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CYP1B1 EXPRESSION, A POTENTIAL RISK FACTOR FOR BREAST CANCER¹

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Footnotes

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³ The abbreviations used are : B[a]P, benzo[a]pyrene; bp, base pair; CYP1A1, cytochrome P4501A1; CYP1B1, cytochrome P4501B1; HMEC, human mammary epithial cells; met, metastasis; ln, lymphnode; PAHs, polycyclic aromatic hydrocarbons; SD, standard deviation.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are metabolically activated to ultimate carcinogens by the cytochrome P450 isozymes CYP1A1 and CYP1B1. High levels of these enzymes may increase DNA adduct formation and initiation of cancer. We investigated whether expression of *CYP1B1* in breast tissue compared with *CYP1A1* is a risk factor for breast cancer. Expression of *CYP1B1* and *CYP1A1* was measured in a collection of histologically normal breast tissue specimens from breast cancer patients and from cancer-free individuals. Using a semiquantitative RT-PCR assay, *CYP1B1* and *CYP1A1* expression levels relative to the constantly expressed β -actin gene were determined. In the study sample of 61 non-tumor epithelial breast specimens, we found about 300-fold and 400-fold interindividual variation in expression for *CYP1B1* and *CYP1A1*, respectively. For most samples, CYP1B1 transcript levels were 2-7 times higher than CYP1A1, suggesting that CYP1B1 plays a greater role than CYP1A1 in activation of PAHs in the breast. The mean CYP1B1 transcript level in normal breast tissue was 60% higher in mastectomy patients compared with cancer-free individuals ($p = 0.0517$). Differences in CYP1B1 expression between cases and controls was greater among subject aged ≥ 50 years. These data suggest that CYP1B1 plays a role in breast cancer etiology, particularly among women ≥ 50 years of age.

INTRODUCTION

Ingested or inhaled PAHs are metabolized to water-soluble derivatives through oxidative activation primarily by the cytochrome P450 enzymes CYP1A1 and CYP1B1. CYP1A1 has been studied extensively for over 25 years. Much less is known about the more recently discovered CYP1B1, which is related in DNA sequence and substrate specificity to CYP1A1 (1). The CYP1B1 enzyme is involved in the activation of a number of lipophilic environmental carcinogens, including PAHs and aromatic amines (2). CYP1B1 also has an endocrine regulatory role. It hydroxylates 17 β -estradiol at the C-4 position to the potentially carcinogenic 4-hydroxyestradiol (3,4), which imparts estrogenic activity (5). In addition, CYP1B1 has an as yet undefined endogenous role, as truncating *CYP1B1* mutations have been linked to primary congenital glaucoma (6). CYP1B1 shows a tissue-specific expression that is distinct from CYP1A1. CYP1B1 is not expressed in liver, the major site for metabolism of xenobiotic compounds, but is constitutively expressed in some extrahepatic tissues, including steroid-responsive tissues such as breast, uterus, and prostate (2).

Recent evidence suggests that CYP1B1 might be more important than CYP1A1 in PAH carcinogenesis. Experiments with recombinant human enzymes showed that CYP1B1 catalyzes oxidation of benzo[a]pyrene (B[a]P) to the diolepoxide ten times more efficiently than CYP1A1 (7). In 7,12-dimethylbenz(a)anthracene-treated mice, the rate of lymphomas in CYP1B1 null mice was one tenth the rate in wild-type mice (8). These data demonstrate the importance of extrahepatic metabolism in determining susceptibility

to PAHs and establish CYP1B1 as an important contributor to PAH carcinogenesis in tissues where CYP1B1, in contrast to CYP1A1, is constitutively expressed.

Because the CYP1B1 and CYP1A1 enzymes activate potential carcinogens, they are thought to play an important role in tumor development (9). In a previous analysis of *CYP1A1* expression levels a collection of non-tumor breast specimens from breast cancer cases and controls, no significant differences were found between cases and controls (10). In this analysis, we examine expression of *CYP1B1* in breast tissue of cases and controls to gain insight into its potential role in breast cancer. Expression of *CYP1B1* was higher than *CYP1A1* in approximately 80% of samples and notably higher in specimens from cases compared with those from controls.

