mixed ethnicity. All enrolled healthy subjects had given their informed consent. A thorough, structured questionnaire was completed by subjects to provide information on variables including ethnicity, age, medical history, BMI, smoking, medication, coffee consumption, diet and life style. This population was phenotyped for urinary 2-hydroxyestradiol/16α-hydroxyestradiol estrogen metabolite ratios and a subset of n=168 were phenotyped for caffeine metabolites in urine as a marker of hepatic CYP1A2 activity.

Project II : Quebec French Canadian case-control population (n=737)

Cases include 300 women with newly-diagnosed, invasive and histologically-confirmed breast cancer. Cases were excluded if they had a previous history of breast cancer, had received preoperative chemotherapy or had been shown to have distant metastasis. 437 control subjects were randomly selected from the general population files of the Régie de l'Assurance

Maladie du Québec. Women with a personal history of breast cancer were ineligible. Written informed consent was obtained from all eligible participants. All subjects resided in the Quebec City area and were aged between 30 and 70 years. Controls were frequency-matched ensuring that their distributions by 10-year age groups and by urban/rural residence were similar to that of cases. Each participant donated a sample of blood for which several PCB congeners (App10) (including AH receptor ligands) and pesticides as well as urinary 2-hydroxyestradiol/16α-hydroxyestradiol estrogen metabolite ratios have been analyzed by Drs. Pierre Ayotte and Eric Dewailly. Lymphocyte DNA from these blood samples was provided by Drs Ayotte and Dewailly.

POLYMORPHISM DETECTION STUDIES

Primer design

Polymorphic sites encompassing the respective RFLP sequences were first identified. Accordingly, PCR primers were then designed using the Primer computer program (Whitehead Institute for Biomedical Research). Human *AHR*, *CYP1A2* and *CYP1B1* DNA sequences were downloaded from Genbank (accession # U02993, M31664, U56438). Accepted primer pairs generated from Primer program were subjected to similarity-search against known human DNA sequences in Genbank hence, ensuring primer-specificity for genes of interest. The primers that were most specific were selected and used for subsequent PCR and optimization of novel RFLP assays. In this manner, primers were designed for the detection of *CYP1A2*1F*, *CYP1A2*2*, *CYP1B1*3* and *CYP1B1*4* polymorphisms.

PCR amplification

Genomic or control DNA (200 ng) was first denatured at 95°C for 5 min and was subjected to PCR in 50 µl reactions containing 25 pmol of forward and reverse primers (Table 6), 1X PCR buffer (20 mM Tris-Cl, pH 8.4, 50 mM KCl)

and primer pair-specific $MgCl_2$ concentration with 10 nmol of each dNTP and 2 units of Platinum *Taq* DNA polymerase. PCR conditions for each polymorphism are described in Table 7. All PCR was performed using either 96-well plates or 12 strips of 8 tubes at a time (Marsh). Genomic DNA initially was denatured for 15 min at 95°C and also at the beginning of every cycle. Extension was performed at 72°C, an optimal temperature for Taq polymerase activity.

For the *CYP1A2* intron-1 polymorphism (*CYP1A2*1F*), a nested PCR was performed to increase product specificity (Table 7). Genomic DNA was first amplified by stepback PCR which involves an initial annealing temperature of 64°C to enrich specific products in the first 5 cycles of PCR. This was followed by 30 cycles of PCR at 58°C. 0.5 µl of this PCR product was re-amplified by a different primer set (Table 6) for 37 cycles.

To monitor for contamination, a negative control lacking template DNA was included in all PCR performed. Successful amplification was verified by analyzing 10 µl of PCR products by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. The remaining amplified DNA was used for RFLP or SSCP analysis.

DETECTION OF *AHR* POLYMORPHISMS

AHR 517, 554, 570 polymorphisms were detected by established techniques previously used in our laboratory (Table 7). Human HepG2 wild-type and variant clone DNAs were used as controls for these assays.

AHR 517, 554, 570 control DNA

AHR variant clones containing control DNA for all three polymorphisms were provided by Dr. Judy Wong. The human hepatoma HepG2 cell line has been previously identified as the "wild-type" sequence and was used in our

Table 6

Primers for RFLP and SSCP PCRs.

Primers	Sequences (5'-->3')	Ref. no.
L1	ACCAGCCTCAGGATGTGAAC	(*119*)
L2	GAATCTTGGACATACGTCAG	(*119*)
E102F	TTTCCTGCCATAATGGATCC	(*119*)
E104R	TTGCTGTGGACAATTGAAAG	(*119*)
E1F	CAAAGAGTCACCCTGGGTCTTAGG	
E2R	AACAGACTGGGACAATGCCAT	
J1A2F	AGGTATCAGCAGAAAGCCAGCAC	
J1A2R	GCTGAGGGTTGAGATGGAGACAT	
R2	GCTACACATGATCGAGCTATAC	(*129*)
R3	CAGGTCTCTTCACTGTAAAGTTA	(*129*)
M1F	TCTGCCTTGTATGGGTGACCAG	
BX3F	GTGGTTTTTGTCAACCAGTGG	
BX3R	GCCTCTTGCTTCTTATTGGCA	
ApaIF	ACTGGAAAGCGGGCAGTGAG	
ApaIR	CTTAATGCGCCGCTACAGGG	
Cont-AF	CCATGATTACGCCAAGCTAT	
Cont-AR	CGACTCACTATAGGGCGAAT	

Table 7

Genotyping PCR and restriction digest conditions.

Variation	Forward primer (5'-->3')	Reverse primer (5'-->3')	Fragment size (bp)	[$MgCl_2$] (mM)	Cycling temperatures (°C)	Restriction enzyme
AHR 517	E102F	E104R	743	2.0	95°C 20 min 55°C 20 min 72°C 40 min	*Bsu* 6I
AHR 554	L1	L2	220	2.0	"	none
AHR 570	E102F	E104R	743	2.0	"	*Hinc* II
*CYP1A2*1F*	E1F	E2R	1092	1.0	95°C 30 min 67°C 30 min 64°C 30 min 72°C 30 min	
	J1A2F	J1A2R	380	0.5	95°C 20 min 58°C 20 min 72°C 40 min	*Apa* I
*CYP1A2*1C*	R2	R3	596	1.5	95°C 20 min 55°C 20 min 72°C 40 min	*Dde* I
*CYP1A2*2*	E2B295F	E2B295R	294	0.5	Hot start 95°C 20 min 57°C 20 min 72°C 40 min	*Mbo* II
*CYP1B1*3*	M1F	BX3R	542	1.0	95°C 20 min 57°C 20 min 72°C 40 min	*Msl* I
*CYP1B1*4*	BX3F	BX3R	390	1.0	95°C 20 min 62°C 20 min 72°C 40 min	*Mwo* I

laboratory as a wild-type control for *AHR* 517, 554, 570 polymorphisms. HepG2 cells were cultured in α-minimal essential medium supplemented with 10% heat-inactivated bovine calf serum at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2. Genomic DNA was prepared as described by Gross-Bellard *et al.* (1973).

AHR 554 SSCP analysis

For the *AHR* 554 polymorphism, PCR was carried out as described with 1 μCi [α^{32}P]dCTP using L1 forward primer: 5'-ACCAGCCTCAGGATGTGAAC-3' and L2 reverse primer: 5'-GAATCTTGGACATACGTCAG-3'. Ten μl of radiolabled PCR product was denatured at 95°C for 5 min in 10 μl of formamide loading dye (85% formamide, 0.03% each of bromophenol blue and xylene cyanole FF) and snap cooled in ice before loading. Two μl of denatured PCR product was separated on non-denaturing 6% polyacrylamide gels with a crosslinker ratio of 19:1 and a final glycerol concentration of 10%. PAGE was carried out in 0.5X TBE buffer (0.045 Tris-borate, 0.001 M EDTA, pH 8) at room temperature and a constant power of 5 watts for 16-17 hours. SSCP gels were dried onto Whatmann filter paper with a Bio-Rad gel drier at 80°C for an hour. Visualization of SSCP patterns was achieved by autoradiography against Kodak BioMax film with two intensifying screens for two days. HepG2 and *AHR* 554 variant clones were used as controls for wild-type and variant migration patterns respectively (Figure 12).

AHR 517/570 RFLP Assays

A PCR fragment containing both the *AHR* 517 and the *AHR* 570 polymorphic sites was amplified using E102F forward primer: 5'-TTTCCTGCCATAATGGATCC-3' and E104R reverse primer: 5'-TTGCTGTGGACAATTGAAAG-3'. Fifteen μl of PCR product was subjected to RFLP analysis using *Bsu* 6I and *Hinc* II restriction enzymes to detect *AHR* 517

and *AHR* 570 polymorphisms respectively. The resulting patterns were electrophoretically analyzed by 2% agarose gel followed by staining with ethidium bromide and UV irradiation for visualization (Figure 13).

DETECTION OF *CYP1A2* POLYMORPHISMS

*CYP1A2*1C* 5' flanking sequence polymorphism was screened by the RFLP assay described by Nakajima *et al.* (*129*). A nested PCR-based RFLP assay was developed to detect the *CYP1A2*1F* allele. In addition, another RFLP assay was also designed for the *CYP1A2*2* polymorphism.

*CYP1A2*1C*- 5' sequence polymorphism RFLP

Genomic DNA was amplified by R2 and R3 primers: 5'-GCTACACATGATCGAGCTATAC-3' and 5'-CAGGTCTCTTCACTGTAAAGTTA-3'. Fifteen µl of PCR product was digested with *Dde* I enzyme followed by 2% agarose gel electrophoresis and visualization by ethidium bromide and UV illumination (Figure 14).

*CYP1A2*1F*- intron 1 RFLP

Nested PCR was performed on genomic DNA to generate specific DNA suitable for RFLP analysis (Table 7). Primers E1F and E2R were used in the first PCR: 5'-CAAAGAGTCACCCTGGGTCTTAGG-3', 5'-AACAGACTGGGACAATGCCAT-3' to generate 1092 bp fragment (Figure 15). This product (0.5 µl) was further amplified by J1A2F: 5'-AGGTATCAGCAGAAAGCCAGCAC-3'and J1A2R: 5'-GGGTTGAGATGGAGACAT-3'. The amplified DNA and 5 µl of internal control DNA were digested with *Apa* I enzyme and similarly separated and visualized in 2% agarose gel (Figure 16).

*CYP1A2*2*-exon 2 polymorphism RFLP

Primers used for this PCR were E2B295F and E2B295R; 5'-ATGTCTCCATCTCAACCCTCAGC-3' and 5- GCGGATCTGCAGGACGTC-3'. A manual hot start was performed to enhance product yield and specificity. In this manner, platinum Taq polymerase was only added to the reaction mixture at 72°C. Comparatively, hot start with platinum Taq polymerase gave higher yield of the more specific PCR product. Amplified product (15 µl) was digested with *Mbo* II enzyme and subsequent RFLP patterns were analyzed in 2% agarose gel (Figure 18).

DETECTION OF *CYP1B1* POLYMORPHISMS

Detection of *CYP1B1*3* and *CYP1B1*4* polymorphisms was achieved by designing and implementing two PCR-based RFLP assays. For the *CYP1B1*3* polymorphism, we included an internal DNA control which was concomitantly digested with amplified target DNA.

*CYP1B1*3*- codon 432 polymorphism RFLP

DNA sequence encompassing the location of *CYP1B1*3* polymorphic nucleotide was obtained by PCR using the M1F and BX3R primers: 5'-TCTGCCTTGTATGGGTGACCAG-3', 5'-GCCTCTTGCTTCTTATTGGCA-3'. Inference of genotype was accomplished by digesting the PCR product and 5 µl of internal control DNA with *Msl* I and subsequent separation and visualization in ethidium bromide-stained 2% agarose gel (Figure 19).

*CYP1B1*4*-codon 453 polymorphism RFLP

Genomic DNA was amplified by PCR using the BX3F: 5'-GTGGTTTTTGTCAACCAGTGG and BX3R primers. The amplified products

were cleaved by *Mwo* I restriction enzyme and similarly analyzed by gel electrophoresis and ethidium-bromide visualization (Figure 20).

ESTABLISHMENT OF CONTROLS FOR *CYP1A2* AND *CYP1B1* RFLP ASSAYS

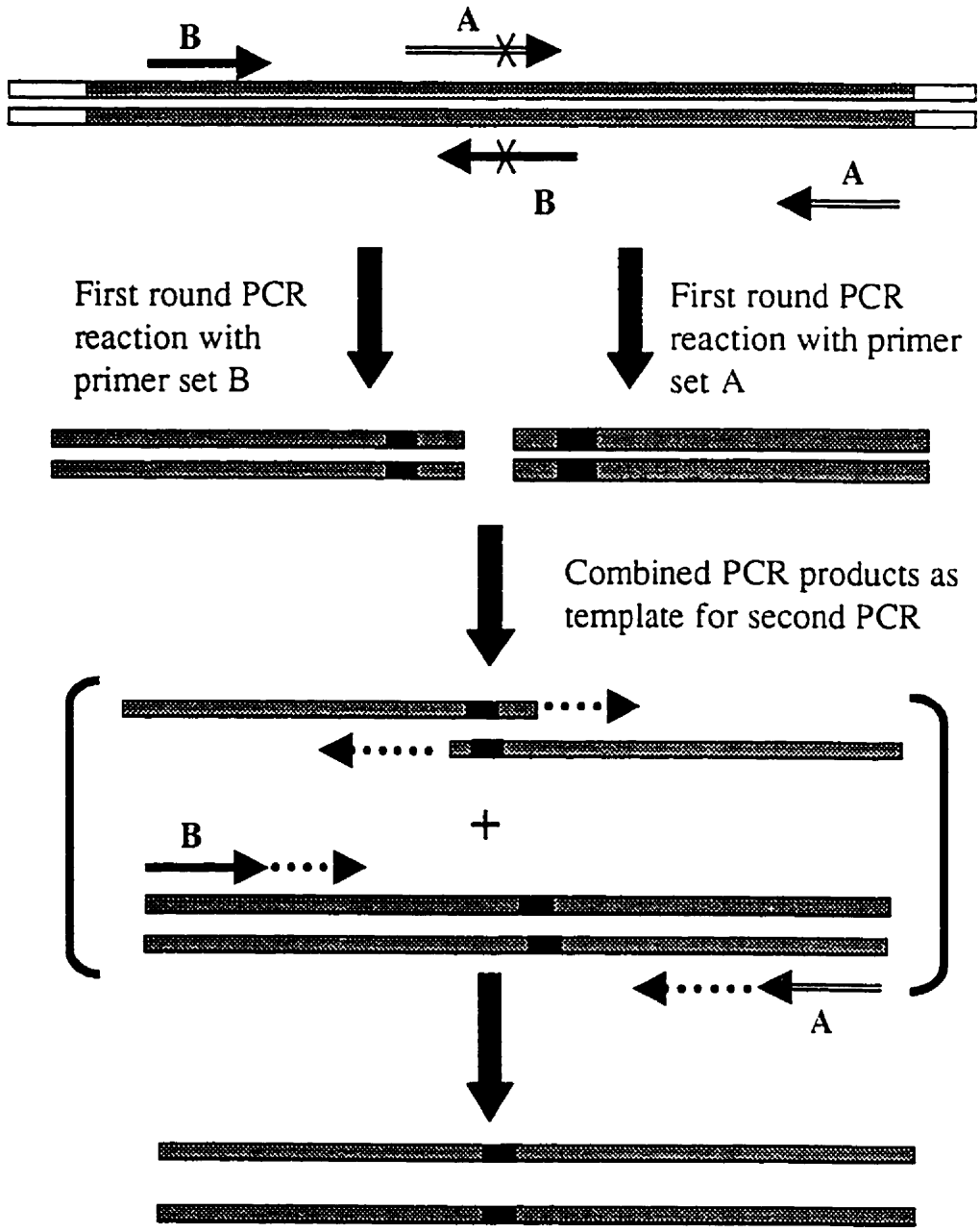
Allelic variant external controls

Human placental DNA samples of variable ethnicity are available at our laboratory. These samples were used for optimization and development of either existing or novel genotyping assays. DNA samples corresponding to wild type, heterozygotes or homozygous variants were amplified by PCR and subcloned into the plasmid vector pCRII with the TA cloning kit. Each PCR of genomic DNA was carried out with either a heterozygous control or a wild type together with a homozygous variant control, representing expected RFLP patterns generated by distinct genotypes. We failed to detect any *CYP1A2*2* heterozygotes or homozygous guanine variant in our collection of placental DNA; therefore, we resorted to PCR-based site-directed mutagenesis to create our own *CYP1A2*2* guanine variant control. The site-directed "mutant" DNA fragment was similarly subcloned into pCRII vector.

