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Polymorphisms in the carcinogen detoxification genes *CYB5A* and *CYB5R3* and breast cancer risk in African American women

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Abstract

Purpose—Cytochrome b_5 (encoded by *CYB5A*) and NADH cytochrome b_5 reductase (encoded by *CYB5R3*) detoxify aromatic and heterocyclic amine mammary carcinogens found in cigarette smoke. We hypothesized that *CYB5A* and *CYB5R3* polymorphisms would be associated with breast cancer risk in women.

Methods—We characterized the prevalence of 18 *CYB5A* and *CYB5R3* variants in genomic DNA from African American (AfrAm) and Caucasian (Cauc) women from the Carolina Breast Cancer Study population (1946 cases and 1747 controls), and determined their associations with breast cancer risk, with effect modification by smoking.

Results—A *CYB5R3* variant, I1M+6T (rs8190370) was significantly more common in breast cancer cases (MAF 0.0238) compared to controls (0.0169, P =0.039); this was attributable to a higher MAF in AfrAm cases (0.0611) compared to AfrAm controls (0.0441, P=0.046; adjusted OR 1.41, CI 0.98-2.04; P=0.062). When smoking was considered, I1M+6T was more strongly associated with breast cancer risk in AfrAm smokers (adjusted OR 2.10, 1.08-4.07; P=0.028) compared to never-smokers (OR=1.21; 0.77-1.88; P for interaction=0.176). I1M+6T and three additional *CYB5R3* variants, -251T, I8-1676C, and *392C, as well as two *CYB5A* variants, 13G and I2-992T, were significantly more common in AfrAms compared to Caucs.

Conclusions—*CYB5R3* I1M+6 C>T should be considered in future molecular epidemiologic studies of breast cancer risk in AfrAms. Further, variants in *CYB5A* and *CYB5R3* should be considered in the evaluation of other tumors in AfrAms that are associated with aromatic and heterocyclic amine exposures, to include prostate, bladder, and colon cancers.

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Conflicts of Interest: The authors assert that they have no relationships that could be construed as resulting in an actual, potential, or perceived conflict of interest relative to the work in this manuscript.

Keywords

Heterocyclic amines; aromatic amines; PhIP; 4-aminobiphenyl; smoking

Introduction

Breast cancer has a high incidence in industrialized countries, and women who move from low-risk to high-risk countries acquire a breast cancer risk of the host country in as little as two generations [1,2]. Environmental factors including diet, smoking, and pollutants appear to play an etiologic role. The aromatic amine 4-aminobiphenyl (4-ABP), is a mammary carcinogen in rodents that is found in cigarette smoke [3,4], and the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP), is another mammary carcinogen found in cigarette smoke and well done meats [5-7]. Both 4-ABP and PhIP lead to DNA adducts that have been found in the breast tissue and milk of women [8,9]. These adducts have also been correlated with smoking exposures [10,11]. Further, some studies have found higher levels of DNA adducts in breast cancer patients versus controls [12,13], although an established relationship between these DNA adducts and breast cancer remains to be proven. Epidemiologic studies of smoking and breast cancer risk have been mixed, with some studies finding modest positive associations [14-20], and other studies yielding no association [21-28]. These inconsistent results could be due, in part, to individual differences in the disposition of tobacco carcinogens after similar smoking exposures.

Both PhIP and 4-ABP are bioactivated to arylhydroxylamine metabolites [29-31], which ultimately form mutagenic DNA adducts (Figure 1). The disposition of these arylhydroxylamines appears to vary among individuals, in that exposure of individual primary human mammary epithelial cell lines to standard concentrations of PhIP hydroxylamine results in more than 75-fold variability in DNA adduct formation [32]. The hydroxylamines of PhIP and 4-ABP are reduced back to their parent compounds, which are not directly mutagenic, by cytochrome b_5 (b5) and NADH cytochrome b_5 reductase (b5R) [33]. Reduction of arylhydroxylamines by this pathway is substantially more efficient than generation of these metabolites by oxidation of the parent compound [34], which suggests that variability in the b5/b5R pathway could modulate the amount of arylhydroxylamine available for DNA adduct formation.