MATERIALS AND METHODS

Materials. Primers for PCR were synthesized by Life Technologies (Rockville, MD). Reverse transcriptase, (oligo dT)₁₆, dNTPs and Rnase inhibitor were purchased from Promega (Madison, WI). Tri Reagent, agarose, and Taq DNA polymerase were obtained from Sigma Chemical Co. (St. Louis, MO). Electrophoresis chemicals were purchased from Bio-Rad (Richmond, CA). Molecular weight standards were obtained from New England BioLabs (Beverly, MA). SYBR Gold nucleic acid gel stain was purchased from Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma Chemical Co. and were molecular biology grade when available.

Specimens. Two collections of non-tumor breast tissue from breast cancer patients and cancer-free individuals were used; one was established by M. Stampfer (11); and the

other was obtained from Aeron Technology and was collected using the same protocol. These tissue banks contain specimens from reduction mammoplasties and mastectomies, including tumor and peripheral non-tumor tissue. In a few cases, contralateral tissue and lymph node metastatic tissue was available. The specimens were dissected and isolated from adipose and connective tissue, so that only epithelial material was stored frozen as organoids. The pathological diagnosis of the excised tumors was intraductal carcinomas for 2 cases and infiltrating ductal carcinoma for the other 23 cases. In 4 of the 25 cases, metastasis to axillary lymph nodes was observed indicating more advanced disease. Samples were collected without respect to age or race. Only the age and disease status of the specimen donors are known. No information is available on donors' race, lifestyle, smoking habits, or other potential confounding factors. Individuals undergoing reduction mammoplasty ranged in age from 15 to 68 years. The age of mastectomy patients ranged from 30 to 87 years.

Cell Culture. cDNA from human mammary epithelial cells (HMEC) in culture was included in each PCR reaction as a positive control. Primary cultures of reduction mammoplasty tissue specimen 184 were grown in the serum-free medium MCDB170 as described by Stampfer (11). Ten 100-mm culture dishes with proliferating 184 cells (passage12) were treated with 10 μ M B[a]P for 20 hours, then washed twice with cold PBS before lysing with Tri Reagent as described below.

RNA, DNA Preparation, and cDNA synthesis. Total RNA and genomic DNA were simultaneously isolated from the specimens with TRI Reagent following the procedure recommended by the manufacturer. Before reverse transcription, RNA was heated at 72° C for two minutes then chilled on ice to remove secondary structure. Two μ g of total

RNA was diluted to a final volume of 25 μ l with 0.1 μ g (oligo dT)₁₆, 0.5 mM dNTPs, 10 units of RNase inhibitor, 100 units of Moloney murine leukemia virus reverse transcriptase, 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM MgCl₂, and 5 mM dithiothreitol. The samples were incubated at 37°C for 1 hour followed by heat inactivation at 98°C for 3 minutes. The cDNA was stored as several small aliquots at -80°C.

Measurement of CYP1B1 and CYP1A1 expression by semiquantitative RT-PCR.

CYP1B1 and *CYP1A1* expression were measured in parallel by a semiquantitative method developed in our lab (10; Russell *et al.*, in preparation). cDNA obtained from the breast epithelial cells was serially diluted, then amplified three times: first with CYP1B1 primers (12), then with CYP1A1 primers (13), and finally with β -actin primers (14). All primer sets are designed to span an intron, thus excluding amplification of any contaminating genomic DNA and generated products of 545 bp for CYP1B1, 320 bp for CYP1A1, and 273 bp for β -actin. As PCR is quantitative only in the exponential phase of product accumulation, PCR conditions and cycle numbers for each target were optimized separately for each primer pair. The cycle numbers for each target were chosen to give appropriate sensitivity while avoiding PCR plateau phase. The CYP1B1 RT-PCR assay was optimized to give the same amplification efficiency as the CYP1A1 reaction. All necessary procedures were used to avoid PCR product contamination in the set-up of the reaction mixtures (15). Each 50 μ l PCR contains 5 μ l of cDNA dilution, 10 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM each dNTP and 1.5 units of Taq polymerase. All targets are amplified on the same day in an MJ Research model PTC-100 thermal cycler using “hot start” conditions. β -actin was amplified using 25 pmoles of each primer, 2 mM MgCl₂, and 18 cycles as follows: 30 sec. at 96°C, 30 sec. at 58°C and 30 sec. at 72°C for 2 cycles