*CYP1A2*2* variant control site-directed mutagenesis

Overlapping E2B295CF: 5'-CTGCCATCTTGTGCCTGGTA-3' and E2B295CR: 5'-CAAGATGGCAGAGGCCAGG-3' mutated primers were designed to anneal specifically on both ends of the polymorphic site. Both primers contained the appropriate complementary nucleotide at the overlapping sequence corresponding to the "mutant" sequence variation. First round PCR was carried out using 200 ng of DNA each with either primer set E2B295CF and E2B295R, or second primer set E2B295CR, E2B295F. These two PCR reactions generate 184 bp and 119 bp respectively. Both PCR reactions were

Figure 11. PCR-based site-directed mutagenesis. A forward or reverse mutated internal primer is used with its corresponding external primers for the first round of PCR. First round products consists of two fragments with an overlapping sequence. Overlapping PCR products are pooled and serve as template for second round PCR. External primers were used to generate a full-length mutated fragment in the second round.



pooled (0.1 µl each) as templates for subsequent second round amplification with E2B295F and E2B295R primers (Figure 11).

Generation of internal controls

In some cases, internal controls were included to ensure complete restriction digestion. For the *CYP1A2*1F* polymorphism, an amplified fragment from pCRII plasmid, harbouring an *Apa* I restriction site, was concurrently digested with the gene-specific PCR product. For *CYP1B1*3* RFLP, an external variant clone control was used as a template to PCR-amplify a fragment containing the variant insert with short flanking sequence on both sides. This fragment encompasses the variant *Msl* I site; thus, it was useful as an internal control to ensure completion of restriction digestion (Table 8).

Control plasmid DNA preparation

Small-scale plasmid DNA purification was performed according to the method described by Sambrook *et al.* (*137*). All purified control DNA samples were resuspended in 40 µl Tris-EDTA, pH 8 with 20 µg/ml RNase A.

Verification of control plasmids

Isolated external and internal plasmid clones were amplified by PCR accordingly. This was followed by digestion with their appropriate restriction enzymes. Correct RFLP patterns were confirmed in each case before applying these preparations as controls for the RFLP experiments.

ANALYSIS OF A NEWLY DISCOVERED *AHR* SEQUENCE VARIATION

One French Canadian DNA sample showed a distinct DNA migration pattern in the *AHR* 554 SSCP experiments (Figure 21). PCR products of three distinct amplification reactions were sequentially subcloned into pCRII plasmid by TA

Table 8

PCR conditions for RFLP internal controls.

Variation	Forward primer (5'–>3')	Reverse primer (5'–>3')	Fragment size (bp)	[$MgCl_2$] (mM)	Cycling temperatures (°C)	Restriction enzyme
*CYP1A2*1C*	ApaIF	ApaIR	919	0.5	95°C 20 min 57°C 20 min 72°C 40 min	*Apa* I
*CYP1B1*3*	Cont-AF	Cont-AR	742	1.0	95°C 20 min 57°C 20 min 72°C 40 min	*Msl* I

cloning kit for DNA analysis to determine the specific base change and the precise location of this sequence variation by sequencing.

Plasmid purification

DNA plasmids containing the insert which harbored the putative novel *AHR* polymorphism were purified by midi-scale plasmid DNA preparation as described by the manufacturer of the Qiagen plasmid maxi kit. The DNA pellets were resuspended in Tris-EDTA, pH 8. Purified plasmid DNA was quantitated spectrophotometrically at 260 nm.

DNA sequencing

DNA sequences of the above plasmid preparations were determined by automated sequencing provided by the Hospital for Sick Children Biotechnology (HSC) Service.

GENOTYPE-PHENOTYPE CORRELATIONS

Project I: Effects of *AHR*, *CYP1A2* and *CYP1B1* polymorphisms on estrogen metabolism

Dr. Helena Jernstrom at the Center for Research in Women's Health conducted the genotype-phenotype correlations to investigate the influence of polymorphisms in *AHR* and P450 genes on estrogen metabolism.

Project II: Genetic variations in *AHR*, *CYP1A2* and *CYP1B1* in relation to breast cancer risk

Our French Canadian breast cancer case-control project is a collaborative study with epidemiologists Drs. Dewailly, Ayotte and Demers at Quebec City. Our collaborators analyzed four polymorphisms (*CYP1A2*1F*, *AHR* 554, *CYP1B1*3* and *CYP1B1*4*) for their relationship to breast cancer susceptibility.

RESULTS

DECTECTION OF *AHR* POLYMORPHISMS

AHR 554 polymorphism

The *AHR* 554 polymorphism was detected by SSCP using the "wild-type" HepG2 and *AHR* variant DNAs as negative and positive controls. The differential patterns corresponding to the three genotypes were readily detectable under the conditions described in the Material and Methods section. Typical SSCP patterns for "wild-type", heterozygotes and homozygous variants are indicated in Figure 12. The variant lysine allele occurred at a frequency of 0.181 in our Toronto ethnically-mixed population. A lower frequency of 0.095 was detected in the French Canadian case-control population (Tables 9-11).

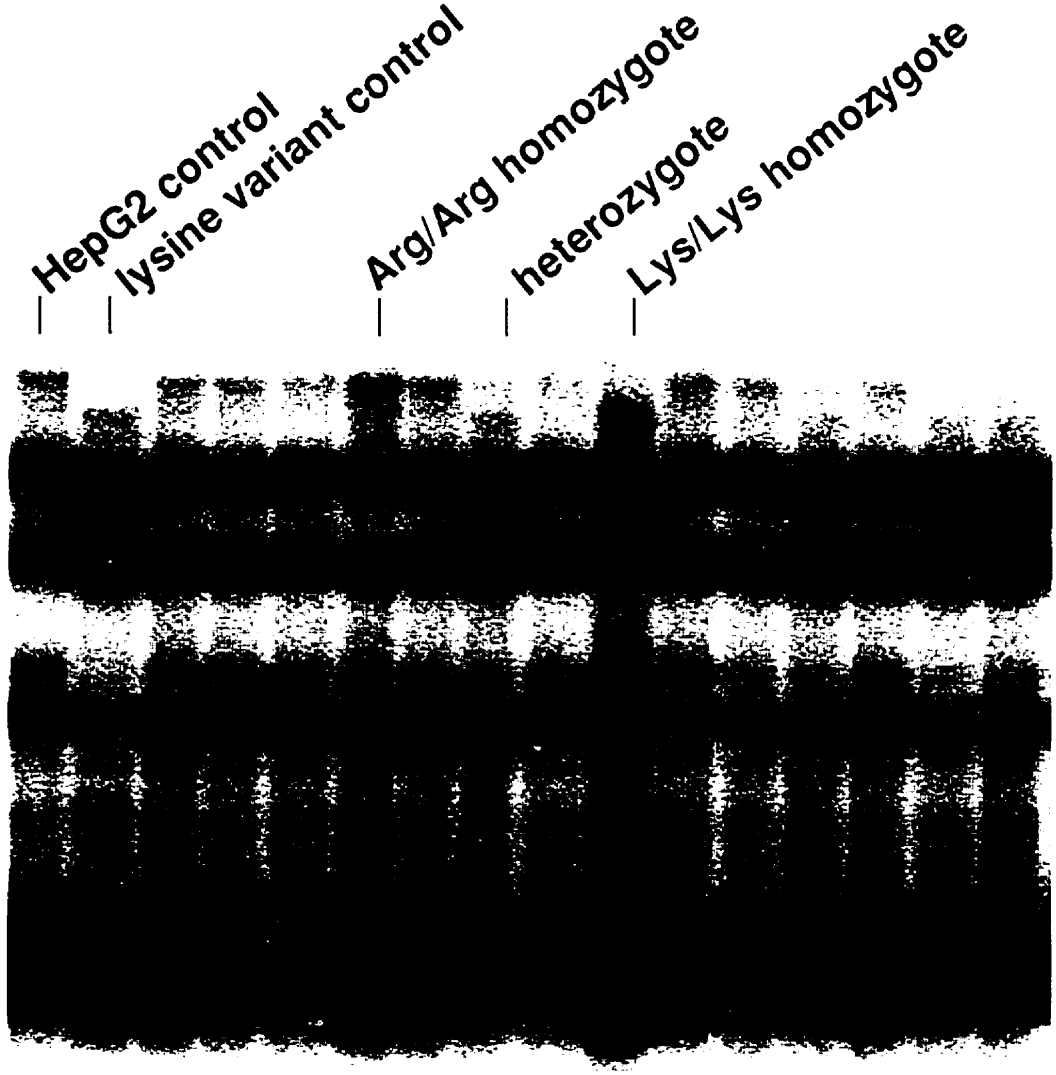
AHR 517/570 polymorphisms

Both the *AHR* 517-serine and *AHR* 570-isoleucine alleles were conveniently detected by PCR amplification of a 750 bp DNA fragment encompassing both polymorphic sites. This DNA fragment was subsequently digested with *Bsu* 6I and *Hinc* II restriction enzymes to determine the individual genotype at the *AHR* 517 and 570 loci. In this case, wild-type DNA contains the appropriate cleavage sites for both enzymes yielding two bands of 300 and 450 bp. Therefore, the RFLP pattern of a heterozygote contains three bands of 300 bp, 450 bp and 750 bp while a homozygous variant is represented by a single uncut 750 bp fragment (Figure 13).

As expected from previous populations (*119*), the frequencies of the codon 517 and codon 570 variants were very low in both populations (< 0.01). The codon 517-serine allele was not detected in the Toronto population (Project I, n=509). On the other hand, 3 heterozygotes were detected for the *AHR* 570 polymorphism, giving a frequency of 0.003. Of these three heterozygotes, 2 were Caucasian and the other was of mixed ancestry. For the French Canadian

Figure 12

SSCP of *AHR* 554 polymorphism.



The *AHR* 554 polymorphism was detected by separating radioactive PCR product in 6% non-denaturing polyacrylamide gel followed by autoradiography.

Table 9

Allele frequencies of *AHR* polymorphisms in the Toronto ethnically-mixed population.

AHR 517	White	Asian	Black	Indian-Pakistani	Other	Total
Pro/Pro	346	76	14	26	41	503
Pro/Ser	0	0	0	0	0	0
Ser/Ser	0	0	0	0	0	0
Total	346	76	14	26	41	503
Ser allele *f.*	0	0	0	0	0	0
***AHR* 554**						
Arg/Arg	266	30	6	18	22	342
Arg/Lys	72	35	8	8	15	138
Lys/Lys	6	12	0	1	3	22
Total	344	77	14	27	40	502
Lys allele *f.*	0.122	0.383	0.286	0.185	0.263	0.181
***AHR* 570**						
Val/Val	338	77	14	26	41	496
Val/Ile	2	0	0	0	1	3
Ile/Ile	0	0	0	0	0	0
Total	340	77	14	26	41	499
Ile allele *f.*	0.0029	0	0	0	0.012	0.003

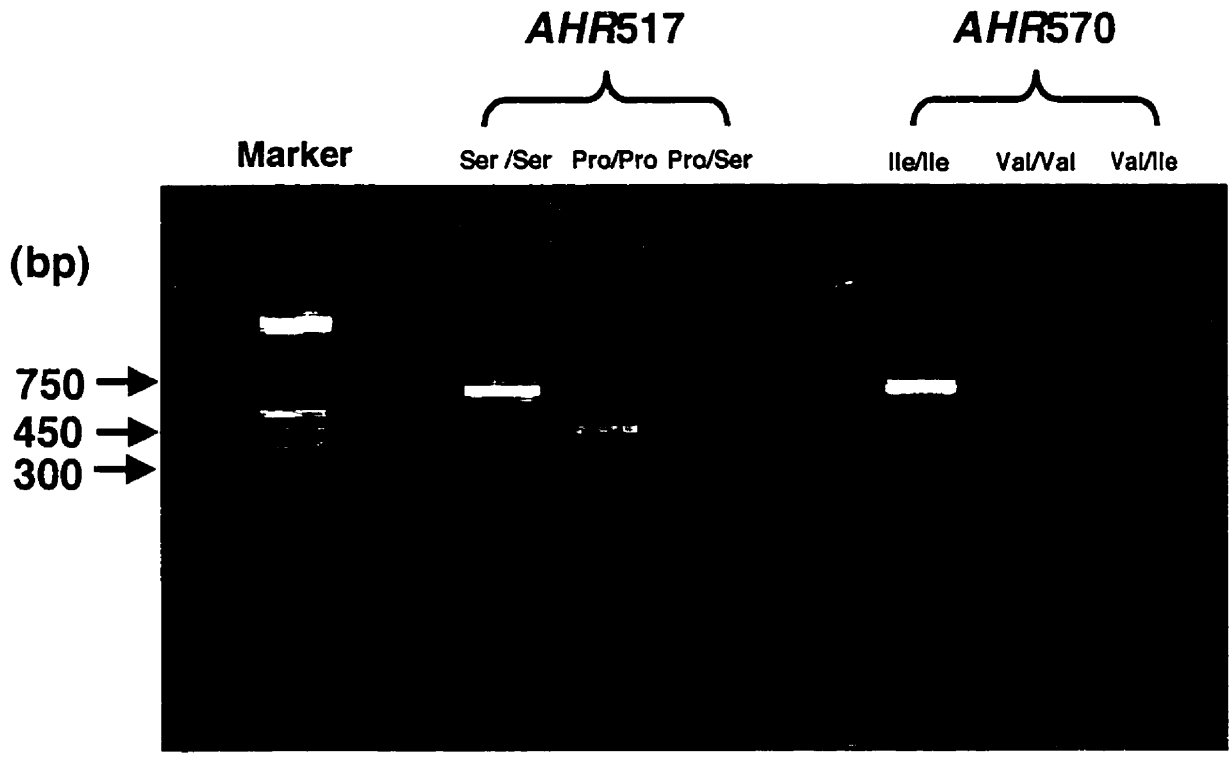
Table 10

AHR variant frequencies of Toronto ethnically-mixed healthy young women study (Project 1).

Gene	Variation	Nucleotide change	Amino acid change	CYP1A1 induction	Allele freq. in Toronto mix (N=509)
AHR	codon 517	C1549T	Pro-->Ser	unknown	0.00
AHR	codon 554	G1661A	Arg-->Lys	increased	0.181
AHR	codon 570	G1708A	Val-->Ile	abolished	0.003

Figure 13

RFLP of *AHR* 517 / 570 polymorphisms.



Pro/Pro (*AHR* 517) & Val/Val (*AHR* 570): 300 bp, 450 bp

Pro/Ser (*AHR* 517) & Val/Ile (*AHR* 570): 300 bp, 450 bp, 750 bp

Ser/Ser (*AHR* 517) & Ile/Ile (*AHR* 570): 750 bp

The *AHR* 517 and 570 polymorphisms were detected by separating *Bsu*6I and *Hinc* II- digested PCR product in 2% agarose gel electrophoresis. This is followed by ethidium bromide staining and visualization by UV illumination.

Table 11

AHR variant allele frequencies of Quebec breast cancer case-control study (Project 2).

Gene	Variation	Nucleotide change	Amino acid change	CYP1A1 induction	Allele freq. in (N=737) French population
AHR	codon 517	C1549T	Pro-->Ser	unknown	0.0007
AHR	codon 554	G1661A	Arg-->Lys	increased	0.095
AHR	codon 570	G1708A	Val-->Ile	abolished	0.0007

population (Project II, n=737), one heterozygote was detected for each polymorphism. Thus, the frequencies of this French Canadian population were 0.0007 for both the *AHR* 517 and *AHR* 570 allele (Tables 11).

Previously, both the *AHR* 517 and 570 polymorphisms have been detected only in various African descendants at a relatively low frequency. The allele frequencies are representative of the known ethnic composition of our studies.

DETECTION OF THE *CYP1A2* POLYMORPHISMS

*CYP1A2*1C*- 5' sequence polymorphism RFLP

A polymorphic site has been identified in the upstream 5' regulatory sequence of the *CYP1A2* gene. To detect this polymorphism in our two populations, I adopted the RFLP method described by Nakajima *et al.* (*129*) For a control, a heterozygote control DNA was included in all PCRs sets. This method results in either an undigested G/G fragment of 596 bp and/or the A/A variant bands with sizes 132 bp and 464 bp (Figure 14).