We have found individual differences in cytochrome b_5 and NADH cytochrome b_5 reductase protein expression and arylhydroxylamine detoxification activities in both human liver and breast samples, with more than 75-fold variability in activities in 70 breast samples from women undergoing reduction mammoplasty or lumpectomy [35,36]. We further found polymorphisms in the genes *CYB5A* and *CYB5R3*, which encode b5 and b5R, in tissues with outlier low protein expression and activities [35,36]. Many of these variants were found only or predominantly in African American samples.

Based on these findings, we hypothesized that polymorphisms in *CYB5A* or *CYB5R3* contribute to breast cancer risk, particularly in women who smoke. We further hypothesized that such polymorphisms would be more common in African American compared to Caucasian women. The purpose of this study, therefore, was to screen for the prevalence of

18 *CYB5A* and *CYB5R3* variants in African American and Caucasian women with breast cancer (invasive or carcinoma *in situ* (CIS)), compared to age-matched unaffected controls, using the Carolina Breast Cancer Study population [37], and to determine whether *CYB5A* or *CYB5R3* polymorphisms were associated with breast cancer risk in relationship to smoking.

Materials and Methods

Carolina Breast Cancer Study population

The Carolina Breast Cancer Study (CBCS) is a case-control study population of women with breast cancer from 24 counties of central and eastern North Carolina [37]. Women who were diagnosed with invasive breast cancer or CIS between the ages of 20-74 were identified through the North Carolina Central Cancer Registry; population-based controls were frequency matched to cases by age (within 5 years) and race [37]. Race was self-reported, and 144 ancestry informative markers (AIMs) were used to estimate West African genetic ancestry and control for population stratification [38]. The majority of subjects in the CBCS were Caucasian (Cauc) or African American (AfrAm) [39], with 1.5% of the population from Native American, Asian, Hispanic, or multi-racial groups. Only Cauc and AfrAm subjects were analyzed in the present study, to include DNA samples from 1946 breast cancer cases (742 AfrAm and 1204 Cauc) and 1747 controls (658 AfrAm and 1089 Cauc). Smoking history was recorded for all subjects, to include active status, packs per day, duration, and time since cessation [27]. Characteristics of CBCS participants included in the study are listed in Table 1.

Genotyping

Genomic DNA samples from all women were genotyped for 18 single nucleotide polymorphisms (SNPs), 8 in *CYB5A* (Table 2) and 10 in *CYB5R3* (Table 3). SNPs were selected based on the following criteria: 1) a variant previously observed in breast or liver samples with low protein expression or arylhydroxylamine reduction activities [35,36]; 2) a reported non-synonymous cSNP (NCBI SNP database; www.ncbi.nlm.nih.gov/); or 3) a reported SNP in a region predicted to affect protein function, splicing, or transcription factor or miRNA binding, even if apparently rare. For the latter predictions, the possible effects of coding polymorphisms on protein function were evaluated using PMut (mmb.pcb.ub.es/PMut/), SIFT (sift.jcvi.org/), and PolyPhen-2 (genetics.bwh.harvard.edu/pph2/ index.shtml) software. The effects of intronic variants on splicing were modeled with the Human Splicing Finder suite of software (HSF, MaxEnt, and ESE Finder; www.umd.be/HSF/). The influence of variants on transcription factor binding was predicted with the MATCH program (www.bioinfo.de/isb/gcb01/poster/goessling.html) and the ENCODE data base [40] (http://genome.cse.ucsc.edu/encode/), and the effects of 3′UTR (untranslated region) SNPs on miRNA binding were predicted with UTRscan (itbtools.ba.itb.cnr.it/utrscan).

The PCR-based Taqman Genotyping Assay (Applied Biosytems, Foster City, CA) was utilized for most polymorphisms, and was performed at the University of North Carolina at Chapel Hill. SNPs that failed the Taqman assay were evaluated by pyrosequencing, using the PSQ^{TM}96MA System (Biotage AB, Uppsala, Sweden), at the University of Wisconsin

Carbone Comprehensive Cancer Center. Both SNP screening techniques were validated by running positive and negative genomic DNA controls from liver or breast samples, where available, in which the allele of interest had been previously established by direct sequencing [35,36]. A total of 144 ancestry informative markers (AIMs) were also genotyped to estimate African and European ancestry [41]. These AIMs were selected from a panel that has been used by others to estimate ancestry in African Americans [42,43].