followed by 30 sec. at 94°C, 45 sec. at 56°C and 1 min at 72°C for 16 cycles followed by 10 min. at 72°C. CYP1A1 was amplified using 50 pmoles of each primer, 6 mM MgCl₂, for 20 cycles. The CYP1A1 product obtained by this procedure has been confirmed by sequencing. CYP1B1 was amplified using 40 pmol of each primer and 2mM MgCl₂ by the same thermal cycling conditions as CYP1A1. Thermal cycler parameters for CYP1A1 and CYP1B1 were 30 sec. at 96°C, 30 sec. at 63°C with a 1°C per cycle decrease, and 30 sec. at 72°C for 7 cycles followed by 30 sec. at 94°C, 45 sec. at 56°C and 1 min. at 72°C for 20 cycles followed by 10 min. at 72°C. Product was stored at 4°C overnight. For each set of samples analyzed, cDNA obtained from 184 HMEC (induced with B[a]P) was included as a positive control and sterile water was included as a negative control.

After amplification, the products were mixed together before electrophoresis on a 10% native polyacrylamide gel. The gel was stained for 10 minutes using SYBR Gold nucleic acid stain (Figure 1). Gels were scanned without destaining on a Molecular Dynamics STORM 860 laser scanner. The fluorescent signal for each band was quantitated using ImageQuant software after background correction included in the software. CYP1A1 and CYP1B1 levels are expressed as a ratio to β -actin values.

Data Analysis. The linear range of the Storm 860 scanner and the SYBR Gold signal intensity were determined by analysis of a dilution series of a mass standard (data not shown). All of the fluorescent signals generated by the PCR products remained in this range or they were discarded and the specimen was re-amplified using a lower cDNA dilution. Specimens were processed in groups of ten. Each group included a positive and a negative control. Four cDNA dilutions (of a 4-fold dilution series) were amplified for

each specimen. Each run was considered valid if the negative control showed no bands at 273 and 320 and 545 bp and if the positive control showed bands at 273 bp and 320 and 545 bp at a predetermined acceptable signal intensity. For each specimen, the fluorescent signal of the cDNA dilution was accepted for analysis if the signal was at least twice the signal of the negative control and if the signal was not in plateau. PCR plateau was determined by examining the signal increase of the dilution series. If the signal intensity no longer increased by 4-fold, that dilution was not used in the analysis. After the signal for a dilution was accepted to be valid, the CYP1B1 level of each specimen was expressed as:

$$\text{CYP1B1 level} = (\text{CYP1B1 fluor. signal} \times \text{DF}) / (\beta\text{-actin fluor. signal} \times \text{DF}),$$

where DF is the dilution factor. Samples analyzed in different groups of 10 were compared after assuring that values of the positive control were in the previously measured range. CYP1A1 levels were determined in a similar fashion. After all the specimens were analyzed, 15% of the cDNA was re-amplified and the CYP1B1 and CYP1A1 levels were found to repeat within two-fold.

The linear relationship between age and CYP1B1 level was evaluated using Pearson's product moment correlation coefficient. Descriptive analyses of CYP1B1 levels by case-control status and age group (< 50 years versus \geq 50 years) included calculation of arithmetic means, geometric means, standard deviations, and medians. Means were compared using the two-tailed Student's t-test.

RESULTS

The control group consisted of 32 reduction mammoplasty specimens. The case group consisted of 25 peripheral non-tumor tissue specimens obtained from mastectomy patients. Four tissue specimens contralateral to the tumor from cases were also analyzed.

CYP1B1 transcript levels versus CYP1A1 transcript levels. In HMEC 184 (passage 12 cells treated with 10 μ M B[a]P for 20 hours), which are included as a positive control, CYP1B1/ β -actin ranged from 8.97 - 12.61 (mean = 9.68) in the 7 measurements, CYP1A1/ β -actin ranged from 1.09 - 1.79 (mean = 1.44). The CYP1B1 to CYP1A1 ratio was 6.8. In untreated 184 cells CYP1B1/ β -actin was almost 50 times higher than CYP1A1/ β -actin (data not shown). In breast tissue specimens from cases and controls, there was a more than 300-fold variation between individuals in the CYP1B1/ β -actin ratio (range 0.43 to 132.50), and a more than 400-fold variation in the CYP1A1/ β -actin ratio (range 0.16 to 66.81) as seen in Figure 2. *CYP1B1* was expressed at lower levels than *CYP1A1* in only one specimen, at similar levels as *CYP1A1* in 10 specimens (ratio 0.8-1.4), and at higher levels than *CYP1A1* (ratio 2 - 82) in the remaining 50 specimens (82% of the sample). The mean CYP1B1 to CYP1A1 ratio was 8.69 and the median ratio was 3.9. The mean ratio did not differ between cases (mean = 8.48, SD = 16.98) and controls (mean = 8.48, SD = 11.39). Seven specimen donors (3 cases and 4 controls) had a CYP1B1 : CYP1A1 ratio \geq 20; all were under 50 years of age.