This pre-established method was optimized to efficiently and specifically amplify the desired DNA fragment with high yield. With subsequent DNA digestion by *Dde I* enzyme, we found a frequency of 0.078 in our Toronto population (Project I) and 0.01 in French Canadian women (Project II) (Tables 12-14). Interestingly, only heterozygote individuals were detected in the Quebec population, there were no homozygous variants.

*CYP1A2*1F*-intron 1 RFLP

I was unable to reproduce the PCR amplification with the primers described by Sachse *et al.* (*115*). In fact, Sachse *et al.* had indicated that the products amplified by their primers were not adequately specific for DNA sequence analysis. In light of this, I developed a novel PCR and RFLP assay to

Table 12

Allele frequencies of *CYP1A2*1C and CYP1A2*1F* in the Toronto ethnically-mixed population.

*CYP1A2*1C*	White	Asian	Black	Indian-Pakistani	Other	Total
G/G	335	36	6	24	30	431
G/A	8	35	4	3	12	62
A/A	0	5	3	0	0	8
Total	343	76	13	27	42	501
"A" allele *f.*	0.012	0.296	0.385	0.056	0.0143	0.078
*CYP1A2*1F*						
C/C	37	5	1	4	8	355
C/A	145	33	7	15	18	218
A/A	163	36	6	8	16	229
Total	345	74	14	27	42	502
"A" allele *f.*	0.683	0.709	0.679	0.574	0.595	0.673

Table 13

CYP1A2 variant allele frequencies of Toronto ethnically-mixed healthy young women study (Project 1).

Gene allele	Variation	Nucleotide change	Amino acid change	Effects on enzyme activity	Allele freq. in Toronto mix (N=509)
*CYP1A2*1F*	intron 1	C2640A	none	higher inducibility	0.673
*CYP1A2*1C*	5' flanking sequence	G-2964A	none	decreased inducibility	0.078

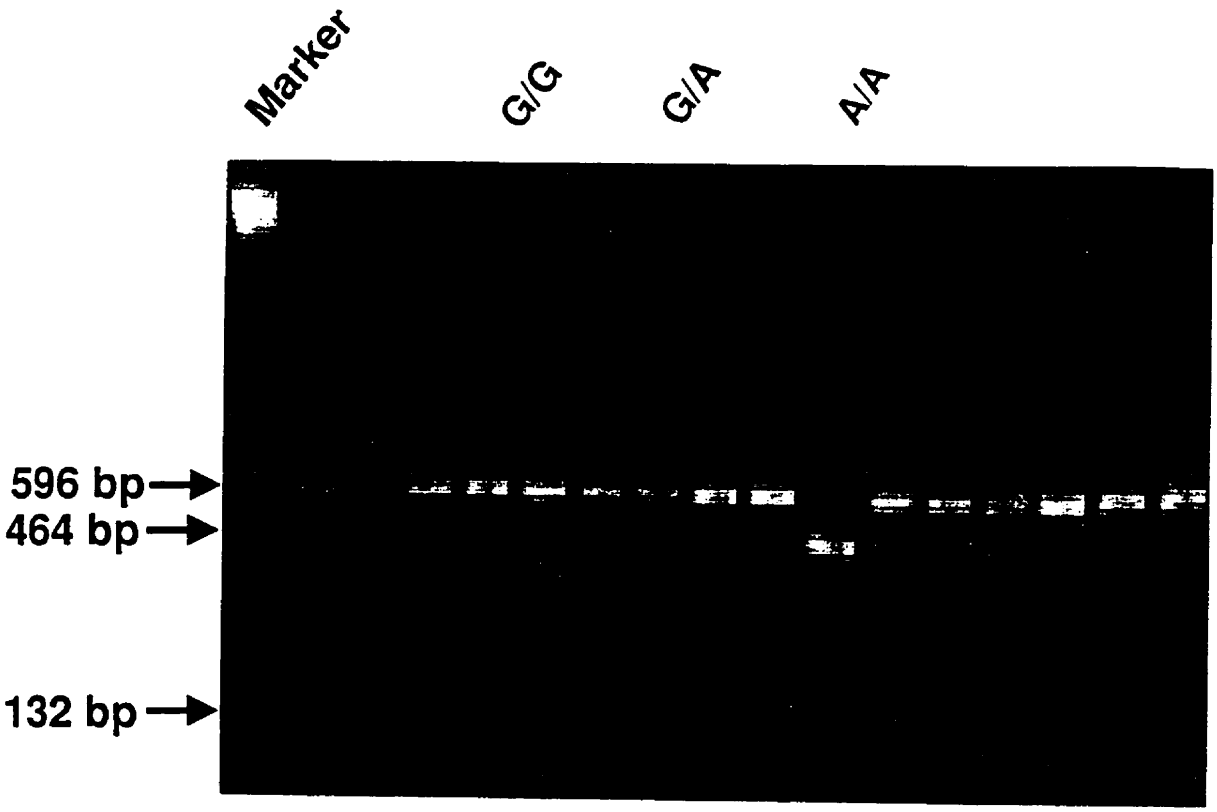
Table 14

CYP1A2 variant allele frequencies of Quebec breast cancer case-control study (Project 2).

Gene allele	Variation	Nucleotide change	Amino acid change	Effects on enzyme activity	Allele freq. in (N=737) French population
*CYP1A2*1F*	intron 1	C2640A	none	higher inducibility	0.719
*CYP1A2*1C*	5' flanking sequence	G-2964A	none	decreased inducibility	0.01

Figure 14

RFLP of *CYP1A2*1C* polymorphism.



G/G: 596 bp

A/G: 132 bp, 464 bp, 596 bp

A/A: 132 bp, 464 bp

The *CYP1A2*1C* polymorphism was detected by separating *Dde* I- digested PCR product in 2% agarose gel electrophoresis followed by ethidium bromide staining and visualization by UV illumination.

reliably detect the intron 1 polymorphism. In addition, I used my validated wild-type and variant clones as controls for my assays.

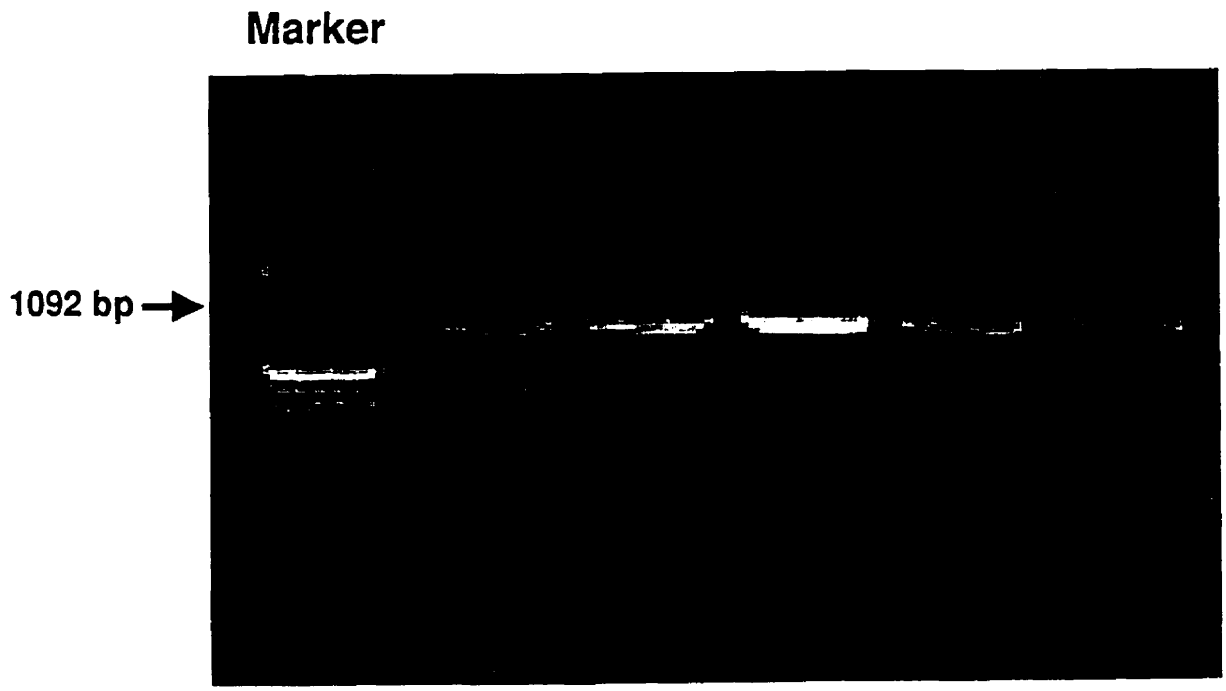
In my hands, specific amplification of *CYP1A2* was difficult perhaps owing to its extensive sequence similarity with other genes including *CYP1A1*. Consequently, I employed nested PCR and step-back PCR to achieve specific amplification. First step-back PCR using a forward primer that localizes to the 3' end of exon 1 and a reverse primer at the 5' end of exon 2, a prominent 1092 bp fragment of interest was amplified (Figure 15). Nevertheless, other non-specific band(s) also were present with faint intensity following ethidium bromide staining. This product was further amplified by primers that locate within this 1092 bp fragment such that a specific 380 bp DNA band is obtained in the second PCR.

For RFLP, a 989 bp control DNA fragment containing an *Apa* I site was co-digested with the 380 bp PCR product. The cytosine allele of this polymorphism contains an *Apa* I restriction site which is lost in the adenine variant sequence. Therefore, upon restriction digestion, an uncut 380 bp fragment would represent the *CYP1A2*1F* adenine allele while homozygosity for the *CYP1A2*1F* cytosine allele would be represented by two bands of 128 bp and 252 bp. A heterozygote would have all three bands. In all cases, restriction enzyme activity and reaction completion were confirmed by the absence of the control 989 bp band and the presence of the digested 200 bp and 789 bp fragments (Figure 16).

Allele frequency of the Toronto ethnically-mixed population (Project I) was 0.673 for the adenine allele and a frequency of 0.719 was obtained in the Quebec case-control population (Project II). The results generated confirm that this adenine variant is prevalent in the general population at frequencies above that of cytosine allele which was the allele originally sequenced (Tables 12-14).

Figure 15

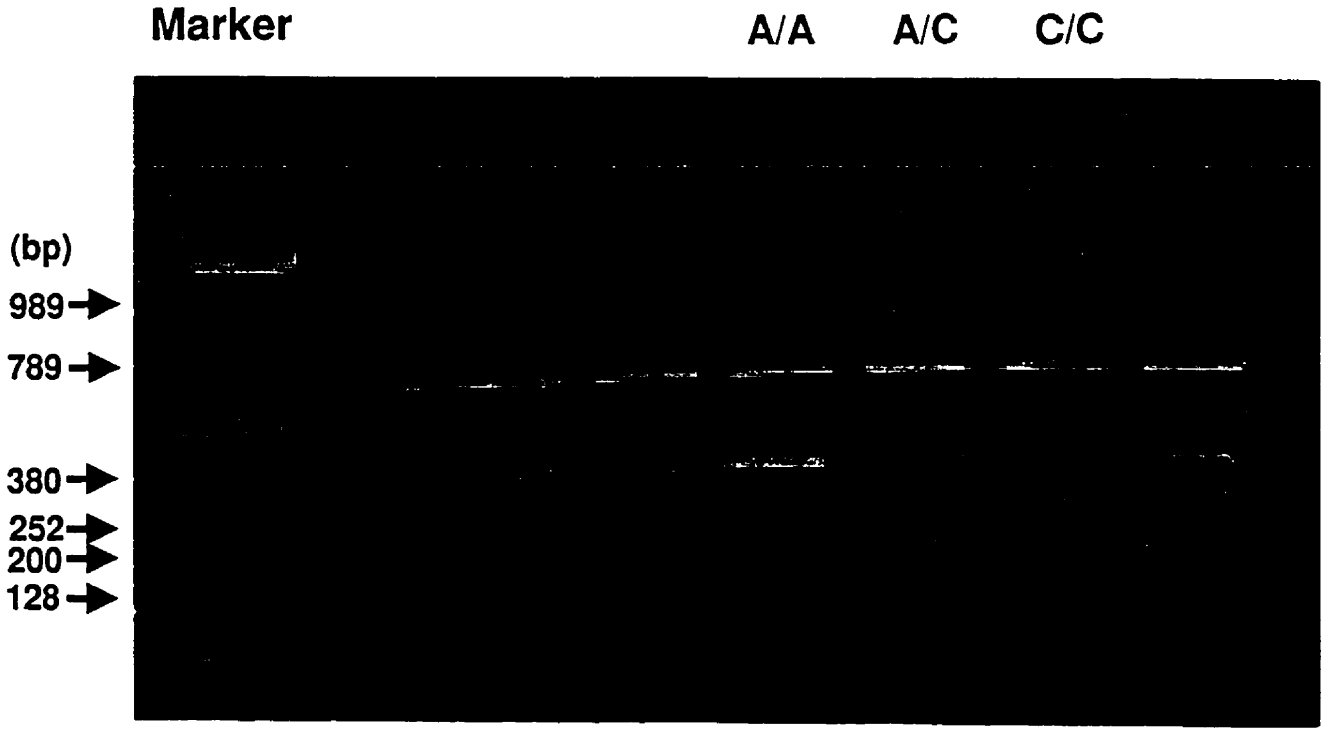
PCR of *CYP1A2*1F* polymorphism.



*CYP1A2*1F* polymorphism was detected by a 2-step PCR assay. First round PCR generated a 1092 bp fragment.

Figure 16

RFLP of *CYP1A2*1F* polymorphism



C/C: 128 bp, 252 bp and internal control DNA of 789 bp and 200bp

A/C: 128 bp, 252 bp, 380 bp and internal control DNA of 789 bp and 200bp

A/A: 380 bp and internal control DNA of 789 bp and 200bp

Internal control: 789 bp, 200 bp

The *CYP1A2*1F* polymorphism was detected by using a 1092 bp first round PCR product as template for subsequent second round nested PCR which generated a 380 bp DNA fragment. *Apa* I-digested PCR product and internal control were separated in 2% agarose gel electrophoresis followed by ethidium bromide staining and visualization by UV illumination.

*CYP1A2*2*-exon 2 polymorphism RFLP

I developed a novel PCR-RFLP assay aimed to detect the *CYP1A2*2* polymorphism. Using the appropriate primers, a 294 bp specific fragment can be amplified for subsequent digestion with *Mbo* II enzyme (Figure 17).

Unfortunately, this genotyping assay was optimal only when high-quality genomic DNA was available to use as template. Although amplification was achieved using DNA available in our laboratory, inconsistent amplification was observed when the same conditions were applied to DNA samples obtained from our collaborators. Furthermore, a hot-start PCR was required for specific amplification. These factors render the assay more cumbersome to perform and its application was limited to suitable DNA preparations. Thus, this assay could not be used for large-scale genotype screening. Our site-directed "mutant" clone DNA was available as a positive control for this assay.

Due to the presence of multiple *Mbo* II sites in this fragment, RFLP patterns of a homozygote with both cytosine alleles is reflected in three bands of 126 bp, 118 bp and 50 bp. Patterns of a homozygous variant with two *CYP1A2*2* guanine alleles is exemplified by a 244 bp and a 50 bp DNA fragment (Figure 18). All these bands are expected to be present in a heterozygous genotype.