Data analyses

Prior to association analysis, SNPs were checked for genotyping efficiency and tested for deviation from genotype proportions expected within control groups, stratified by race under Hardy-Weinberg equilibrium (HWE) conditions using Chi Square testing. Minor allele frequencies (MAFs) and genotypes for each SNP were then compared between AfrAm and Cauc women in the control groups, and between women with breast cancer and controls (both across all subjects and within the two racial groups), using Chi Square testing with $P < 0.05$. Breast cancer risk was calculated using unconditional logistic regression (SAS software program) in order to quantify the association with *CYB5A* and *CYB5R3* genotypes, with adjustment for age and African ancestry. An offset term was included in models to account for randomized recruitment sampling. The sample size provided 80% power to detect odds ratios of 1.2 or greater for alleles with a frequency of 5% or greater in the population overall. We also estimated odds ratios stratified by smoking status (ever, never). As an exploratory analysis, we modeled multiplicative interactions between genotype and smoking using a likelihood ratio test; however, power for testing interactions between genotype and smoking, stratified by race, was fairly limited.

Results

Allele detection

Five previously reported *CYB5A* variants, rs36082929 (-206G>-), rs74339771 (65A>G), rs1803366 (155G>A), rs78009726 (178A>G, Thr60Ala), and rs76241580 (*246T>C) were not detected in the CBCS population. These rare variants have not been found in population screens reported in either the NCBI or the 1000 Genomes databases (www.1000genomes.org/). Two *CYB5R3* variants, rs111154229 (176G>A), and rs76458556 (890G>A), previously found by direct resequencing of liver cDNA samples [35], were also not detected in the CBCS population. Both appear to be rare; 176G>A has not been reported in other SNP databases (either NCBI or 1000 Genomes), while 890G>A was reported with an MAF of only 0.002 overall (browser.1000genomes.org). All detected alleles were in HWE in the AfrAm population. Two variants, rs7284807 (*392 G>C) and rs1790894 (I2 -992 C>T) were not in HWE in Cauc controls, and were not analyzed further.

Allele frequencies by cancer outcome

Allele frequencies for the remaining 11 variants were compared between breast cancer cases and controls, both overall and within racial groups. *CYB5R3* I1M+6C>T was significantly more common in breast cancer cases compared to controls (MAF 0.0238 versus 0.0169, P=0.039), with an odds ratio of 1.42 (0.99-2.04), P=0.057. This difference was attributable to a significantly higher prevalence in AfrAm cases compared to AfrAm controls (MAF

0.0611 versus 0.0441, P=0.046; OR 1.41 (0.98-2.04); P=0.062, Tables 4 and 5). An elevated, but very imprecise odds ratio was found between I1M+6C>T and breast cancer in Cauc women (OR 1.57 (0.13-19.71), P=0.726)), due to very low allele frequencies. No other variant was significantly different between cases and controls, either in the population overall or as stratified by race.

Breast cancer risk was further evaluated for I1M+6C>T in relationship to smoking status (Table 5). Because of relatively low numbers of carriers for most alleles, smoking status was collapsed by necessity into two separate categories, ever smokers and never smokers (defined as less than 100 lifetime cigarettes). The *CYB5R3* I1M+6T allele was significantly associated with breast cancer risk in ever smokers (OR 1.97, 95% CI 1.03-3.77), and in particular in AfrAm ever smokers (OR 2.10, 95% CI 1.08-4.07). This relationship was not as strong among AfrAm never smokers (OR 1.21, 95% CI 0.77-1.88; P = 0.4096). In Cauc women, allele frequencies for I1M+6T were too low to determine risk when stratified by smoking status (Table 5). Interactions between the I1M+6 genotype and smoking for breast cancer risk were also modeled. The P value for interactions was 0.162 for all subjects and 0.176 for the AfrAm population.