***CYP1B1* expression in cases versus controls and as function of age.** The difference in CYP1A1 levels between cases and controls was not statistically different (10, and Table 1). When *CYP1B1* expression in cases and controls was compared (Table 1), the mean CYP1B1 transcript level was about 60 % higher in cases than in controls (and $p = 0.0517$). This difference was attenuated when evaluating the log transformed data (Table

1). As shown in Figure 3, the range of *CYP1B1* expression is much greater in cases than in controls.

CYP1A1 expression in breast tissue has been found previously to be independent of age (11). *CYP1B1* expression does not appear to be linearly related to age ($r = 0.124$, $p = 0.3437$). However, when the data were split into two age groups (< 50 and ≥ 50), which roughly corresponds to menopausal status, a *CYP1B1* age association became apparent (Figure 4). Among older controls, the *CYP1B1* range of values was limited compared to the other 3 groups, indicating that endogenous estrogen levels might influence *CYP1B1* expression.

***CYP1B1* and *CYP1A1* expression in non-tumor versus tumor tissue.** For 5 cases, non-tumor tissue, tumor tissue, and/or metastasis to the lymph nodes from the same individual were available. We compared *CYP1B1* expression in tumor tissue versus normal tissue to evaluate whether higher *CYP1B1* levels might be related to the disease process. The results were ambiguous (Table 2). For one individual, non-tumor tissue had higher *CYP1B1* expression than tumor tissue. For 3 individuals, expression was higher in tumor than non-tumor tissue. In one individual, where 2 different tumors and 2 metastases to the lymph node were available, *CYP1B1* expression in nontumor tissue, 1 tumor, and 1 metastasis was comparable, but in 1 tumor and the other metastasis *CYP1B1* expression was increased 3-fold and 9-fold, respectively. *CYP1A1* expression varied less between non-tumor and tumor tissue.

DISCUSSION

Interindividual variation in carcinogen metabolism is a determinant of susceptibility to various cancers (16). Variability in carcinogen metabolism can be due to the level of the enzymes or to the catalytic activity of the enzymes. Genetic polymorphisms can alter the catalytic properties of an enzyme. Two common genetic polymorphisms consisting of single base changes have been described for *CYP1B1* (17). A change in catalytic activity towards steroid hormones has been observed in these *CYP1B1* variants (18). The role of these polymorphisms as risk factors for cancer is not yet clear. Two studies on *CYP1B1* polymorphism and breast cancer risk ~~done~~ conducted in different ethnic groups gave conflicting results (17,19). A recent study found that the *CYP1B1* variant with increased activity for 4-hydroxyestradiol formation is a susceptibility factor for ovarian cancer (20).

Whereas a polymorphism represents a lifelong rigid category with a defined effect, the expression level of a gene is quite variable and measuring expression provides a more differentiated response. Our primary goal in this study was to analyze *CYP1B1* expression in normal breast tissue and determine whether it could have a role in breast cancer development. *CYP1A1* expression in response to PAHs and organochlorines and its regulation by the Ah receptor has been studied extensively. It is generally accepted that *CYP1A1* transcript levels mirror enzyme levels (21). *CYP1B1* mRNA levels also have been found to correlate with comparable changes in the protein levels as a result of transcriptional activation (22, 23, 12). In mouse fibroblasts, *CYP1B1* levels are regulated not only by the Ah receptor, but can also involve protein stabilization (24). No such regulatory mechanism has been described in human cells. Therefore, *CYP1B1* expression is assumed to be a proxy measure for *CYP1B1* protein levels.

CYP1B1 is expressed constitutively in human mammary epithelial cells in culture, whereas CYP1A1 is detected only after cellular exposure to environmental chemicals (12). We found that *CYP1B1* expression was much higher than *CYP1A1* expression in the cultured HMEC 184. For 184A1, an immortalized cell line derived from 184 after treatment with B[a]P the opposite was observed (25). The 184A1 cells, however, were grown in different cell culture media, and cell-specific expression depends on culture conditions (26). CYP1B1 transcript levels were higher than CYP1A1 transcript levels in more than 80% of the samples tested in our study. Of the known PAH-activating enzymes in the breast, CYP1B1 appears to be the predominant one. *CYP1B1* expression varied more than 300-fold among the 61 specimens tested. Interindividual variation in *CYP1B1* expression has been observed by others: 2.5-fold variation in early passage HMEC from 7 women (12); and 30-fold variation in CYP1B1 transcript levels in uncultured lymphocytes obtained from 10 individuals (27).