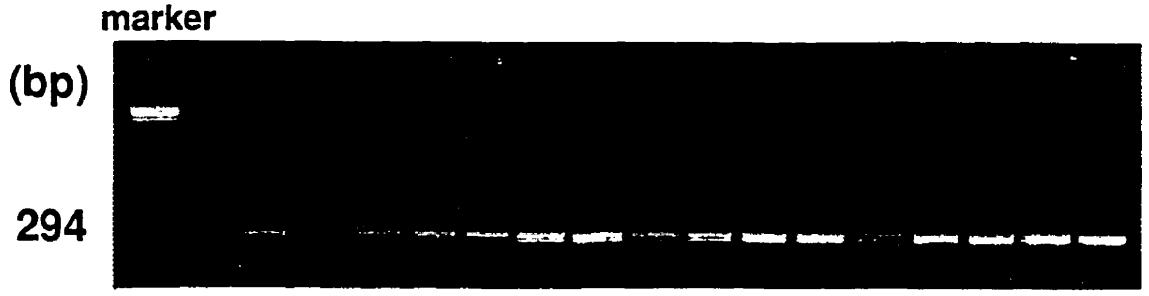
DETECTION OF *CYP1B1* POLYMORPHISMS

*CYP1B1*3*-codon 432 polymorphism

Our novel PCR and RFLP assay was used to ascertain the existence of the *CYP1B1*3* polymorphism in our populations. Further, I included an internal control which is a 790 bp DNA fragment that can be cleaved by *MsI* I to its 330 bp and 460 bp fragments. Genotypes for the samples were entered into the record only when this control DNA was completely digested. For RFLP, the gene-specific 542 bp PCR product was co-digested with control DNA by *MsI* I

Figure 17

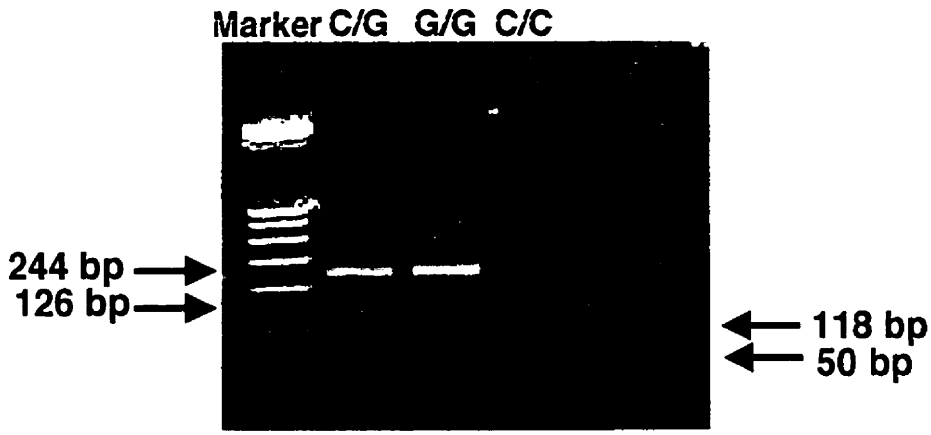
PCR of *CYP1A2*2* polymorphism.



A 294 bp PCR product encompassing the *CYP1A2*2* polymorphism was separated and visualized in ethidium bromide-stained 2% agarose gel.

Figure 18

RFLP of *CYP1A2*2* polymorphism.



C/C (Phe/Phe): 118 bp, 126 bp, 50 bp

C/G (Phe/Leu): 118 bp, 126 bp, 244bp, 50 bp

G/G (Leu/Leu): 244 bp, 50 bp

The *CYP1A2*2* polymorphism was detected by separating *Mbo* II-digested PCR product in 2% agarose gel electrophoresis followed by ethidium bromide staining and visualization by UV illumination.

enzyme. If the Val432 allele is present, an uncut 542 bp PCR band remains after digestion. In contrast, the variant nucleotide introduces an enzyme recognition sequence; thus the DNA with Leu432 is digested to 190 bp and 352 bp fragments (Figure 19). Again, various genotyped DNAs from our collection of Toronto placental tissues were subcloned to serve as positive and negative controls. The variant leucine allele was more prevalent in our Toronto population (Project I) at an allele frequency of 0.658. Its frequency in our French Canadian population (Project II) was 0.548 (Tables 15-17).

*CYP1B1*4* -codon 453 polymorphism

A PCR-based RFLP assay was similarly developed to identify the *CYP1B1*4* polymorphism. DNA with and without the *CYP1B1*4* Ser453 allele was also subcloned and the RFLP patterns were assessed in parallel with the DNA samples. In this manner, the 390 bp PCR product containing both *CYP1B1*4* Ser453 alleles is digested by the *Mwo* II enzyme yielding its characteristic pattern with the 100 bp and 290 bp fragments. Alternatively, the Asn453 *CYP1B1*4* allele is evident by an uncut 390 bp DNA band (Figure 20).

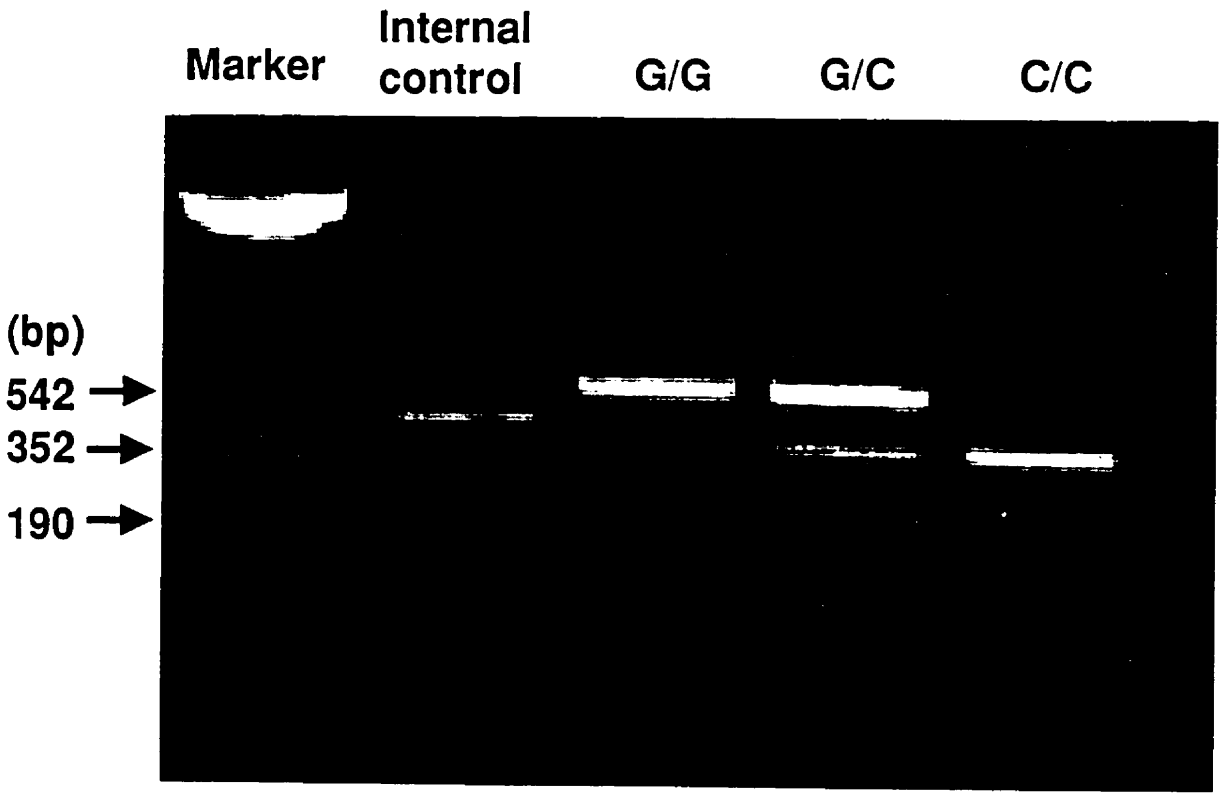
Using the novel PCR and RFLP procedure, *CYP1B1*4* serine variant allele was present at a frequency of 0.156 in the Toronto population (Project I). For the French Canadian population (Project II), a higher frequency of 0.182 was obtained (Tables 15-17).

ANALYSIS OF A NEWLY DISCOVERED *AHR* SEQUENCE VARIATION

A unique *AHR* 554 SSCP pattern was observed in 1/737 French Canadian DNA samples (Figure 21). I confirmed the validity of this distinct pattern by repeating the PCR and SSCP analysis on the genomic DNA. The same unique pattern was observed suggesting that this 200 bp contains a specific sequence variation. To determine the location and nucleotide change

Figure 19

RFLP of *CYP1B1*3* polymorphism.



G/G (Val/Val): 542 bp

G/C (Val/Leu): 190 bp, 352 bp, 542 bp

C/C (Leu/Leu): 190 bp, 352 bp

Internal control: 330 bp, 460 bp

The *CYP1B1*3* polymorphism was detected by separating *Msl* I- digested PCR product in 2% agarose gel electrophoresis followed by ethidium bromide staining and visualization by UV illumination.

Table 15

Allele frequencies of *CYP1B1*3 and CYP1B1*4* in the Toronto ethnically-mixed population.

*CYP1B1*3*	White	Asian	Black	Indian-Pakistani	Other	Total
Val/Val	57	2	10	0	6	75
Leu/Val	149	19	4	7	16	195
Leu/Leu	139	56	0	20	20	235
Total	345	77	14	27	42	505
Leu allele *f.*	0.619	0.851	0.143	0.870	0.667	0.658
*CYP1B1*4*						
Asn/Asn	37	5	1	4	8	355
Asn/Ser	145	33	7	15	18	218
Ser/Ser	163	36	6	8	16	229
Total	345	74	14	27	42	502
Ser allele *f.*	0.186	0.007	0	0.346	0.110	0.156

Table 16

CYP1B1 variant allele frequencies of Toronto ethnically-mixed healthy young women study (Project 1).

Gene allele	Variation	Nucleotide change	Amino acid change	Effects on enzyme activity	Allele freq. in Toronto mix (N=509)	Ref. no.
*CYP1B1*3*	codon 432	G1294C	Val-->Leu	decreased 4OH:2OH	0.658	(132)
				increased Km		(133)
				increased 4OH:2OH		(134)
*CYP1B1*4*	codon 453	A1358G	Asn->Ser	elevated 4OH:2OH	0.156	

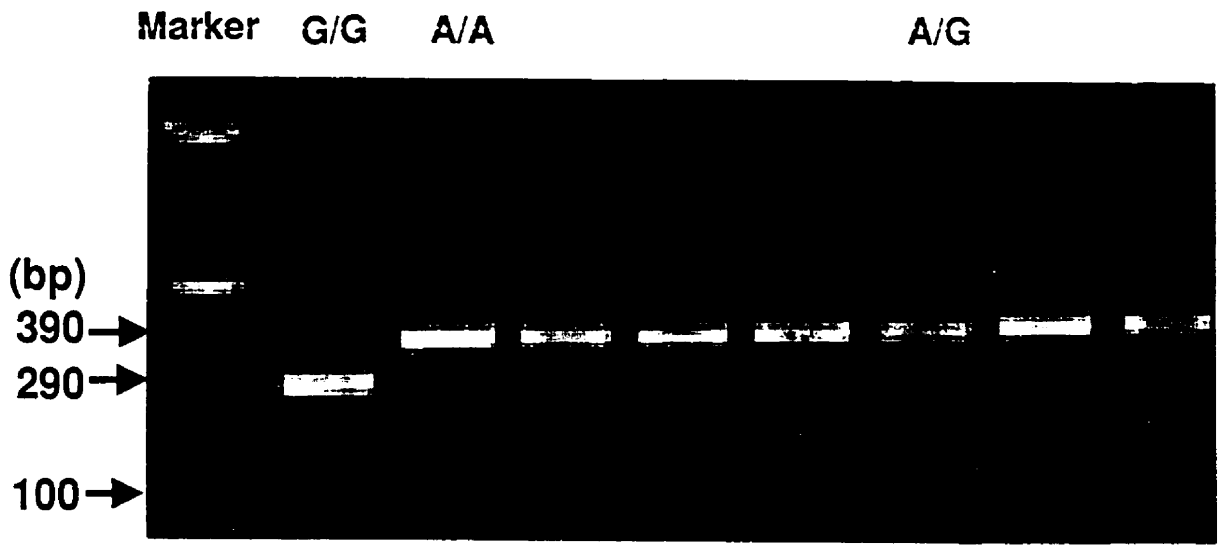
Table 17

CYP1B1 variant allele frequencies of Quebec breast cancer case-control study (Project 2).

Gene allele	Variation	Nucleotide change	Amino acid change	Effects on enzyme activity	Allele freq. in (N=737) French population	Ref. no.
*CYP1B1*3*	codon 432	G1294C	Val->Leu	decreased 4OH:2OH	0.548	(132)
				increased Km		(133)
				increased 4OH:2OH		(134)
*CYP1B1*4*	codon 453	A1358G	Asn->Ser	elevated 4OH:2OH	0.182	

Figure 20

RFLP of *CYP1B1*4* polymorphism.



A/A(Asn/Asn): 390 bp

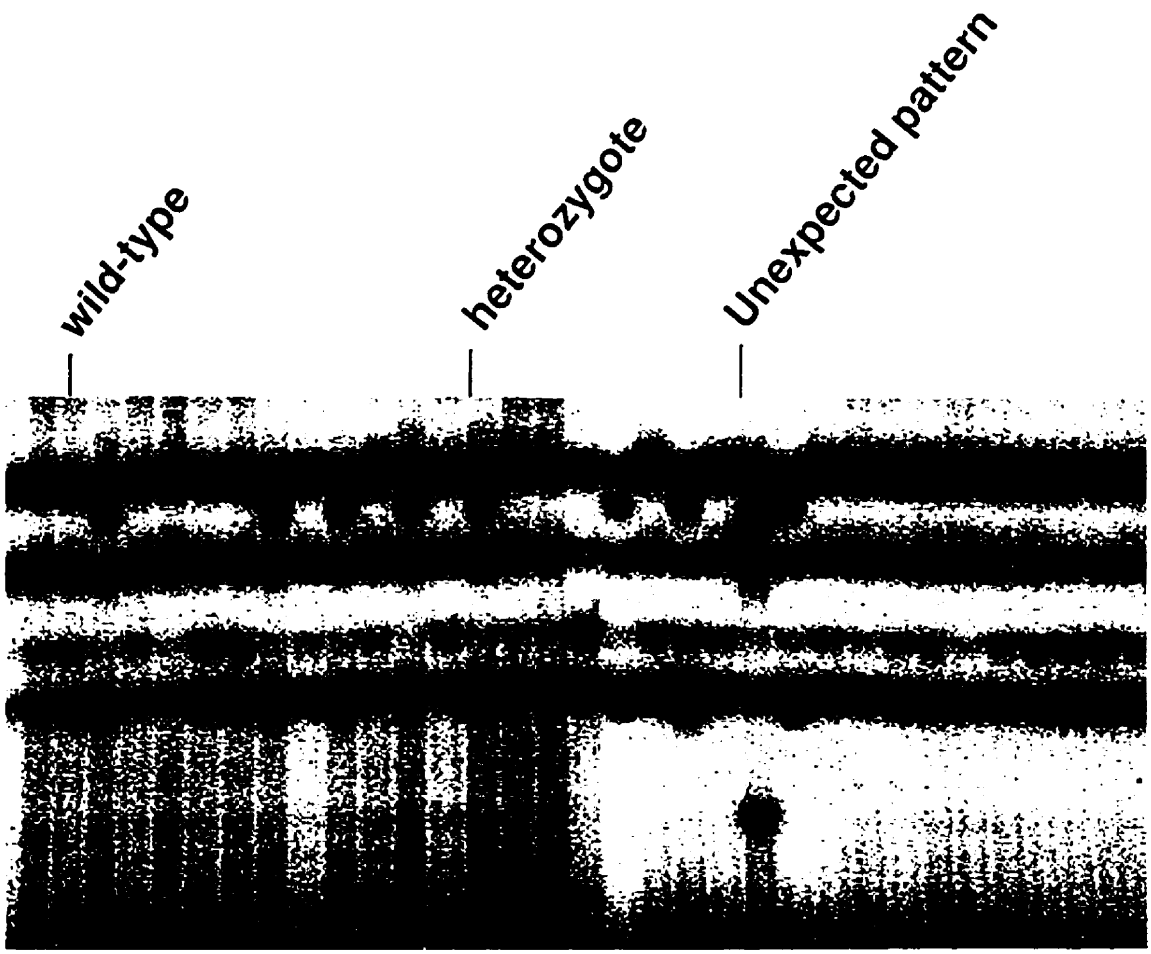
A/G (Asn/Ser): 100 bp, 290 bp, 390 bp

G/G (Ser/Ser): 100 bp, 290 bp

The *CYP1B1*4* polymorphism was detected by separating *Mwo* I- digested PCR product in 2% agarose gel electrophoresis followed by ethidium bromide staining and visualization by UV illumination.

Figure 21

AHR new sequence variation.



A unique *AHR* 554 SSCP pattern was detected by separating radioactive PCR product in 6% polyacrylamide gel followed by autoradiography.

involved, I performed three independent PCRs to amplify this 200 bp DNA fragment. These fragments were subcloned into pCRII for DNA sequence analysis. DNA sequencing revealed that the actual base change occurs in the exon-10 transactivation domain and was associated with an amino acid substitution.