In silico analysis of the 1M+6 locus

In silico analyses were performed on the IM+6 polymorphic locus. The reference C allele was conserved among primates, rodents, and rabbits (UCSC Genome Browser; genome.ucsc.org). The T variant was predicted by Human Splicing Finder to create a cryptic splice site 4 nucleotides downstream from the natural donor site at the junction between alternative exon 1M and intron 1. The resulting mRNA was predicted to be 4 bp longer than wild type (NCBI Ref Seq ID: NM_000398), and to create a frame-shift leading to a premature stop codon. Further, this transcript was predicted to undergo nonsense-mediated decay, since the predicted stop codon was located more than 50 nucleotides upstream of the exon 2/3 splice junction [44].

Allele frequencies by race

Minor allele frequencies for *CYB5A* and *CYB5R3* SNPs by race are shown in Table 4. I1M +6C>T was almost 90-fold more common in AfrAm compared to Cauc controls (MAF 0.0441 versus 0.0005, P < 0.0001). Three other *CYB5R3* variants were also over-represented in AfrAm versus Cauc controls. The promoter variant -251G>T was ∼140-fold more prevalent in AfrAm women (MAF 0.0727; P < 0.0001), and was predicted to delete binding sites for several transcription factors (Table 3). The intronic SNP I8-1676T>C was more than 100-fold more prevalent in AfrAm women (MAF 0.1531, P < 0.0001), and was within an experimentally demonstrated binding site for the DNA repair protein RAD21. The 3′UTR variant *392G>C was found with an MAF of 0.1353 in AfrAm women, but is of unknown functional significance.

As for *CYB5A*, two variants were more prevalent in AfrAm women (Table 4). The intronic SNP I2-992T was found with a MAF of 0.1689 in AfrAm controls, and was predicted to be within a binding site for NF-κB. The non-synonymous cSNP 13T>G (Ser5Ala) was found only in AfrAm subjects, but with a very low frequency (MAF 0.0069).

Discussion

4-ABP and PhIP are mammary carcinogens that are found in tobacco smoke. These chemicals are bioactivated to their arylhydroxylamine metabolites, which lead to DNA adducts that are thought to initiate cancer [29-31]. Cytochrome b_5 and its reductase oppose this bioactivation step; this pathway, found in both liver and breast tissues, is a potential source of individual variability in response to these carcinogens [33,34]. We hypothesized that variants in the *CYB5A* and *CYB5R3* genes encoding this pathway would be associated with breast cancer risk in women who smoke. We further hypothesized that several *CYB5A* and *CYB5R3* variants that we previously found only in African American liver and breast tissues (Table 2) would be over-represented in AfrAm women in this larger population.

Of the 18 variants screened, an intronic variant in *CYB5R3*, I1M+6T (rs8190370) was significantly more common in breast cancer cases compared to controls, and this was attributable to a significantly higher allele frequency in AfrAm cases compared to AfrAm controls. When stratified by smoking, I1M+6T was more strongly associated with breast cancer, particularly in AfrAm women where the allele was most prevalent. When we further analyzed for an interaction between the I1M+6T allele and smoking, the P values were not significant for an interaction on the multiplicative scale. However, given the relatively low allele frequencies overall, the study as underpowered to detect such an interaction.

In the control subjects in this CBCS survey, I1M+6C>T was observed with an MAF that was almost 90-fold higher in AfrAm versus Cauc women. This variant was previously observed in the heterozygous state in 1 of 69 livers (unpublished data from Sacco et al. 2010), and 2 of 70 breast samples [36] all samples were from AfrAm subjects, with b5R immunoreactive protein expression below the 95% confidence interval for each tissue. The reference C allele is conserved among mammals, and the T variant is predicted to change the splice site at the first intron-exon splice junction of *CYB5R3*, leading to a truncated transcript. The *CYB5R3* I1M+6C>T variant may therefore lead to impaired b5R expression, and this polymorphism merits further functional characterization.

The risk of breast cancer in African American versus Caucasian women has been the subject of a number of studies, most of which have focused on differences in clinical presentation, tumor behavior, and dietary and hormonal exposures [50,51]. As for interactions between smoking and race, a 1992 study found an association between smoking and breast cancer in white women, but not black women [52]. In the CBCS population used in the present study, smoking was previously found to be a more significant risk factor for breast cancer in AfrAm than Cauc women, based on both duration of active smoking and time since smoking cessation [53,54]. This relationship was found to be stronger when considering multiple polymorphisms in nucleotide excision repair (NER) genes [54], which mediate repair of smoking-induced DNA adducts. These studies, together with our data, suggest that the higher risk associated with smoking among AfrAm women may be partly due to genetic variation in pathways that mediate both the detoxification of arylamine and heterocyclic amine tobacco carcinogens and the repair of resulting DNA adducts.