The observed interindividual variation in *CYP1B1* and *CYP1A1* expression may be due to environmental factors, such as exposure to PAHs and organochlorines. Since the Ah receptor induces expression of both *CYP1A1* and *CYP1B1*, simultaneous high CYP1A1 and CYP1B1 transcript levels in breast tissue may be indicative of environmental exposure to Ah receptor ligands. In 7 specimens from cases and 3 from controls, both CYP1A1 and CYP1B1 transcript levels were above the 75% quantile. Possible environmental exposures might be from smoking or involuntary exposure in ambient air contaminants or diet. High levels of CYP1A1 and CYP1B1 have similar effects on the activation of environmental procarcinogens, both lead to increased

activation, though some stereoselective differences in activation by CYP1A1 and CYP1B1 have been observed (12).

Increased levels of CYP1A1 and CYP1B1 have a different effect on estrogen metabolism. Even though both CYP1A1 and CYP1B1 can metabolize 17 β -estradiol, a high level of CYP1A1 will result in conversion to an inactive metabolite, 2-hydroxyestradiol, whereas a high level of CYP1B1 will lead to enhanced formation of the potentially carcinogenic 4-hydroxyestradiol. This metabolite has similar estrogenic activity as 17 β -estradiol and can be further oxidized to a quinone that can depurinate DNA or lead to formation of reactive oxygen species (5, 28, 29)

The difference in *CYP1B1* expression between cases and controls suggests a role for this enzyme in the etiology or process of the disease. CYP1B1 activity has been found to be higher in breast cancer tumors than in adjacent normal tissue (30). In an immunohistochemical study, CYP1B1 activity was shown to be expressed in a wide range of tumors, but was not detectable in normal tissue by the method used (31). In contrast, a recent study found strong immunohistochemical staining of the CYP1B1 protein in all breast tissues tested and the staining was both nuclear and cytoplasmic (32). We observed some variation in CYP1B1 levels of non-tumor and tumor tissue from the same individual, though no consistent pattern was apparent (Table 2). This might indicate that changes in *CYP1B1* expression are not part of the disease process, but rather a sign of the multiple, random changes occurring in tumor progression. While there was no clear linear relationship between CYP1B1 levels and age, there does appear to be a relationship between CYP1B1 levels and menopausal status. In controls ≥ 50 years of age CYP1B1 transcript levels were markedly lower, about a third of levels in cases ≥ 50

years of age. Caucasian breast cancer patients with the *CYP1B1* variant with increased activity for 4-hydroxyestradiol formation have a significantly higher percentage of estrogen receptor positive/progesterone receptor positive tumors (17). Increased CYP1B1 metabolism through high enzyme levels or through the high activity *CYP1B1* variant might be correlated with development of estrogen receptor positive tumors, the predominant tumor type in postmenopausal women (33). Even though this study is limited by the small number of unselected specimen groups available, the finding of an association of CYP1B1 transcript levels with disease warrants further investigation. Since human CYP1B1 is capable of activating diverse xenobiotic procarcinogens and also hydroxylate 17 β -estradiol to 4-hydroxyestradiol, it could play a role in breast cancer etiology by activating either environmental chemicals or endogenous substrates to carcinogens.

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Table 1: CYP expression summary statistics for cases and controls

CYP	Summary Statistic	Cases	Controls	p-value
CYP1A1	arithmetic mean (SD)	9.61* (12.60)	6.92* (8.64)	0.3319
	geometric mean (SD)	4.66 (4.13)	3.18 (4.42)	0.3093
	median	7.45	4.61	N.A.
	range	0.24 - 66.81	0.16 - 38.35	N.A.
CYP1B1	arithmetic mean (SD)	33.85 (34.61)	20.01 (18.05)	0.0517
	geometric mean (SD)	19.16 (3.32)	13.30 (2.79)	0.2056
	median	21.4	12.23	N.A.
	range	1.52 - 132.5	0.43 - 79.3	N.A.