SUMMARY OF ALLELE FREQUENCIES FOR PROJECT I

With respect to the allele frequencies (Table 18), our results show similar allele frequencies for *AHR* 517, 554, 570 to a previous Toronto population studied by Wong (*119*). Allele frequency of the*CYP1B1*3* polymorphism was found to be higher at a 0.658 compared to 0.519 reported by Bailey *et al. (131).* Frequency of the*CYP1B1*4* variant allele was similar to those previously reported by Bailey *et al. (131).* Interestingly, the *CYP1A2*1F* polymorphism is prevalent in this population with an allele frequency of 0.673 compared to 0.68 observed by Sachse *et al.* (*115*) in a Caucasian population study. Lastly, the frequency of the *CYP1A2*1C* polymorphism was substantially lower in the Toronto population (f=0.078) than originally detected in a Japanese population by Nakajima *et al.* (f=0.23) (*129*).

GENOTYPE-PHENOTYPE CORRELATIONS OF PROJECT I

To assess any differences in CYP1A2 activity among the different *AHR* and *CYP1A2* genotypes, a caffeine-phenotyping study was performed by Dr. Bing Tang for a subset of this population (n=168). Our collaborators correlated the different *AHR* and *CYP1A2* genotypes with the natural logarithm of the mean urinary caffeine index (ln caffeine index) as a measurement of CYP1A2 activity. Only the *AHR* 554, *CYP1A2*1C* and *CYP1A2*1F* were analyzed since allele frequencies of the other *AHR* polymorphisms were too low. We did not find any statistically significant changes in caffeine indexes related to any of the genotypes tested (Tables App1-3). However, a decrease in the mean caffeine index was observed for the *CYP1A2*1F* A/A genotype (ln mean caffeine

index=1.56±0.35) when compared to the C/A genotype (ln mean caffeine index=1.67±0.38). The observed difference was at the borderline of statistical significance (p=0.056) (Table App3).

To determine the effects of the different *AHR*, *CYP1A2* and *CYP1B1* polymorphisms on endogenous 17β-estradiol metabolism, my genotyping data were also correlated with the natural logarithm of the mean urinary 2-hydroxyestradiol to 16α-hydroxyestradiol metabolite ratio (ln mean 2OH:16αOH) as a marker for estradiol metabolism (Tables App4-9). Comparisons were made between the mean estradiol metabolite ratios of all the genotypes examined excluding *AHR* 517 and *AHR* 570 because of their rarity in the population. Intriguingly, the *AHR* 554 Lys/Lys genotype correlated with a dramatic decrease in the 2OH:16αOH metabolite ratio (ln mean 2OH:16αOH=0.032±0.52) when compared with the Arg/Arg "wild-type" genotype (ln mean 2OH:16αOH=0.31±0.58). The correlation was statistically significant with p=0.026 (Table App4). We also detected a difference in the estradiol metabolite ratio between *CYP1B1*3* Leu/Leu genotype (ln mean 2OH:16αOH=0.24±0.59) and the genotypes with at least one Val allele (Val/Leu and Val/Val) (ln mean 2OH:16αOH=0.33±0.57). The decrease in 2OH:16αOH ratio of the Leu/Leu genotype was approaching statistical significance with p=0.062 (App8).

Lastly, comparison between the mean caffeine index and the mean 2OH:16αOH showed a positive correlation with a correlation coefficient (r_s) of 0.28 and p=0.0002.

SUMMARY OF ALLELE FREQUENCIES FOR PROJECT II

All allele frequencies of our French Canadian population approximate those in previous studies of other Caucasian women (Table 19). For the *AHR*

517 and *AHR* 570 polymorphisms, only one heterozygote was detected for each polymorphism. The *CYP1A2*1F* adenine polymorphism was highly prevalent in our Quebec French Canadian population at a frequency of 0.719. On the contrary, *CYP1A2*1C* was only present at frequency of 0.01. Allele frequencies of the *CYP1B1*3* leucine and *CYP1B1*4* serine alleles occurred at similar frequencies as reported by Bailey *et al.* (*131*).

BREAST CANCER CASE-CONTROL STUDY

In this study, only *CYP1A2*1F*, *AHR* 554, *CYP1B1*3* and *CYP1B1*4* polymorphisms were retained for statistical analysis. Allele frequencies of *CYP1A2*1C*, *AHR* 517, *AHR* 570 were not sufficiently high in this population to allow any statistical analysis. Most polymorphisms failed to show any relationship with breast cancer risk. However, several associations were detected when stratified for menopausal status, family history of breast cancer, personal history of breast benign disease, maternity, body mass index (BMI), cigarette smoking, alcohol consumption or organochlorine exposures (Table App10). Individuals with the Val/Val genotype for *CYP1B1*3* polymorphism, having a family history of breast cancer, have elevated breast cancer risk (OR=1.85; 95% CI=1.04-3.28). Heterozygosity for the Val allele also was associated with breast cancer risk but was not statistically significant (Table App11). Conversely, the *AHR* 554 wild-type Arg/Arg genotype was associated with a decreased breast cancer risk in those exposed to low levels of PCB congener no.153 (OR=0.40; 95% CI=0.20-0.79; p=0.03) (Table App12). This association was not found in moderately and highly exposed individuals with the same genotype. Furthermore, homozygosity for the cytosine allele of the *CYP1A2*1F* was associated with lower breast cancer risk in individuals with low or high serum levels of PCB congener no.183 (Tertile 1: OR=0.25; 95% CI=0.07-0.90; p=0.005) (Tertile 3: OR=0.31 95% CI=0.10-0.99; p=0.005). However, the same genotype also was linked to increased relative risk in moderately exposed individuals (Tertile 2: OR=2.57, 95% CI=1.08-6.11;

p=0.005) (Table App13). Finally, individuals homozygous for the Asn453 *CYP1B1*4* allele also appear to have decreased relative breast cancer risk (OR=0.43 95% CI=0.22-0.83; p=0.02), in body mass index (BMI) ≥ 27 kg/m^2 stratum. A relation was found between reduced breast cancer risk in average women (21<BMI<27) with either the heterozygous or the homozygous genotype for the serine allele (OR=0.36; 95% CI=0.18-0.71; p=0.02) (Table App14).

Table 18

Variant allele frequencies of Toronto ethnically-mixed healthy young women study (Project 1) compared to previous studies of various ethnic groups.

Gene & allele	Allele freq. in Toronto mix (N=509)	Allele freq. in previous literature	Ref. no.
AHR 517	0.00	0.035-0.05	*(119)*
AHR 554	0.181	0.11	*(119)*
AHR 570	0.003	0.07	*(119)*
*CYP1A2*1F*	0.673	0.68	*(128)*
*CYP1A2*1C*	0.078	0.23	*(129)*
*CYP1B1*3*	0.658	0.305-0.591	*(131)*
*CYP1B1*4*	0.156	0.03-0.174	*(131)*

Table 19

Variant allele frequencies of Quebec breast cancer case-control study (Project 2) compared to previous studies of Caucasian populations.

Gene & allele	Allele freq. in (N=737) French population	Allele freq. in previous literature	Ref. no.
AHR 517	0.0007	0.00	*(119)*
AHR 554	0.095	0.12	*(119)*
AHR 570	0.0007	0.00	*(119)*
*CYP1A2*1F*	0.719	0.68	*(128)*
*CYP1A2*1C*	0.01	unknown	
*CYP1B1*3*	0.548	0.591	*(131)*
*CYP1B1*4*	0.182	0.174	*(131)*

DISCUSSION

Virtually all diseases involving environmental toxicity and cancer susceptibility represent the combined manifestation of "major" genes and "modifier" genes. For instance, BRCA1 is considered by many to be "the" gene responsible for human breast cancer. Upon the discovery of *BRCA2* gene and additional epidemiological studies, it is now believed that there may be as many as 4-12 "major" genes and a dozen or more "modifier" genes increasing breast cancer risk. Genes encoding drug-metabolizing enzymes and the aryl hydrocarbon receptor are among the modifying factors that affect cancer susceptibility or toxicity from endogenous, environmental and dietary agents.

The preeminent role of cytochrome P450 enzymes in bioactivating procarcinogenic xenobiotics and endogenous hormones to carcinogenic forms has been well established (*37, 40, 59, 63*). Thus, the polymorphic nature of many P450 enzymes has led to the hypothesis that allelic variant forms of certain P450 enzymes can confer altered susceptibility to chemical carcinogens over the lifetime of individuals.

The relationship of P450 genetic polymorphisms to cancer susceptibility has been evaluated in many studies comparing cancer cases to controls (ie. "case-control" studies). These studies suggest associations but cannot elucidate the causal relationship between chemical exposure, P450 polymorphisms and the disease outcome. The majority of these studies have been focused on lung cancer. Notably, the *CYP1A1*2A* polymorphism, which confers a higher enzyme inducibility phenotype, was reported to be inherited in lung cancer patients more often than in noncancer controls in a Japanese population (*105*). The valine/valine *CYP1A1*2C* homozygote was found to occur two to three fold more frequently in the lymphocytes of Japanese lung cancer patients compared to healthy controls (*138*). For human breast cancer, *AHR* and its downstream P450 enzymes which catalyze the bioactivation of environmental procarcinogens and endogenous steroid hormones may also be related to breast cancer risk. No definite increase of *CYP1A1*2A* or *CYP1A1*2C* polymorphisms was observed in breast cancer cases in the large,

prospective Nurses' Health Study in the United States (*139*). However, Taioli *et al.* (*140*) reported an association of the *CYP1A1*2A* homozygous variant genotype with breast cancer in African-Americans but not in Caucasians.

The roles of *CYP1A2* in bioactivating dietary chemical procarcinogens such as heterocylic aromatic amines and *CYP1B1* in the metabolism of many carcinogenic xenobiotics have been clearly established both *in vitro* and in animal models (*72*). However, estrogens also have been implicated in the initiation and promotion phases of breast cancer. Three P450 enzymes have been shown to catalyze estrogen catabolism *in vitro*. CYP1A2 catalyzes the formation of 2-hydroxyestradiol while CYP1B1 is responsible for the generation of the 4-hydroxyestradiol metabolite (*56, 58, 59*). The 16α-hydroxylation of 17β-estradiol has been reported to be catalyzed by CYP3A4 (*56, 141*). The 4-hydroxyestradiol metabolite has been implicated in breast cancer by virtue of its potential to participate in metabolic redox cycling (*55, 63-65*). The 16α-hydroxylated metabolites of 17β-estradiol and estrone, induce covalent binding to DNA causing genotoxicity and transformation (*56*).

Overall, our study aimed to assess the role of *AHR*, *CYP1A2* and *CYP1B1* polymorphisms in modifying breast cancer risk and 17β-estradiol metabolism. We did not include *CYP1A1* and *CYP3A4* in our studies since these are the subjects of investigation by our collaborators. We could not reliably detect the *CYP1A2*2* polymorphism in the DNA samples provided. Consequently, this polymorphism was not genotyped in our studies.

Allele frequencies in the Toronto population

By examining the allele frequencies of all seven polymorphisms in the multi-ethnic Toronto population we conclude that *CYP1A2*1C* and *CYP1A2*1F* are present in all ethnic groups examined. For *CYP1A2*1C*, we found the guanine allele to be the predominant allele in all ethnic populations examined (Table 12). The *CYP1A2*1C* adenine variant was present much more frequently in non-Indian Asians and Blacks compared to other ethnic groups

(Table 12). Similarly, the *CYP1A2*1F* adenine allele was more prevalent than the cytosine allele in all groups examined (Table 12). It is therefore reasonable to consider the adenine allele as the "wild-type" allele.

*CYP1B1*3* and *CYP1B1*4* were originally discovered in Caucasian populations (*136*). Allele frequencies of these polymorphisms were initially assessed by Bailey *et al.* Our study indicates that inheritance of these two polymorphisms is not restricted to Caucasians but also is found in other populations. For *CYP1B1*3*, the leucine allele is predominant in all ethnic groups (f>0.60) except in Blacks (f=0.143). A major disparity is observed between our study and that of a previous study. Bailey *et al.* (*131*) reported an allele frequency of 0.305 for their African-American population (n=118). This discrepancy likely is the result of limited number of Black subjects (n=14) enrolled in our study. Unfortunately, our Black population was too small to generate a reliable frequency. A future study with a larger Black population will be useful in determining the "actual" allele frequency for this ethnic group. Our data demonstrate that the Leu432 is the predominant *CYP1B1*3* allele in all of our populations (Table 15). The Leu432 allele was also shown to be the predominant allele in the Caucasian population studied by Bailey *et al.* Our Toronto population showed the prevalence of this polymorphism at f=0.658 while a somewhat lower frequency was reported by Bailey *et al.* (f=0.591). My initial validation of the Bailey *et al.* RFLP assay demonstrated the difficulty in differentiating between the small band sizes (61, 82 and 143 bp) in the expected RFLP pattern. More importantly, I could not achieve complete restriction digestion despite sequential optimization. Indeed, a product catalogue provided by the sole supplier of the *Eco57* I enzyme, MBI Fermentas, stated that "the cleavage of DNA by *Eco57* I is never complete". Therefore, I developed a novel assay and used *MsI* I restriction endonuclease instead. Perhaps the slightly lower frequency reported by Bailey *et al.* (f=0.591) compared to our Toronto population (f=0.658) is an artifact of their experimental error. Since the leucine allele introduces a cleavage site, the inability of the

Eco57 I enzyme to complete its reaction would lead to many false negatives (uncut bands representing the valine allele). For this reason, my novel *CYP1B1*3* genotyping assay will be invaluable for future studies. Overall, we provide evidence to support the leucine allele as the "wild-type" allele for these populations.

The *CYP1B1*4* serine allele was found in all groups except for Blacks likely due to the small population size (n=14). The frequency of this polymorphism in Blacks is expected to be very low since Bailey *et al.* reported a frequency of only 0.03 (n=118) in their African-American population (*131*). Similar to that observed by Bailey *et al.* the Asn453 allele was the predominant allele for all ethnic groups observed (Table 15).

As expected from previous studies in our laboratory (*119*), the *AHR* 517 and 570 variants were extremely rare in all populations ($f \leq 0.01$) evaluated. The *AHR* 554 polymorphism was present in all groups and the arginine allele consistently dominated in all ethnic populations (Table 9). For this reason, the arginine allele can be considered to be the "wild-type" allele. Finally, the differing allele frequencies of the *AHR*517, *AHR*554, and *AHR*570 polymorphisms in our populations do not support a "linkage disequilibrium" relationship between these polymorphisms.

Project I: Effects of polymorphisms on CYP1A2 activity

Effects of the *AHR* 554 (n=168), *CYP1A2*1C* (n=166) and *CYP1A2*1F* (n=168) polymorphisms on CYP1A2 metabolic activity were tested by caffeine-phenotyping. We did not detect any significant differences in CYP1A2 activity (measured by the mean caffeine indexes) among the various genotypes for any polymorphism (Tables App1-3). Larger sample sizes may be needed for further assessment of the relationship. Nevertheless, comparison between the mean caffeine index of *CYP1A2*1F* C/A and A/A genotypes revealed a decrease in CYP1A2 activity in individuals with the A/A genotype. This difference was at borderline statistical significance with p=0.056 (Table App3). Such difference

was not detected by *Sachse et al.* (*115*). The discrepancy between our finding and that of Sachse *et al.* may reflect the differences in ethnicity, diet and environmental exposures of the populations studied. We have not stratified for smoking status which may potentially influence our preliminary results since it has been shown that higher CYP1A2 inducibility was detected in smokers with the A/A genotype (*115*). Controlling for smoking status would allow the comparisons between genotypes and CYP1A2 inducibility. Nevertheless, with limited smokers in our sample population (n=29), this stratification may not be useful to generate any meaningful results. This is also true for our analysis of the *CYP1A2*1C* and *AHR* 554 polymorphisms. Overall there is a trend for the decreased caffeine metabolite ratio with the *CYP1A2*1F* A/A genotype but we may not have enough statistical power to detect it (n=168). A larger caffeine-phenotyping study is required. Further grouping and separation of known variables such as ethnicity and diet are currently underway.

Project I: Effects of polymorphisms on estrogen metabolism

To date, *in vivo* studies on the effects of *AHR*, *CYP1A2* and *CYP1B1* polymorphisms on human estrogen metabolism have been lacking. With the presence of known polymorphisms in these genes, involvement of these polymorphic enzyme variants in endogenous estrogen metabolism were determined in our Toronto healthy young women study (Project I). To examine this, urinary 2-OH:16α-OH estrogen metabolite ratios were correlated with the different *AHR*, *CYP1A2* and *CYP1B1* genotypes to ascertain their relationship to estrogen metabolism.