Overall, *CYB5A* and *CYB5R3* polymorphisms were fairly uncommon, with MAF values ≤ 0.15 for most variants. This may reflect evolutionary pressure to conserve the function of this pathway, which has an important endogenous role in maintaining hemoglobin in its functional, reduced state [45-47]. However, several variants were more prevalent in AfrAm women compared to Cauc women. In addition to I1M+6T, the *CYB5R3* promoter SNP -251G>T was more than 140-fold more prevalent in AfrAm women, with an MAF (∼0.070) that was similar to that reported for native African populations (browser.1000genomes.org). We previously observed this variant in a heterozygous liver with outlier low b5R expression and hydroxylamine reduction activity (Table 3). This SNP is predicted to eliminate VDR/CAR/PXR binding sites, and functional analysis of this SNP using a luciferase reported assay showed a 58% decrease in expression compared to the wild type promoter (data not shown).

A third *CYB5R3* variant, I8-1676T>C, which was found with > 100-fold higher allele frequency in AfrAm women, is within a binding site for RAD21, which could affect DNA double-strand-break repair. A final *CYB5R3* variant, *392G>C, was found with >30-fold higher frequency in AfrAm women. This variant was previously observed in haplotype configuration in 8 breast samples, all from AfrAm women. [36] Although it was not predicted by UTRscan to affect miRNA binding, this 3′UTR variant merits functional characterization because of its relatively high prevalence.

Two *CYB5A* variants also had significantly higher allele frequencies in AfrAm women. One coding SNP, 13T>G (Ser5Ala) was exclusively found in AfrAm subjects, albeit at a very low frequency. This variant was also reported with low frequency in native Africans (MAF 0.020) and was absent in European subjects in the 1000 Genomes Project (browser.1000genomes.org). Another *CYB5A* variant, I2-992C>T, was observed with >20-fold higher allele frequency in AfrAm women (MAF 0.169); this frequency is intermediate between those reported for African (0.201) and European (0.007) populations (browser.1000genomes.org). I2-992C>T was predicted to be within a binding site for NF-κB, which could affect gene expression [48,49]. There are several limitations to this study. Because of low allele frequencies, some of the estimates were imprecise and required that the smoking data be categorized as ever or never smokers. This did not allow consideration of age at smoking initiation, cigarette dosage and duration, or exposure to passive smoke [54,55]. Our findings should be confirmed in a larger AfrAm population to allow inclusion of more detailed smoking categories. In addition, samples were not fully re-sequenced, so other, potentially novel polymorphisms or haplotype associations within *CYB5A* and *CYB5R3* were not evaluated. Finally, we have previously found substantial individual variability in b5 and b5R protein expression, as well as 4-ABP hydroxylamine reduction activities in human breast, which could not be attributed to genetic polymorphisms alone [36]. This suggests that there may be tissue-specific, environmentally induced, or epigenetic regulation of this pathway that may also influence breast detoxification of carcinogenic arylhydroxylamines. This possibility deserves further study in breast tissues from women with and without breast cancer.

In summary, we found that the *CYB5R3* variant I1M+6 C>T was significantly over-represented in women with breast cancer, in particular AfrAm women. Further, there was a

suggestion of an interaction of this variant and smoking among AfrAm women. This intronic variant is predicted to cause aberrant splicing of the *CYB5R3* transcript, which may lead to decreased b5R expression and impaired detoxification of carcinogenic arylhydroxylamine metabolites. The *CYB5R3* I1M+6 C>T variant should be considered in the molecular epidemiology studies of breast cancer risk in AfrAms. In addition, this and other variants in *CYB5A* and CYB5R3 that are over-represented in AfrAm subjects should be considered in etiologic studies of prostate, bladder, and colon cancers, which are also associated with exposure to arylamine and heterocyclic amine carcinogens [56-58].