* These values are obtained from repeat measurements of CYP1A1 expression simultaneously with CYP1B1 expression of specimens analysed in ref. 10 for all of the controls and 24 of the 29 cases. The published measurement of the CYP1A1 mean for cases was 9.55 and for controls 6.31.

Table 2: Comparison of CYP1B1 and CYP1A1 expression in non-tumor versus tumor tissue for five individuals

ID	Specimen	CYP1B1/ β -actin (relative difference)		CYP1A1/ β -actin (relative difference)	
380	peripheral/non-tumor	4.46	(1.00)	0.74	(1.00)
	tumor in left breast	11.45	(2.57)	0.77	(1.04)
	tumor in right breast	4.45	(1.00)	0.45	(0.61)
	met to ln L	4.83	(1.08)	0.35	(0.47)
	met to ln R	41.87	(9.39)	6.21	(8.39)
383	peripheral/non-tumor	10.41	(1.00)	7.56	(1.00)
	tumor	6.31	(0.61)	1.33	(0.18)
407	peripheral/non-tumor	1.65	(1.00)	0.25	(1.00)
	tumor	6.92	(4.19)	1.73	(6.92)
412	peripheral/non-tumor	15.45	(1.00)	4.45	(1.00)
	tumor	66.88	(4.33)	10.80	(2.43)
426	peripheral/non-tumor	4.60	(1.00)	5.75	(1.00)
	met to ln	71.40	(15.52)	9.51	(1.65)

Figure Legend

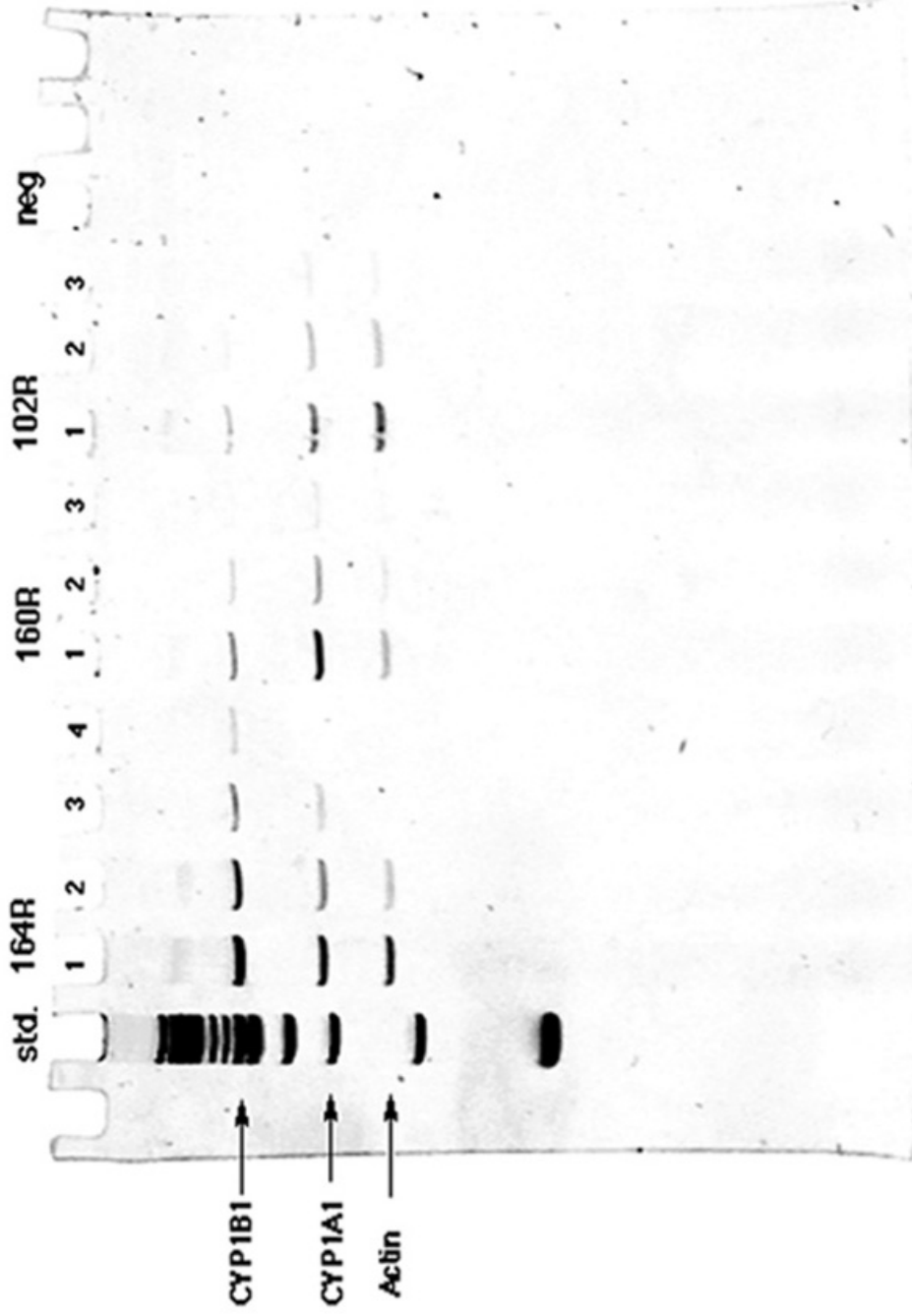
Fig. 1: Polyacrylamide gel of quantitated CYP1B1, CYP1A1 and β -actin PCR products for 3 specimens (CYP1B1 545 bp fragment, CYP1A1 320 bp fragment, and β -actin 273 bp fragment). The cDNA from each specimen was diluted serially 4-fold and several of these dilutions were amplified for each specimen. The cDNA dilution chosen for each gene target varied in order to keep the PCR signal in the quantitative range. Lane 1, molecular weight standard; lane 2-5, lane 6-8, lane 9 -11 represent the serial dilutions of 3 different specimens; lane 12, negative control.