Our laboratory previously showed that there was no change between the two variant *AHR* 554 alleles in AHR-mediated CYP1A1 induction in transfection experiments in cell culture. A recent study suggested that *AHR 554* lysine allele confers higher CYP1A1 inducibility in 3-methylcholanthrene-induced lymphocytes (*118*). Interestingly, data from our current study indicate

that the *AHR 554* Lys/Lys variant genotype confers a significant 9.7 fold decrease in the mean 2OH:16αOH metabolite ratio (p=0.026) compared to the Arg/Arg "wild-type" (Table App4). The Lys/Arg heterozygous genotype displayed an intermediate mean 2OH:16αOH ratio but was not statistically different from the "wild-type". The 2OH:16αOH ratio has been postulated as the susceptibility marker for breast cancer and has been used for susceptibility assessment. Others have argued that the generation of the 16α-hydroxymetabolite constitutes a minor pathway relative to the other estradiol-hydroxylation pathways. Regardless, our data indicate that the *AHR* 554 polymorphism has a functional impact at the level of estradiol metabolism. The decrease in 2OH:16αOH ratio could not be ascribed to changes in CYP1A2 activity since our preliminary results in the caffeine-phenotyping analysis for *AHR* 554 did not show any significant change in CYP1A2 activity (Table App1). However, the possibility of an involvement of decreased CYP1A2 activity cannot be excluded since there were only 4 individuals phenotyped for caffeine metabolism who also had the Lys/Lys genotype. Since CYP1A1 primarily oxidizes 17β-estradiol to 2-hydroxyestradiol, the potential role of CYP1A1 in this observation may be ruled out in that regardless of whether *AHR*554 has no change or increased *CYP1A1* transactivation, the 2OH:16αOH ratio should either remain the same or increase. Further or different stratification and perhaps a larger population size is required before any conclusions can be drawn. With its influence on 17β-estradiol metabolism, the *AHR* 554 polymorphism may potentially alter individual susceptibility of estradiol-dependent cancers.

For *CYP1B1*3*, we also detected a decreased mean 2OH:16αOH for the Leu/Leu genotype when compared to the genotypes with at least one valine allele (Table App8). The difference in the estrogen metabolite ratio was approaching statistical significance (p=0.062). Very recently, Li *et al.* (*133*) have shown that the Leu432 CYP1B1 variant enzyme, in an optimized

reconstitution system, exhibits at least a 3-fold increase in its K_m for 17β-estradiol 2-hydroxyation and 17β-estradiol 4-hydroxylation activities. Our current data appear to support their finding since the *CYP1B1*3* Val/Val and Leu/Val genotypes have higher mean 2OH:16αOH than the Leu/Leu genotype. Our observations may be due to the relatively lower affinity of the Leu432 variant for 17β-estradiol CYP1B1-dependent metabolism compared to the Val432 variant enzyme. The statistical power of our study may not be sufficient to detect a significant trend but a future study with a larger population size will help evaluate the relationship.

A positive correlation (r_s=0.28) was observed between the caffeine indexes and the 2OH:16αOH ratios (p=0.0002). This indicates that the two metabolic processes are linked and thus interrelated. In essence, our results indicate that CYP1A2 mediates both 17β-estradiol 2-hydroxylation as well as caffeine 3-demethylation. Therefore, our result is consistent with the literature supporting the role of CYP1A2 in both metabolic pathways.

Project II: French Canadian breast cancer case-control study

Several associations among the different genotypes and breast cancer risk were detected in our study. The statistically significant relationships observed in our study may have an underlying biological connection. However, with the large number of comparisons between the genotypes and other variables, it is possible that some of these associations are merely due to chance. Consequently, further research on these relationships is required before any firm conclusions can be drawn.

Results from our case-control study suggested a relation between breast cancer risk and individuals with family history of breast cancer as well as the *CYP1B1*3* Val/Val genotype (Table App11). Interestingly, Zheng *et al.* (*68*) recently reported an association of the Leu/Leu genotype with breast cancer in

a Chinese women population in Shanghai (OR=2.3; 95% CI=1.2-4.5). Although the functional significance of the *CYP1B1*3* polymorphism has not been thoroughly studied, Bailey *et al.* (*131*) reported that Caucasian patients with the Val/Val genotype had a significantly higher percentage of estrogen receptor (ERs) or progesterone receptor-positive (PRs) breast cancers. These results suggest that this polymorphism may be functionally related to steroid receptor expression in breast cancer. In the same study, Bailey et *al.* did not detect any significant association of the Val/Val genotype with breast cancer. This may reflect the differences between the populations studied or possibly due to the inherent problems of their *CYP1B1*3* RFLP assay. Furthermore, Shimada and colleagues (*132*) demonstrated that the Val432 variant has an increased metabolic activity for 17β-estradiol 4-hydroxylation which yielded an elevated 4-hydroxy to 2-hydroxyestradiol metabolite ratio (4OH:2OH). Moreover, Li *et al.* (*133*) very recently demonstrated that the Leu432 variant confers a higher K_m for 17β-estradiol 2- and 4-hydroxylation activities. Thus, Leu432 is thought to have lower affinity for 17β-estradiol compared to Val432. Based on these functional data, our finding of the elevated risks of breast cancer in individuals homozygous for the valine allele with family history of breast cancer may be mechanistically related to enhanced 17β-estradiol 4-hydroxylation, hence increased exposure to the genotoxic 4-hydroxyestradiol metabolite. Interestingly, a report by Hanna *et al.* (*134*) showed a 3 fold increase in estrogen 4-hydroxylation activity by the Leu432 variant. Conflicting results between these *in vitro* studies may be attributed to the use of different recombinant protein expression systems and the sensitivity of their different estrogen metabolite detection methods. Moreover, frequency of the leucine allele is more prevalent in this French Canadian population (f=0.548). This suggests that French Canadian women with family history of breast cancer and the more common leucine allele have a lower chance of developing breast cancer compared to their Val/Val counterpart. The underlying molecular mechanism governing the association of the Val/Val genotype with breast

cancer risk of the present study may be different from that observed between the Leu/Leu genotype and breast cancer risk in Chinese women (*68*). The Leu432 has been shown to metabolize various HAs slightly more efficiently than the Val432 variant (*132*). Several of these HAs are mammary carcinogens in animal models (*31*) and their DNA adducts have been detected in both normal and cancerous human breast tissue as well as primary cultures of human mammary epithelium (*34*). Studies have suggested that several Asian women populations, including Chinese women in Shanghai (*52*), have lower mean serum estrogen levels than age-matched North American Caucasian women. It is possible that activation of dietary and environmental chemicals by mammary CYP1B1 is relatively more important than 17β-estradiol 4-hydroxylation in women with lower serum levels of estrogens. Perhaps French Canadian women also have higher levels of serum estrogens compared to Chinese women in Shanghai rendering enhanced 17β-estradiol 4-hydroxylation (associated with the Val/Val genotype) as a risk factor for breast cancer in this French Canadian population. Alternatively, there may be in interaction between family history of breast cancer and the *CYP1B1*3* valine allele, which further elevates breast cancer risk. On the basis of our finding and conflicting results between the two functional studies, it is worthwhile to re-investigate the functional impacts of these *CYP1B1*3* variant enzymes in both 17β-estradiol hydroxylation and activation of mammary procarcinogens. Such studies will shed light on the role of *CYP1B1*3* Leu432 variant in elevating breast cancer risk in women with a family history of breast cancer.

Data from our case-control study also revealed a link between *AHR* 554 Arg/Arg homozygous genotype and decreased risk of breast cancer in women with low exposure to PCB congener no. 153 (tertile 1). To our knowledge, this is the first study suggesting any relationship between *AHR* polymorphism with human breast cancer susceptibility (Table App12). PCB congener no. 153 corresponds to a non-planar di-ortho chlorinated 2,2',4,4',5,5'-hexachlorobiphenyl with affinity for rat and guinea pig AHR. Bunce and

coworkers (*142*) showed that PCB no. 153 is an antagonist of the AHR and competes with TCDD at high concentrations. Binding of PCB no. 153 to AHR induces minimal EROD activity (*142*). PCBs occur in complex mixtures. For example, evaluation of PCB congener profiles in the serum of humans consuming Great Lakes fish revealed PCB no. 153 as one of the four major congeners that accounted for 55-64% of the total PCB load (*143*). Another study which analyzed levels of 9 PCBs in 36 human breast adipose tissue samples found that PCB no. 153 was present in all 36 samples at the highest concentration range (*29*). It is unknown whether PCB no. 153 is also an antagonist of the human AHR. If so, PCB no. 153 may have different receptor activation capability than other agonistic PCBs that exist in the PCB mixtures. Alternatively, as Smart *et al.* suggested, Lys554-encoded AHR may truly have higher CYP1A1 inducibility such that, at the same concentration (tertile 1) of PCB no.153, the higher CYP1A1 inducibility and/or other downstream events conferred by Lys554-AHR compensate for the antagonistic effects of PCB no. 153. Hence, association was observed with the Arg/Arg genotype but not the Lys/Arg or Lys/Lys genotypes. It is also possible that this Arg554 polymorphism is linked to other polymorphism(s) in the same gene or in other genes that are involved in mammary carcinogenesis. Such association was not observed in those moderately (tertile 2) and highly (tertile 3) exposed to PCB congener no. 153. The reason for this is not understood. One possibility is that at low levels of PCB no. 153, Arg554 variant AHR response is reduced by this PCB inhibitor. At higher levels of PCB no.153 which also usually reflects higher exposure to other PCBs, the overall competition between the PCBs and subsequent metabolic events render the Arg/Arg genotype no longer "protective" at these higher concentrations (tertile 2 & 3). Perhaps a future study with a larger sample size is needed to clarify our results. Nevertheless, our finding merits further investigation in the functional consequence of this *AHR* 554 polymorphism in the presence of this PCB congener alone and in PCB mixtures, which may provide insight into the underlying mechanism(s) that confer(s) the associated protective effect or breast cancer.

When stratified for low and high serum levels of PCB congener no. 183 (2,2'3,4,4',5,6'-heptachlorobiphenyl), homozygosity for the cytosine allele of the *CYP1A2*1F* intron 1 polymorphism was found to be significantly associated with lower breast cancer risk. Unfortunately, the toxicological significance of this PCB has not been thoroughly studied. Unexpectedly, the same genotype also was related to elevated breast cancer risk in those with moderate levels of serum PCB congener no. 183 (Table App13). Based on the data obtained from an *in vivo* caffeine-phenotyping study, Sachse *et al.* (*115*) reported that the A/A genotype may either directly represent a high CYP1A2 inducibility or it is linked to polymorphisms conferring high inducibility. Normal hepatic function is essential in the detoxification of numerous carcinogens. Previous studies comparing metabolic activation of PAHs and other heterocyclic amines have shown that CYP1A2 preferentially bioactivates heterocylic amines found in the diet. Therefore, hepatic CYP1A2 activity is central to the detoxification of numerous dietary mutagens. This process involves the initial formation of reactive chemical species catalyzed by CYP1A2 which are immediately detoxified by phase II conjugating enzymes. Effective coupling of these two processes is critical for efficient elimination of xenobiotics (Figure 22). It is possible that the induction profile and levels of phase II enzymes have evolved to complement that conferred by the *CYP1A2*1F C/C allele* with low inducibility phenotype. Consequently, the association between the C/C low inducibility genotype with decreased cancer risk may be explained by the more efficient coupling between CYP1A2 and phase II enzymes in chemical inactivation. In contrast, phase II enzymes may be limiting for the A/A genotype conferring a high inducibility phenotype thus, reactive metabolites may escape from conjugation and induce mutagenicity in liver or peripheral tissues (ie. mammary tissue) via circulation through the blood stream. In addition to bioactivation of dietary chemicals, CYP1A2 also plays an important role in 17β-estradiol 2-hydroxylation. In effect, the relative importance between these two metabolic processes may differ depending on the extent and nature of induction of hepatic

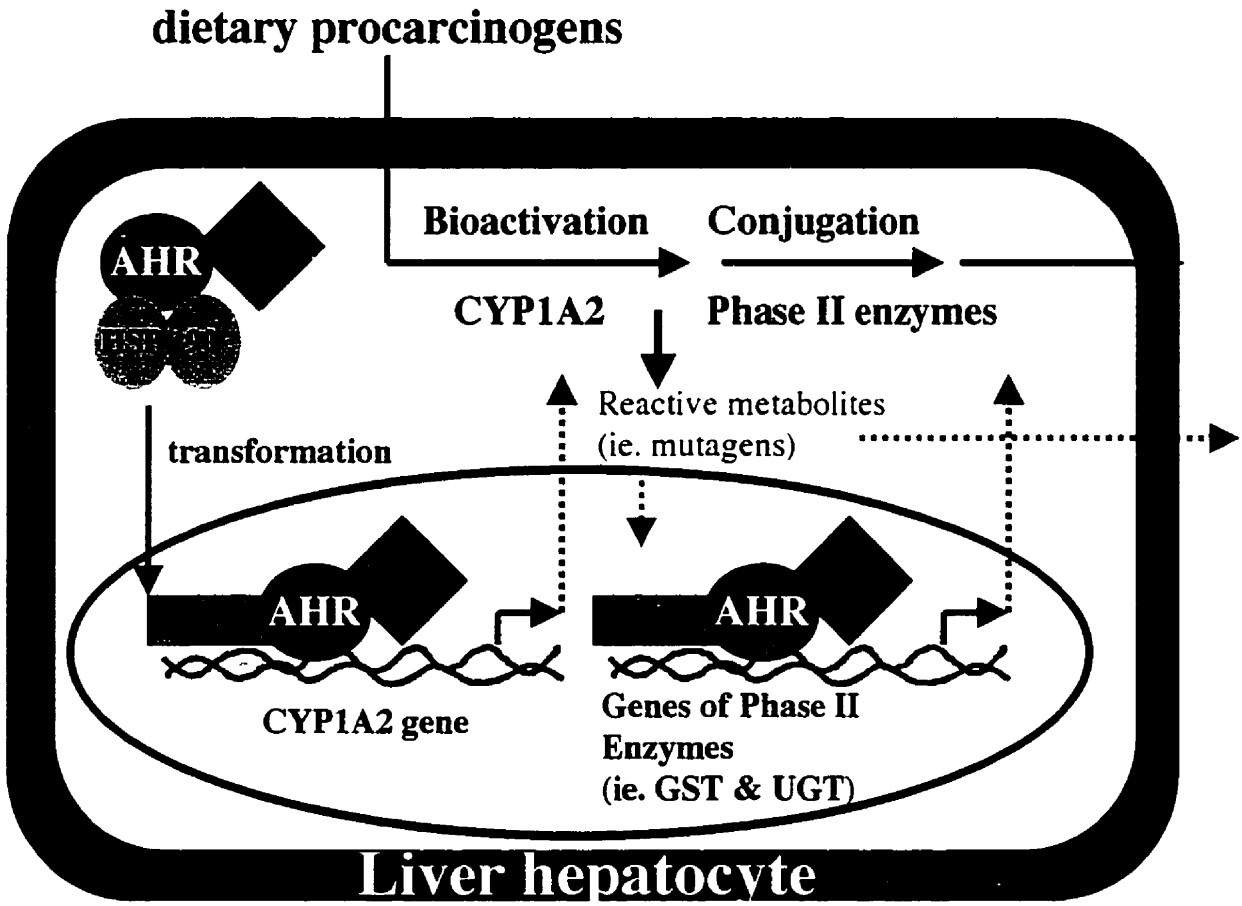
and mammary P450 enzymes and the resulting effects in these tissues. This in turn is determined by varying degrees of exposure to PCB no. 183. and possibly other constituents present in PCBs mixtures.

At low levels of PCB congener no. 183 (tertile 1), liver CYP1A2 induction may be the primary determinant of cancer susceptibility since extrahepatic CYP1A1 and CYP1B1 may be minimally induced at this concentration. Therefore, the more efficient inactivation of dietary chemicals by the variant C/C CYP1A2 renders this genotype beneficial against breast cancer at this exposure level (Figure 22).