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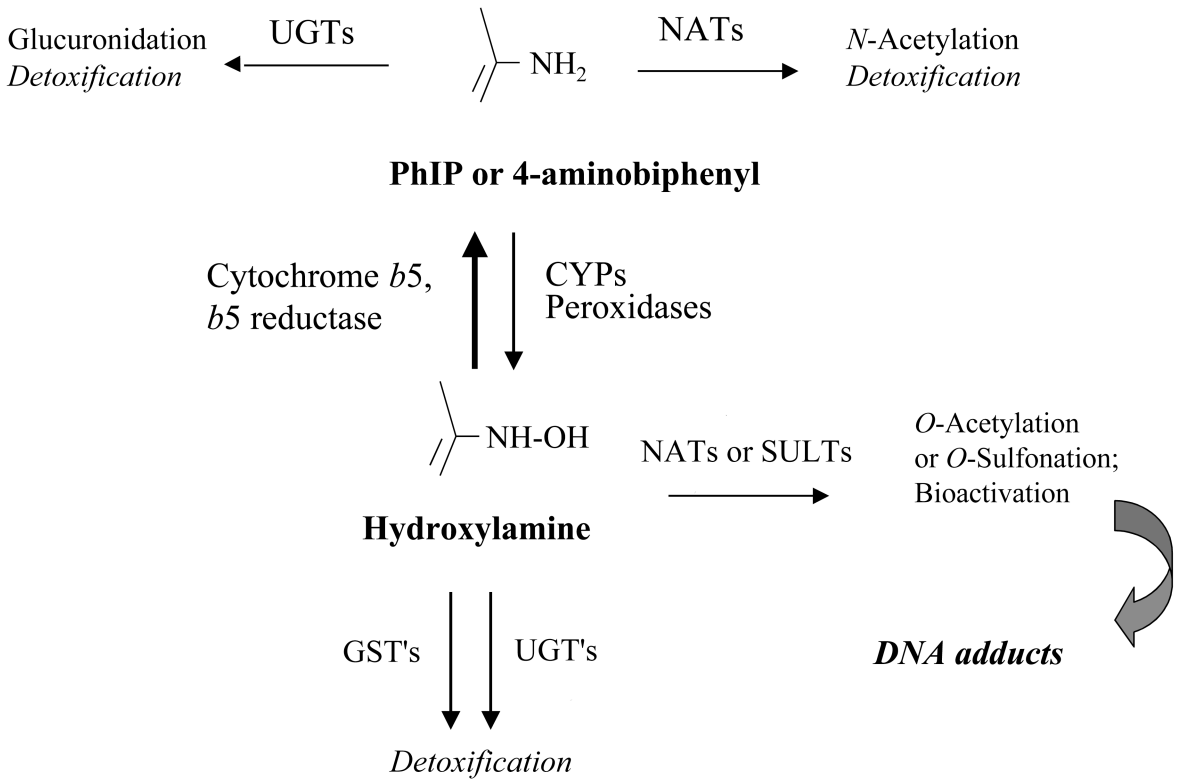

Figure 1.

Metabolism and bioactivation of carcinogenic aromatic and heterocyclic amines. Aromatic and heterocyclic amines may initially be detoxified by either glucuronidation or *N*-acetylation (by NAT1 or NAT2). Alternatively, the parent amines can be bioactivated to their hydroxylamine metabolites via cytochrome P450′s (CYP1A2, 1A1, and 1B1), myeloperoxidases, or lactoperoxidases. Hydroxylamine metabolites are reduced back to their parent compound by NADH cytochrome b_5 reductase (b5R) and cytochrome b_5 (b5) [33]. Reverse reduction by the b5/b5R pathway is up to 55 times more efficient than forward oxidation by P450′s [34]. Hydroxylamine metabolites may also be detoxified by glutathione S-transferases (GST′s) or UDP-glucuronosyltransferases (UGT's), or can be further bioactivated by *O*-acetylation or *O*-sulfonation. This final bioactivation step can lead to DNA adducts via arylnitrenium ion formation.

Table 1

Characteristics of Carolina Breast Cancer Study (CBCS) patients with invasive carcinomas and carcinoma *in situ*, and unaffected controls included in *CYB5A* and *CYB5R3* genotyping.