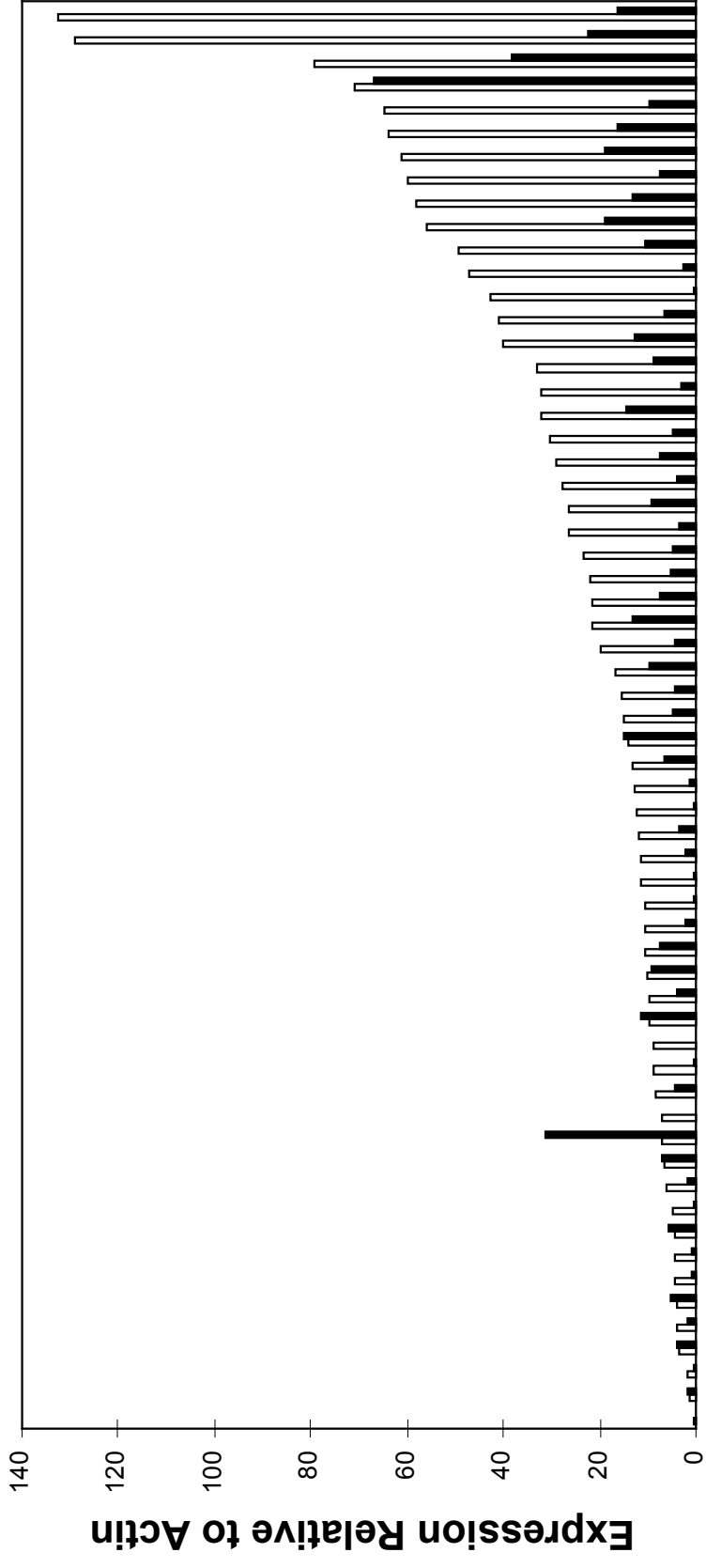
Fig. 2: CYP1A1/CYP1B1 to β -actin ratio in non-tumor breast tissue for all individuals ranked by increasing CYP1B1 values. Open bars represent the CYP1A1. Solid bars represent CYP1B1.