The rationale behind the association of the C/C genotype with elevated cancer risks at moderate serum levels of this PCB may be more complicated. Moderate exposure (tertile 2) to PCB congener no. 183 may trigger the AHR pathway causing induction of hepatic CYP1A2 activity as well as extrahepatic breast epithelial CYP1A1 and CYP1B1, all of which bioactivate procarcinogens and play preeminent roles in estrogen metabolism. In the breast, elevated CYP1A1 and CYP1B1 enhance local bioactivation of BaP and other PAHs as well as the formation of the non-estrogenic 2-hydroxyestradiol metabolite of CYP1A1 and the genotoxic 4-hydroxyestradiol metabolite of CYP1B1. In addition, this concentration of PCB no. 183 may not be necessarily sufficient to induce mammary phase II enzymes to adequate levels for complete deactivation of local reactive chemicals and estradiol metabolites. Failure of adequate detoxification of these reactive chemicals would cause potential DNA damage and cellular toxicities of the mammary tissue (Figure 23). Under these conditions, mammary growth stimulation by 17β-estradiol and other estrogenic metabolites would promote mammary cancer development. This may be a plausible mechanism to explain the connection between CYP1A2 C/C genotype and increased breast cancer risk at moderate level of PCB.no183. With the *CYP1A2*1F* C/C genotype representing lower CYP1A2 inducibility (hence decreased 17β-estradiol 2-hydroxylation and serum estrogen clearance) increased bioavailability of the most potent 17β-estradiol may

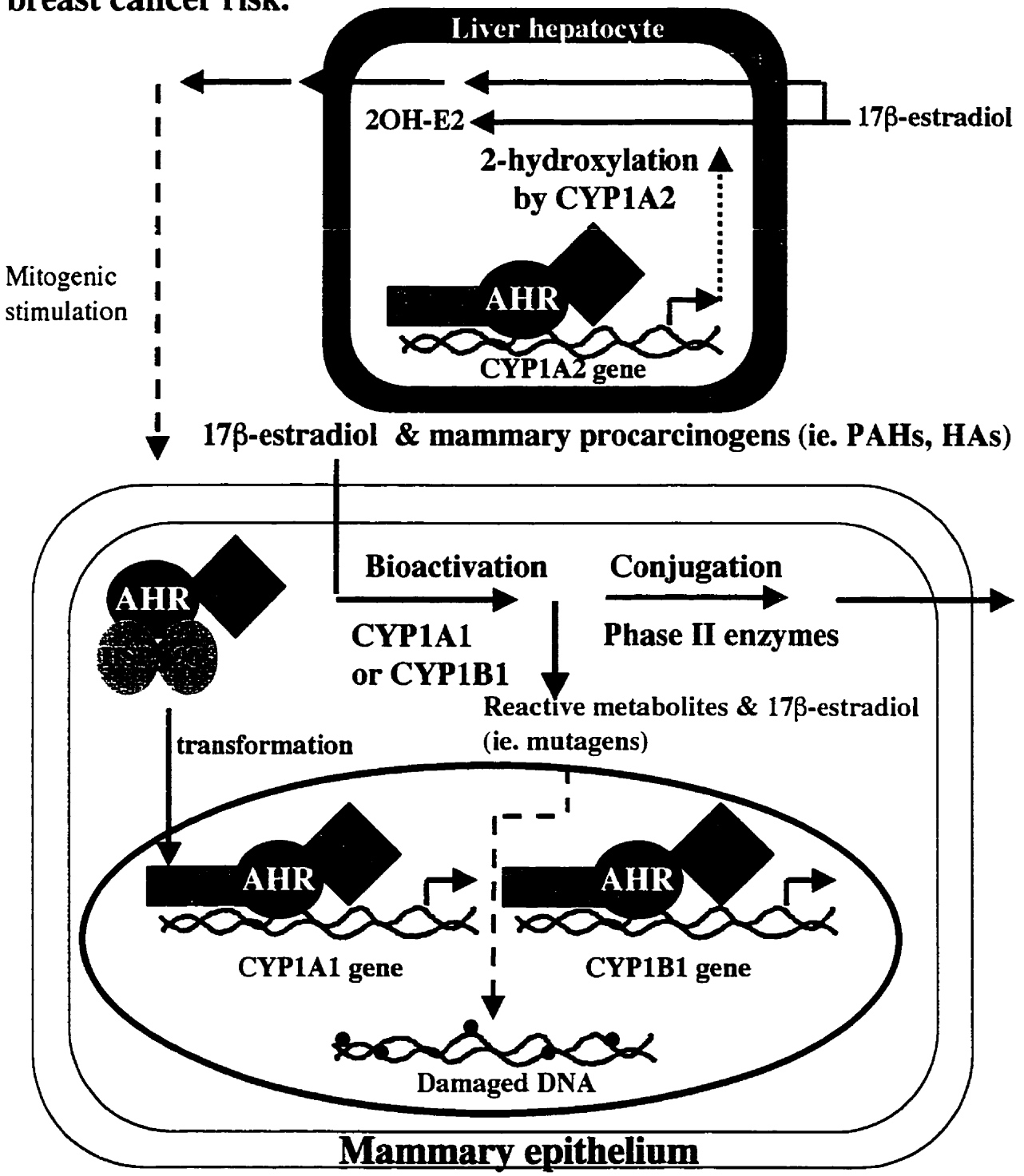
Figure 22

Plausible mechanism of action underlying the relationship between low and high exposure to PCB no. 183 in those with *CYP1A2*1F* C/C "low inducibility" genotype and decreased breast cancer susceptibility.



Complete deactivation of procarcinogens is dependent on a balance between phase II enzymes and that of CYP1A2 gene following exposure to AHR agonists. Regulation of Phase II genes may have evolved to couple levels of CYP1A2 conferred by the C/C "low inducibility" genotype but less with that conferred by the A/A "high inducibility" genotype thus, enhancing the formation of toxic reactive metabolites capable of damaging DNA and other macromolecules.

Figure 23: A possible mechanistic explanation for the association between the *CYP1A2*1F* C/C "low inducibility" genotype in combination with moderate exposure to PCB no. 183 and elevated breast cancer risk.



Lower inducibility of the *CYP1A2*1F* C/C genotype following exposure to PCB no. 183 may result in decreased hepatic CYP1A2-mediated inactivation of 17β-estradiol (2-hydroxylation). This leads to higher levels of circulating 17β-estradiol which stimulates proliferation of estrogen-responsive tissues (i.e. breast epithelium). Induction of mammary CYP1A1 and CYP1B1 enhances local activation of procarcinogens and subsequent DNA damage. Proliferation of such damaged cells promotes mammary carcinogenesis.

enhance cancer development of the "damaged" mammary tissue either by its mitogenic stimulation or by its mammary CYP1B1-dependent activation.

At higher levels of this PCB, extrahepatic mammary phase II conjugating enzymes may be induced to levels capable of counteracting the effects of induced mammary CYP1A1 and CYP1B1, thus once again making hepatic CYP1A2 activity the major influence of cancer susceptibility (Figure 22). It is not known whether this relative balance of P450s and phase II enzymes truly accounts for the observed positive and negative association of the C/C genotype with breast cancer risk at different serum levels of PCB congener no.183. Similarly, activation of the AHR pathway not only causes induction of drug-metabolizing enzymes but also differential regulation of other growth-related genes that may interact with P450 enzyme metabolism and contribute to altered cancer susceptibility. There is no clear linear dose-response relationship in our finding here. This may be attributed to the very low number of subjects having been exposed to this PCB and inherited the C/C genotype. However, we cannot rule out the possible existence of other kinds of dose-response relationship contributing to our observation. It is noteworthy that my interpretation is largely speculative because of the large number of factors involved and it is impossible to draw any firm conclusions. Further investigation is required to unravel the intricate molecular mechanism contributing to our finding.

The Asn/Asn homozygous genotype of the *CYP1B1*4* polymorphism was found to associate with reduced breast cancer risk when stratified for BMI≥27 kg/m^2 (Table App14). This "protective" association also was found in leaner women either with the Asn/Ser or Ser/Ser genotype (21<BMI<27). Body mass index of greater than 27 kg/m^2 is considered obese. Obesity is known to increase breast cancer risk among postmenopausal women (*144*) likely due to greater peripheral conversion (in adipose tissue) of precursor androgen steroid hormones to estradiol and estrone (*145, 146*). Subsequently, 17β-estradiol exerts growth-promoting effects and induces hormone-responsive cell

proliferation by binding to intracellular growth receptors and regulating gene transcription (*147, 148*). CYP1B1 is expressed both in normal and breast cancer tissues. Homozygosity for *CYP1B1*4* Asn allele may be related to decreased formation of the 4-hydroxyestradiol metabolite either through diminished CYP1B1 activity or altered regio-selectivity of the asparagine-encoded CYP1B1 enzyme leading to reduced 17β-estradiol 4-hydroxylation. The protective effect conferred by the Asn/Asn genotype may be more pronounced in obese women with elevated 17β-estradiol. Conversely, this Asn453 polymorphism itself may not directly affect breast cancer susceptibility. Instead, it may be linked to other polymorphisms in the *CYP1B1* gene that either abolish its activity or diverge its activity from estrogen 4-hydroxylation. Alternatively, reduced cancer risk may be due to decreased ability of the Asn453 variant enzyme to bioactivate PAHs that preferentially accumulate in adipose tissues. The differences between the Asn453 and Ser453 variants in their ability to bioactivate procarcinogens have not been assessed and hence are currently unknown. Given the same chemical exposure levels, obese individuals with higher fat content will have more PAHs and PCBs accumulated in their fat and adipose tissues. This may render obese individuals with decreased CYP1B1 bioactivating activity less susceptible to cancer such as that of the breast. Moreover, the polymorphism may also be interacting with other genes that reduce breast cancer risk. Nonetheless, these interpretations alone cannot explain the association in women with 21<BMI<27 and either a heterozygous or a homozygous genotype for the serine allele. It is possible that decreased bioactivation of xenobiotics accounts for the "protective" association of one genotype while lower 17β-estradiol 4-hydroxylation accounts for the "protective" association with the other genotype at a different BMI. In this case, the variant enzyme may exhibit increased activities towards PAH transformation but decreased ability to metabolize steroid hormone substrates (or vice versa). Consequently, these associations remain obscure and further functional studies will be required to clarify our results. Interestingly, a 2.4 fold higher 17β-

estradiol 4-hydroxylation activity of the Ser453 variant enzyme was recently reported (*134*). This finding may in part explain our observed "protective" effect of the Asn/Asn genotype due to reduced 4-hydroxyestradiol levels conferred by this genotype.

Overall, results of our current study support a relation between *AHR* and P450 polymorphisms to breast cancer risk. In particular, interactions between gene, environment and obesity are important areas that require further research.

Discovery of a new *AHR* sequence variation

I found a new *AHR* sequence variation in the exon-10 transactivation domain. The sequence variation was initially detected in our SSCP experiments. DNA sequencing revealed that this nucleotide change results in a non-conserved amino acid substitution. The frequency of this potential new polymorphism has not been examined in any other population. Our laboratory will assess its allele frequency prior to any further studies on this novel mutation. The human *AHR* appears to be remarkably conserved with very few genetic polymorphisms. This may be attributed to its crucial function in maintaining homeostasis following chemical insults. Any changes in receptor function may in turn, predispose individuals to elevated or reduced cancer risk. The overall biological function of this variant AHR remains to be elucidated. If this variation alters receptor function, it may also have important implications in cancer biology and dioxin-mediated toxicity. This is exemplified in rodents where *Ahr*-null mice were completely protected from tumor formation under conditions that induced tumors in nearly all *Ahr* wild-type and heterozygous mice injected with BaP (*85*).

CONCLUSION

The high incidence of breast cancer prompted investigators to focus their search on the genetic and environmental causes of the disease. Despite tremendous research in this area, clear definitive associations with many of the postulated human risk factors are scarce. Our study of genetic susceptibility to environmental and endogenous factors, such as estrogen metabolism, contribute to the identification of individuals at higher risk of the disease and to whom future preventive possibly pharmacological intervention, should be targeted.

The strengths of our study include the relatively large sample sizes, the design of genotype-phenotype correlations and association of genotypes with breast cancer risk, as well as the detailed collection of estrogen and caffeine metabolite ratio and other variables. To our knowledge, our large sample sizes and the combination of genotypes (*AHR*, *CYP1A2*, *CYP1B1*) studied represent the most comprehensive breast cancer case-control study and 17β-estradiol metabolism in relation to *AHR* and P450 genetic polymorphisms. Moreover, our case-control study was conducted in a Quebec French Canadian population, a population that is relatively homogenous in genetic background. Therefore, the potential confounding effect by ethnicity should not affect our results. Furthermore, our new, rapid and simple detection assays for *CYP1A2*1F*, *CYP1A2*2*, *CYP1B1*3* and *CYP1B1*4* genetic polymorphisms have great potential for their application to further epidemiologic studies on these genes and other disease outcomes. Finally, our case-control results are consistent with previous observations by other laboratories, with implications on the potential role of *AHR*, *CYP1A2* and *CYP1B1* in the etiology of human breast cancer.

A limitation of our case-control study is the lack of data on the PCB concentrations in the mammary tissue since serum PCBs levels may or may not correlate with levels in mammary adipose tissue. For instance, certain PCBs

may be mostly sequestered to the mammary tissue leaving undetectable residues in the serum making their associations with disease outcome elusive. Zheng *et al.* (*28*) have shown that serum levels of PCB no. 153 significantly correlate with levels in the breast tissue. Indeed, we found a link between PCB congener no.153 and breast cancer risk. PCB no. 183 was not examined in the Zheng *et al.* (*28*) study; hence whether serum levels of PCB no. 183 also reflect those in adipose tissue remains obscure. Moreover, for our Toronto healthy young women estrogen metabolism study, correlations between *CYP1B1* genotypes and the possible changes in the 4OH:2OH ratio were hampered by the lack of information on 17β-estradiol 4-hydroxylation. Measurement of the 4OH:2OH ratios would facilitate a correlation analysis, results of which could be invaluable in our understanding of the changes in *in vivo* estrogen metabolism conferred by the variant *CYP1B1* genotypes.

The AHR pathway is involved in multiple cellular functions including the regulation of growth-related genes, 17β-estradiol metabolism, bioactivation and detoxification of xenobiotics. AHR agonists not only induce the metabolism of endogenous 17β-estradiol but also compete with estradiol for their own metabolism. In addition to AHR agonists, there are also AHR antagonists (eg. resveratrol) that compete for the AHR binding site. This is further complicated by the fact that CYP1 genes display different expression patterns with distinct tissue-specificity. Furthermore, the induction profile of these enzymes also differs upon AHR activation by different agonists. Together, the broad and overlapping substrate specificity, the dual function of P450s in the metabolism of endogenous and exogenous chemicals as well as their regulation by the common AHR demonstrate the potential complex interplay between these pathways which ultimately modify breast cancer susceptibility. For this reason, my genotyping data will be further assessed for the relationship between multiple intragenic and intergenic polymorphisms of these three genes to CYP1A2 activity, 17β-estradiol metabolism and breast cancer risk. Our findings

will merit further studies on gene-environment interactions and breast cancer susceptibility.

SUMMARY OF MAJOR FINDINGS

Allele frequencies in the Toronto population

(1) The distribution of the *CYP1A2*1C*, *CYP1A2*1F*, *CYP1B1*3* and *CYP1B1*4* alleles are not restricted to the original populations in which these polymorphisms initially were detected. These polymorphisms are present in all ethnic populations studied.

(2) The predominant allele of each polymorphism was determined based on allele frequency data.

(3) As expected, the allele frequencies of *AHR*517 and *AHR*570 were very low (f<0.01). The *AHR*554 polymorphism is present in all ethnic populations tested but has varying allele frequencies.

Project I: Effects of polymorphisms on CYP1A2 activity and estrogen metabolism

(1) Comparisons between the mean caffeine index of *CYP1A2*1F* C/A and A/A genotypes showed reduced CYP1A2 activity conferred by the A/A genotype with p=0.056, approaching statistical significance.

(2) The *AHR* 554 Lys/Lys genotype was found to be significantly associated with a 9.7 fold decrease in the mean 2OH:16αOH estrogen metabolite ratio.

(3) The *CYP1B1*3* Val/Val genotype was found to confer a decreased mean 2OH:16αOH estrogen metabolite ratio compared to the genotypes with at least one leucine allele. The decrease approached statistical significance, (p=0.062).

(4) A significant correlation was detected between the caffeine indexes and the 2OH:16αOH estrogen metabolite ratios demonstrating the involvement of CYP1A2 in both metabolic processes.

Project II: French Canadian breast cancer case-control study

(1) A relation was found between breast cancer risk and women with family history of breast cancer in combination with the *CYP1B1*3* Val/Val genotype.

(2) The *AHR*554 Arg/Arg genotype was found to be associated with decreased breast cancer risk in women exposed to low levels of PCB congener no. 153.

(3) A connection between *CYP1A2*1F* C/C genotype was found between breast cancer susceptibility and varying serum levels of PCB congener no. 183.

(4) A relationship was detected between reduced breast cancer risk in obese (BMI≥27 kg/m^2) individuals with the *CYP1B1*4* Asn/Asn genotype. Another association was found in leaner women (21<BMI<27) with the Ser/Asn or Ser/Ser genotypes.