	Controls		Cases	
	N	**%**	**N**	**%**
African American	658	100	742	100
Median age in years (range)	50 (26–74)		51 (23-74)	
Mean proportion of African ancestry	0.774		0.778	
Age (years)				
<50	314	47.7	355	47.8
>=50	344	52.3	387	52.2
Menopausal status				
Premenopausal	290	44.1	324	43.7
Postmenopausal	368	55.9	418	56.3
Education				
<High school (HS)	198	30.1	215	29.0
HS & Post HS	348	53.0	406	54.8
>=College	111	16.9	120	16.2
Smoking				
Ever	262	39.8	322	43.4
Never	396	60.2	420	56.6
Caucasian	1089	100	1204	100
Median age in years (range)	51 (21–74)		50 (24-74)	
Mean proportion of African ancestry	0.066		0.064	
Age (years)				
<50	491	45.1	592	49.2
>=50	598	54.9	612	50.8
Menopausal status				
Premenopausal	456	41.9	540	44.9
Postmenopausal	633	58.1	664	55.2
Education				
<High school	115	10.6	103	8.6
HS & Post HS	613	56.3	671	55.7
>=College	361	33.1	430	35.7
Smoking				
Ever	546	50.1	587	48.8
Never	543	49.9	617	51.3

Table 2

Single nucleotide polymorphisms in *CYB5A* selected for genotyping in the Carolina Breast Cancer Study population, along with rationale for selection.

SNP	SNP ID	Rationale for selection
-206G>-	rs36082929	Reported in the NCBI SNP database.Within binding sites of several transcription factors (YY1, NFκ-B, NF-κB, NF- κA, and c-Fos).[a]
13T>GSer5Ala	rs75160992	Found in 2 of 111 liver samples (both heterozygous African Americans) in association with outlier low protein expression and activity [35].
65A>GHis22Arg	rs74339771	Found in 1 of 70 breast samples (heterozygous African American woman) in association with low protein expression and activity [36]. Predicted to be deleterious to protein function. [b]
I2-992C>T	rs1790894	Reported in the NCBI SNP database. Within binding sites for NF κ-B. [a]
155G>A Arg52Lys	rs1803366	Reported in the NCBI SNP database Predicted to be deleterious to protein function. [c]
178A>G Thr60Ala	rs78009726	Found in 1 of 63 leukocyte cDNA samples in a heterozygous African American subject [59]. Variant protein undergoes accelerated proteasomal degradation [59].
390C>ATyr130stop	rs1803364	Reported in the NCBI SNP database.Predicted to be deleterious due to stop codon. [d]
*246T>C	rs76241580	Found in 1 of 69 liver samples (Caucasian heterozygote)in association with outlier low b5 protein expression andactivity; [e] Located in internal ribosomal entry site [60]; may affect translation initiatio [61].

[a] ENCODE database and ChIP experimental data (www.genome.ucsc.edu);

[b] PMut;

[c] SIFT;

[d] Polyphen-2;

[e] Unpublished data from Sacco *et al.* 2010.

Table 3

Single nucleotide polymorphisms in *CYB5R3* selected for genotyping in the Carolina Breast Cancer Study population, along with rationale for selection.

SNP	SNP ID	Rationale for selection
-251G>T	rs73888347	Found in 1 of 69 livers in association with low (outside the 95% confidence interval of the mean) b5R expression and reduction activity; heterozygous African American subject. [a] Predicted deletion of VDR/CAR/PXR binding sites. [b]
-231C>A	rs75133903	Found in 1 of 69 livers in association with low b5R expression; heterozygous Caucasian subject.[a]
I1M+6C>T	rs8190370	Found in 1 of 69 livers, [a] and 2 of 70 breast samples [36], in association with low b5R expression; all heterozygous African American.Predicted to create a cryptic splice site.[c]
I1M+6072C>T	rs8190414	Reported in the NCBI SNP database. Within binding sites of NFκ-B, Max. [d]
176G>AArg59His	rs111154229	Observed in a heterozygous Caucasian liver with low b5R expression [35]. Predicted to be deleterious to protein function.[e,f]
I8-1676T>C	rs751153	Reported in the NCBI SNP database. Within binding sites of Rad21.[d]
890G>AArg297His	rs76458556	Observed in heterozygous Caucasian liver with low b5R expression and activity [35]. Predicted to be deleterious to protein function. [g]
*138G>A	ss159816065	Found in 1 of 69 livers with outlier low b5R expression and activity; heterozygous African American subject. [a]
*392G>C	rs7284807	Found in 5 of 69 livers with predominantly low b5R activity; all African American heterozygotes.[a]
*863T>C	ss159830807	Found in 1 of 69 livers with low b5R expression and activity; heterozygous Caucasian subject.[a]