Fig. 3: Distribution of *CYP1B1* expression levels in non-tumor breast tissue among breast cancer cases (solid bars) and reduction mammoplasty controls (open bars).

Fig. 4: Box plots of *CYP1B1* and *CYP1A1* expression levels (relative to β -actin) for cases and controls stratified by age group.

Figure 1





Individuals

Figure 2

Figure 3

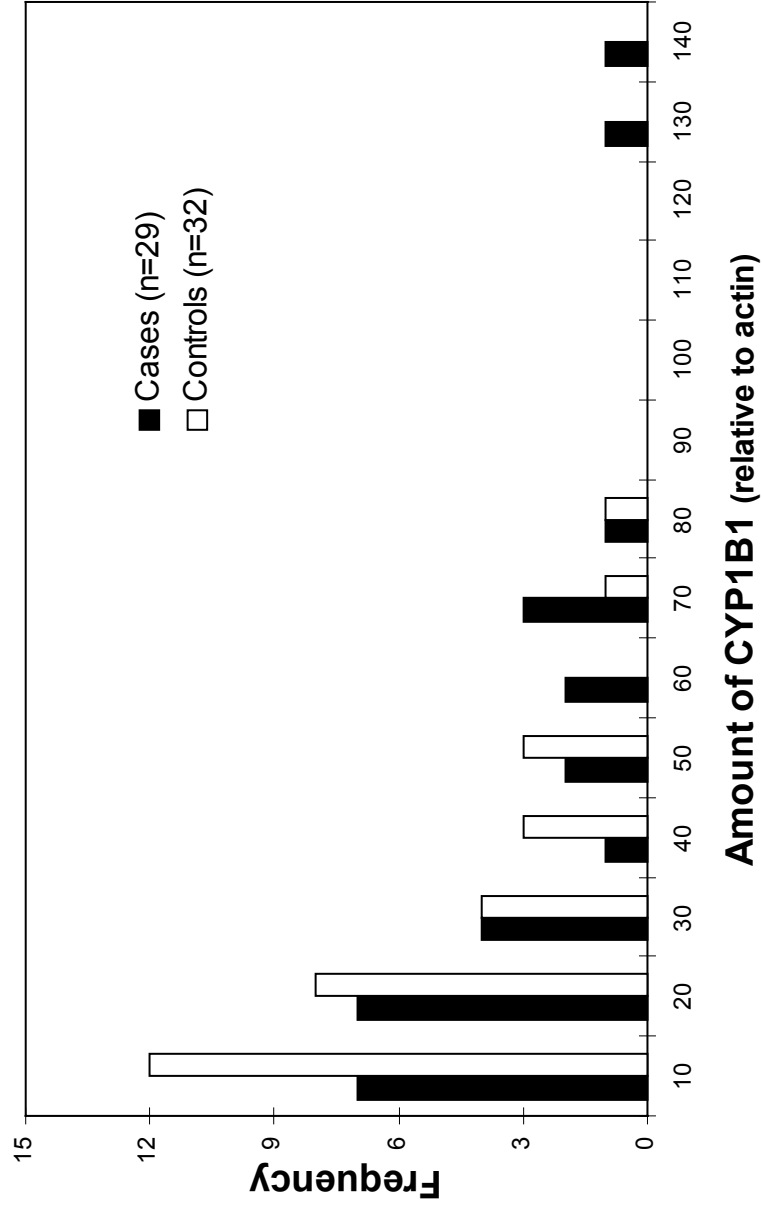


Figure 4