(5) A new sequence variation was found in the exon-10 transactivation domain of AHR with corresponding amino acid substitution by a non-conserved residue.

Appendices

Table App1

Relationship of *AHR* 554 polymorphism and mean natural logarithm of caffeine index.

AHR 554	Arg/Arg n=118	Arg/Lys n=16	Lys/Lys n=4
ln (mean caffeine index ± SD)	1.62±0.37	1.55±0.38	1.88±0.36
	p=0.288		
	p=0.179		
		p=0.107	
	p=0.149		
Correlation coefficient (r_s)	-0.025		
P value	0.749		
Total (n)	168		

Data analyzed by Dr. Helena Jernstrom.

Table App2

Relationship of *CYP1A2*1C* polymorphism and mean natural logarithm of caffeine index.

*CYP1A2*1C*	G/G n=141	G/A n=22	A/A n=3
ln (mean caffeine index ± SD)	1.61±0.38	1.63±0.34	1.33±0.42
	p=0.804		
	p=0.203		
		p=0.165	
	p=0.190		
Correlation coefficient (r_s)	-0.011		
P value	0.887		
Total (n)	166		

Data analyzed by Dr. Helena Jernstrom.

Table App3

Relationship of *CYP1A2*1F* polymorphism and mean natural logarithm of caffeine index.

*CYP1A2*1F*	C/C n=18	C/A n=78	A/A n=72
ln (mean caffeine index ± SD)	1.52±0.42	1.67±0.38	1.56±0.35
	p=0.136		
	p=0.701		
		p=0.056	
Correlation coefficient (r_s)	-0.060		
P value	0.436		
Total (n)	168		

Data analyzed by Dr. Helena Jernstrom.

Table App4

Relationship of *AHR* 554 polymorphism and mean natural logarithm of 2-hydroxyestradiol/16α-hydroxyestradiol urinary metabolite ratio.

AHR 554	Arg/Arg n=340	Arg/Lys n=137	Lys/Lys n=22
ln (mean 2OH:16αOH ± SD)	0.31±0.58	0.28±0.62	0.032±0.52
	p=0.549		
	p=0.026*		
		p=0.077	
Correlation coefficient (r_s)	-0.082		
P value	0.069		
Total (n)	499		

Data analyzed by Dr. Helena Jernstrom.

Table App5

Relationship of *AHR* 570 polymorphism and mean natural logarithm of 2-hydroxyestradiol/16α-hydroxyestradiol urinary metabolite ratio.

AHR 570	Val/Val n=493	Val/Ile n=3
ln (mean 2OH:16αOH ± SD)	0.29±0.59	0.17±0.29
	p=0.73	
Correlation coefficient (r_s)	-0.024	
P value	0.600	
Total (n)	496	

Data analyzed by Dr. Helena Jernstrom.

Table App6

Relationship of *CYP1A2*1C* polymorphism and mean natural logarithm of 2-hydroxyestradiol/16α-hydroxyestradiol urinary metabolite ratio.

*CYP1A2*1C*	G/G n=429	G/A n=61	A/A n=8
ln (mean 2OH:16αOH ± SD)	0.31±0.58	0.18±0.50	0.54±0.94
	p=0.271		
		p=0.088	
Correlation coefficient (r_s)	-0.077		
P value	0.085		
Total (n)	598		

Data analyzed by Dr. Helena Jernstrom.

Table App7

Relationship of *CYP1A2*1F* polymorphism and mean natural logarithm of 2-hydroxyestradiol/16α-hydroxyestradiol urinary metabolite ratio.

*CYP1A2*1F*	C/C n=55	C/A n=216	A/A n=228
ln (mean 2OH:16αOH ± SD)	0.21±0.55	0.31±0.55	0.29±0.63
Correlation coefficient (r_s)	0.019		
P value	0.676		
Total (n)	499		

p=0.262
p=0.377
p=0.822
p=0.298

Data analyzed by Dr. Helena Jernstrom.

Table App8

Relationship of *CYP1B1*3* polymorphism and mean natural logarithm of 2-hydroxyestradiol/16α-hydroxyestradiol urinary metabolite ratio.

*CYP1B1*3*	Val/Val	Leu/Val n=194	leu/Leu n=234
ln (mean 2OH:16αOH ± SD)	0.36±0.60	0.33±0.57	0.24±0.59
	p=0.776		
	p=0.143		
		p=0.109	
	p=0.062		
Correlation coefficient (r_s)	-0.072		
P value	0.109		
Total (n)	502		

Data analyzed by Dr. Helena Jernstrom.

Table App9

Relationship of *CYP1B1*4* polymorphism and mean natural logarithm of 2-hydroxyestradiol/16α-hydroxyestradiol urinary metabolite ratio.

*CYP1B1*4*	Asn/Asn n=352	Asn/Ser n=125	Asn/Ser n=14
ln (mean 2OH:16αOH ± SD)	0.29±0.61	0.31±0.55	0.29±0.39
	p=0.729		
	p=0.964		
		p=0.927	
Correlation coefficient (r_s)	0.000		
P value	1.000		
Total (n)	494		

Data analyzed by Dr. Helena Jernstrom.

Table App10

Factors stratified for in the French Canadian case-control study.

Menopausal status	menopaused / not menopaused
Family history of breast cancer	yes / no
Personal history of breast benign disease	yes / no
Had at least one child	yes / no
Breast fed at least one child	yes / no
Body Mass Index (BMI)	- lean: BMI ≤ 21 - average: 21< BMI < 27 - obese: BMI ≥ 27
Cigarette smoking	never / ever
Alcohol consumption	- no - 0 to 5 drinks / week - more than 5 drinks / week
Organochlorines	DDE, DDT, HCB, oxychlordane, *trans*-nonachlor, HCH, PCB congeners no 99, 118, 138, 153, 156, 170, 180, 183, 187
Genes and polymorphisms	*AHR* 554, *CYP1A2*1F*, *CYP1B1*3*, *CYP1B1*4*

Table App11:

Breast cancer risk of women having family history of breast cancer according to the genotypes.

Genes	Alleles	*(cases/controls-)* OR (95% CI)[a]	
*AHR*554	Lys/Lys + Arg/Lys	*(23/120)*	1.00
	Arg/Arg	*(100/498)*	1.03 (0.63-1.69)
*CYP1A2*1F*	A/A	*(62/343)*	1.00
	C/A	*(54/230)*	1.29 (0.86-1.94)
	C/C	*(11/72)*	0.86 (0.43-1.72)
CYP1B1*3	Leu/Leu	*(27/183)*	1.00
	Leu/Val	*(48/240)*	1.38 (0.83-2.31)
	Val/Val	*(30/114)*	1.85 (1.04-3.28)
*CYP1B1*4*	Ser/Ser + Asn/Ser	*(45/206)*	1.00
	Asn/Asn	*(81/432)*	0.85 (0.57-1.28)

a) Odd ratios (OR) are adjusted for age and region of residence.

This table is provided by Dr. Alain Demers.

Table App12

Modification effect of polymorphisms on the association between PCB congener no. 153 and breast cancer risk.

Genes	Alleles	PCB congener no 153						Total	Chisq (df)	*P* value
		tertile 1 (low)		tertile 2 (moderate)		tertile 3 (high)				
		(cases/controls)	OR (95% CI)[a]	*(cases/controls)*	OR (95% CI)	*(cases/controls)*	OR (95% CI)	OR (95% CI)		
AHR554	Lys/Lys+ Arg/Lys	*(23/25)*	1.00	*(14/32)*	0.42 (0.17-1.02)	*(23/29)*	0.85 (0.36-1.96)	1.00	6.79 (2)	**0.03**
	Arg/Arg	*(50/134)*	**0.40 (0.20-0.79)**	*(78/132)*	0.60 (0.31-1.18)	*(74/135)*	0.54 (0.27-1.08)	0.71 (0.49-1.05)		
	Total		1.00		1.16 (0.77-1.73)		1.22 (0.79-1.87)			
*CYP1A2*1F*	A/A	*(41/84)*	1.00	*(55/87)*	1.23 (0.73-2.09)	*(56/87)*	1.27 (0.74-2.19)	1.00	1.77 (4)	0.78
	C/A	*(29/63)*	1.01 (0.55-1.83)	*(32/67)*	0.95 (0.53-1.71)	*(35/59)*	1.12 (0.61-2.05)	0.87 (0.63-1.21)		
	C/C	*(6/21)*	0.60 (0.22-1.65)	*(10/16)*	1.40 (0.56-3.49)	*(11/22)*	0.94 (0.40-2.19)	0.80 (0.48-1.33)		
	Total		1.00		1.19 (0.80-1.76)		1.23 (0.81-0.87)			
*CYP1B1*3*	Leu/Leu	*(22/44)*	1.00	*(23/43)*	0.92 (0.43-1.96)	*(31/49)*	1.08 (0.52-2.28)	1.00	2.17 (4)	0.71
	Leu/Val	*(27/57)*	0.80 (0.39-1.62)	*(37/69)*	0.94 (0.48-1.85)	*(40/57)*	1.24 (0.62-2.50)	0.99 (0.67-1.45)		
	Val/Val	*(17/35)*	0.79 (0.35-1.77)	*(25/32)*	1.31 (0.61-2.82)	*(13/25)*	0.90 (0.37-2.22)	1.00 (064-1.58)		
	Total		1.00		1.19 (0.78-1.81)		1.31 (0.83-2.08)			
*CYP1B1*4*	Ser/Ser + Asn/Ser	*(23/48)*	1.00	*(34/53)*	1.29 (0.65-2.58)	*(31/62)*	0.98 (0.49-2.00)	1.00	1.29 (2)	0.53
	Asn/Asn	*(54/118)*	1.00 (0.54-1.84)	*(64/115)*	1.12 (0.61-2.07)	*(69/102)*	1.32 (0.70-2.48)	1.05 (0.76-1.46)		
	Total		1.00		1.18 (0.79-1.75)		1.20 (0.79-1.83)			

(3) Odd ratios (OR) are adjusted for age, region of residence, BMI, benign breast disease and breast feeding duration.
(4) The Chi square test tests if OR are homogenous through all stratums.

This table is provided by Dr. Alain Demers.

Table App13

Modification effect of polymorphisms on the association between PCB congener no. 183 and breast cancer risk.

Genes	Alleles	PCB congener no 183			Total	Chisq (df) [b]	*P* value
		tertile 1 (low)	tertile 2 (moderate)	tertile 3 (high)			
		(cases/controls) OR (95% CI)[a]	*(cases/controls)* OR (95% CI)	*(cases/controls)* OR (95% CI)	OR (95% CI)		
*AHR*554	Lys/Lys + Arg/Lys	*(16/27)* 1.00	*(19/30)* 1.01 (0.42-2.45)	*(22/25)* 1.50 (0.61-3.70)	1.00	2.09 (2)	0.35
	Arg/Arg	*(51/122)* 0.71 (0.34-1.48)	*(79/131)* 0.96 (0.47-1.97)	*(59/132)* 0.71 (0.34-1.50)	0.69 (0.47-1.02)		
	Total	1.00	1.26 (0.84-1.90)	1.09 (0.70-1.69)			
CYP1A2*1F	A/A	*(41/78)* 1.00	*(54/89)* 1.09 (0.64-1.86)	*(48/83)* 1.03 (0.58-1.80)	1.00	14.91 (4)	**0.005**
	C/A	*(24/58)* 0.82 (0.43-1.54)	*(34/64)* 0.93 (0.52-1.68)	*(33/57)* 1.04 (0.56-1.92)	0.89 (0.63-1.25)		
	C/C	*(3/22)* **0.25 (0.07-0.90)**	*(17/12)* **2.57 (1.08-6.11)**	*(4/23)* **0.31 (0.10-0.99)**	0.73 (0.43-1.24)		
	Total	1.00	1.37 (0.92-2.04)	1.13 (0.73-1.75)			
*CYP1B1*3*	Leu/Leu	*(20/42)* 1.00	*(24/47)* 0.99 (0.46-2.12)	*(26/39)* 1.31 (0.60-2.86)	1.00	7.98 (4)	0.09
	Leu/Val	*(24/45)* 1.03 (0.49-2.20)	*(38/78)* 0.91 (0.46-1.81)	*(36/52)* 1.36 (0.65-2.81)	0.99 (0.67-1.47)		
	Val/Val	*(14/33)* 0.75 (0.32-1.77)	*(27/26)* 1.94 (0.87-4.30)	*(11/30)* 0.73 (0.29-1.82)	1.01 (0.63-1.61)		
	Total	1.00	1.18 (0.76-1.82)	1.25 (0.77-2.02)			
*CYP1B1*4*	Ser/Ser + Asn/Ser	*(22/45)* 1.00	*(35/50)* 1.39 (0.69-2.79)	*(26/61)* 0.84 (0.41-1.74)	1.00	1.29 (2)	0.53
	Asn/Asn	*(46/111)* 0.89 (0.47-1.69)	*(70/111)* 1.23 (0.67-2.29)	*(58/100)* 1.13 (0.60-2.14)	1.03 (0.73-1.44)		
	Total	1.00	1.39 (0.93-2.06)	1.11 (0.72-1.71)			

(1) Odd ratios (OR) are adjusted for age, region of residence, BMI, benign breast disease and breast feeding duration.
(2) The Chi square test tests if OR are homogenous through all stratums.

This table is provided by Dr. Alain Demers.

Table App14

Modification effect of the BMI on the association between breast cancer risk and the different genotypes.

Genes	Alleles	BMI (kg/m²)						Total	Chisq (df)	*P* value
		≤ 21		21< BMI < 27		≥ 27				
		(cases/controls)	OR (95% CI)[a]	*(cases/controls)*	OR (95% CI)	*(cases/controls)*	OR (95% CI)	OR (95% CI)		
*AHR*554	Lys/Lys + Arg/Lys	*(11/11)*	1.00	*(33/48)*	0.72 (0.28-1.87)	*(16/28)*	0.58 (0.20-1.68)	1.00	0.26 (2)	0.88
	Arg/Arg	*(42/63)*	0.72 (0.28-1.85)	*(104/222)*	0.47 (0.20-1.14)	*(57/118)*	0.48 (0.19-1.18)	0.72 (0.49-1.04)		
	Total		1.00		0.67 (0.44-1.02)		0.65 (0.41-1.04)			
*CYP1A2*1F*	A/A	*(32/32)*	1.00	*(77/146)*	0.49 (0.28-0.87)	*(44/82)*	0.49 (0.26-0.92)	1.00	3.86 (4)	0.42
	C/A	*(18/33)*	0.53 (0.25-1.14)	*(50/99)*	0.48 (0.26-0.87)	*(28/58)*	0.43 (0.22-0.84)	0.85 (0.62-1.18)		
	C/C	*(4/12)*	0.31 (0.09-1.09)	*(17/30)*	0.52 (0.24-1.13)	*(6/17)*	0.37 (0.13-1.07)	0.79 (0.48-1.31)		
	Total		1.00		0.71 (0.47-1.07)		0.66 (0.42-1.04)			
*CYP1B1*3*	Leu/Leu	*(19/26)*	1.00	*(32/75)*	0.62 (0.30-1.30)	*(25/36)*	0.97 (0.44-2.14)	1.00	4.09 (4)	0.39
	Val/Leu	*(17/24)*	1.08 (0.45-2.59)	*(62/102)*	0.83 (0.42-1.63)	*(25/59)*	0.59 (0.28-1.28)	0.99 (0.68-1.44)		
	Val/Val	*(12/14)*	1.33 (0.49-3.57)	*(29/44)*	0.92 (0.43-1.97)	*(15/33)*	0.60 (0.25-1.41)	1.08 (0.70-1.68)		
	Total		1.00		0.71 (0.46-1.11)		0.64 (0.39-1.05)			
CYP1B1*4	Ser/Ser + Asn/Ser	*(26/24)*	1.00	*(38/99)*	**0.36 (0.18-0.71)**	*(24/43)*	0.52 (0.24-1.11)	1.00	7.63 (2)	**0.02**
	Asn/Asn	*(27/52)*	0.52 (0.25-1.08)	*(107/171)*	0.58 (0.31-1.06)	*(54/112)*	**0.43 (0.22-0.83)**	1.04 (0.76-1.43)		
	Total		1.00		0.73 (0.49-1.11)		0.67 (0.43-1.06)			

(1) Odd ratios (OR) are adjusted for age and region of residence.
(2) The Chi square test tests if OR are homogenous through all stratums.

This table is provided by Dr. Alain Demers.

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