[a] Unpublished data from Sacco *et al.* 2010;

[b] MATCH;

[c] Human Splicing Finder;

[d] ENCODE database and ChIP experimental data (www.genome.ucsc.edu);

[e] PMut;

[F] SIFT;

[g] PolyPhen-2.

Table 4

Minor allele frequencies (MAF) for *CYB5A* and *CYB5R3* variants in African American (AfrAm) and Caucasian (Cauc) women from the Carolina Breast Cancer Study [37]. Subjects are cases (with a diagnosis of invasive breast cancer or carcinoma *in situ*) or controls (without a breast cancer or CIS diagnosis and frequency-matched by age and race).

CYB5A	**Minor allele frequencies Cases**		**Minor allele frequencies Controls**	
	AfrAm	**Cauc**	**AfrAm**	**Cauc**
13 T>G(Ser5Ala)	0.0088	0.0008	0.0069 [a]	0.0000 [a]
I2-992 C>T	0.1577	0.0096 [c]	0.1689	0.0069 [c]
390 C>A	0.0000	0.0004	0.0000	0.0000
CYB5R3	**Minor allele frequencies Cases**		**Minor allele frequencies Controls**	
	AfrAm	**Cauc**	**AfrAm**	**Cauc**
-251 G>T	0.0674	0.0017	0.0727 [a]	0.0005 [a]
-231 C>A	0.0000	0.0000	0.0000	0.0009
I1M+6 C>T	0.0611 [b]	0.0008	0.0441[a, b]	0.0005 [a]
I1M+6072 C>T	0.0007	0.0000	0.0000	0.0000
I8-1676 T>C	0.1493	0.0025	0.1531[a]	0.0014 [a]
*138 G>A	0.0027	0.0025	0.0000	0.0028
*392 G>C	0.1154	0.0033 [c]	0.1353	0.0042 [c]
*863 T>C	0.0000	0.0000	0.0000	0.0005

[a] Significantly different between AfrAm and Cauc controls ($P \leq 0.0001$).

[b] Significantly different between cases and controls ($P < 0.05$).

[c] Not in HWE in Caucasians.

Table 5

Odds ratios (OR) with 95% confidence intervals (CI) for breast cancer risk among African American (AfrAm) and Caucasian (Cauc) women from the Carolina Breast Cancer Study population, in relation to *CYB5R3* I1M+6 genotype and smoking status.

	I1M+6 C>T genotype (rs8190370)				**OR (95% CI)***	**P value**
	Cases		**Controls**			
All subjects	CC CT + TT	1847 89	CC CT + TT	1685 57	Referent 1.42 (0.99-2.04)	0.0565
Ever smokers	CC CT + TT	868 34	CC CT + TT	792 15	Referent **1.97 (1.03-3.77)**	**0.0400**
Never smokers	CC CT + TT	979 55	CC CT + TT	893 42	Referent1.25 (0.81-1.95)	0.3155
Caucasians	CC CT + TT	1198 2	CC CT + TT	1084 1	Referent 1.57 (0.13-19.71)	0.7257
Ever mokers	CC CT + TT	583 0	CC CT + TT	544 1	Referent *Not calculated*	
Never smokers	CC CT + TT	615 2	CC CT + TT	540 0	Referent *Not calculated*	
African American	CC CT + TT	649 87	CC CT + TT	601 56	Referent 1.41 (0.98-2.04)	0.0623
Ever smokers	CC CT + TT	285 34	CC CT + TT	248 14	Referent **2.10 (1.08 – 4.07)**	**0.0284**
Never smokers	CC CT + TT	364 53	CC CT + TT	353 42	Referent 1.21 (0.77-1.88)	0.4096

*Adjusted for age, race, and African ancestry. An offset term is included in the model to account for randomized recruitment probabilities.