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Induction of *CYP1A1* and *CYP1B1* and formation of carcinogen–DNA adducts in normal human mammary epithelial cells treated with benzo[a]pyrene

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Abstract

Inter-individual variation in formation of carcinogen–DNA adducts and induction of cytochrome P450 genes was measured in 23 cultured normal human mammary epithelial cell (NHMEC) strains established from reduction mammoplasty tissue. Semi-confluent cells were exposed to 4 μM benzo[a]pyrene (BP) for 12 h and BP–DNA adduct levels were measured by chemiluminescence immunoassay using antiserum elicited against DNA modified with r7, t8-dihydroxy-t-9, 10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE). BP–DNA adduct levels for 22 of 23 different cell strains ranged from non-detectable (three samples) to about 15 adducts/10^8 nucleotides. Increases in levels of *CYP1A1* and *CYP1B1* were detected using both oligonucleotide arrays and reverse transcription/quantitative real-time polymerase chain reactions (RT-PCRs). For *CYP1A1* and *CYP1B1*, the oligonucleotide array data and RT-PCR data were highly correlated ($r=0.73$ and 0.70, respectively), suggesting that oligonucleotide arrays are a suitable gene discovery tool, and demonstrating that the complementary and efficient RT-PCR may be used to confirm microarray data for a specific gene in a large number of samples. As measured by RT-PCR, inter-individual variation in *CYP1A1* induction was 100-fold, while the variation in *CYP1B1* induction was almost 40-fold. On a per-person basis, *CYP1A1* and *CYP1B1* induction were well-correlated ($r=0.88$, $P<0.001$), which is to be expected as they are under the control of a common transcriptional regulation mechanism in response to BP exposure.

Abbreviations: BP, benzo[a]pyrene; BP-7,8-oxide, benzo[a]pyrene-7,8-epoxide; BP-7,8-dihydrodiol, *trans*-7,8-dihydro-7,8-dihydroxy-benzo[a]pyrene; BPDE, r7,t8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydro-benzo[a]pyrene; BPdG; r7,t8,t9-trihydroxy-c-10-(N2deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[a]pyrene; CIA, chemiluminescence immunoassay; GC, GeneChip; MES, 2-[N-morpholino]-ethansulfonic acid; NHMEC, normal human mammary epithelial cell; NIOSH, National Institute for Occupational Safety and Health; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; RT, reverse transcription.

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Inter-individual variation in carcinogen–DNA adduct formation could not be explained only by variation in levels of *CYP1A1* or *CYP1B1* induction, as neither was well-correlated with BPDE–DNA adduct level ($r=0.40$ and 0.50 for *CYP1A1* and CYP1B1, respectively). Evaluation of glutathione-*S*-transferase M1 genotype (*GSTM1* positive or null) revealed an apparent correlation between positive *GSTM1* genotype and BPDE–DNA adduct levels ($r=0.84$ and 0.77 for *CYP1A1* and CYP1B1, respectively); however, after removal of the single outlier this relationship was not significant. Overall the data suggest that BPDE–DNA adduct levels in normal human breast tissue may be modulated by multiple factors that include, but are not exclusive to, *CYP1A1* and *CYP1B1* inducibility and the presence or absence of *GSTM1*.
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Keywords: Polycyclic aromatic hydrocarbons; Chemiluminescence immunoassay; Metabolic activation; Cell culture; Carcinogenesis; DNA damage

1. Introduction

Human exposure to mixtures of environmental pollutants that include polycyclic aromatic hydrocarbons (PAHs) is unavoidable [1]. Because many of these compounds are human carcinogens, elevated cancer rates may occur in individuals subjected to environmental and occupational PAH exposures [2]. Carcinogenic PAHs induce multiple metabolic pathways in human tissues and cells, as evidenced by DNA-microarray studies [3,4]. In humans, carcinogenic PAHs are biotransformed through induction of multiple enzyme systems, particularly the cytochrome P450s [5,6], and wide inter-individual variation in response to PAH exposures has been documented [4]. As breast cancer incidences appear to be increasing in recent years, several molecular epidemiologic studies have focused on normal breast tissue and breast cancer cells, and established associations between patterns of carcinogen–DNA adduct formation and cytochrome P450 polymorphisms [7–9]. These studies suggest that both environmental and genetic factors may contribute to the burden of human female breast cancer.

While *CYP1A1* was cloned in 1985 and its expression had been studied long before that, *CYP1B1* was not cloned until almost a decade later and has been less intensively investigated [10,11]. Both CYP1A1 and CYP1B1 enzymes may be involved in the metabolic activation of exogenous carcinogens that reach human mammary tissues [12–16]. In addition, CYP1B1, known to metabolize steroids, may be involved in the metabolic activation of estrogens to carcinogenic intermediates; consequently, it has been identified as a target for inhibition in anticancer strategies [6,17]. Both CYP1A1 and CYP1B1 are activating enzymes that can metabolize xenobiotics in concert with detoxifying enzymes. Glutathione-*S*-transferase M1 (GSTM1), a detoxication enzyme, contributes to the disposition of PAHs by effectively lowering the concentration of activated xenobiotic available for DNA-binding.

In these experiments, we have used normal human mammary epithelial cells (NHMECs), cultured from normal mammary tissues obtained at reduction mammoplasty from 22 individuals to study gene expression of the carcinogen-metabolizing enzymes *CYP1A1* and *CYP1B1*. The data have been stratified by *GSTM1* genotype. NHMEC strains were exposed to the carcinogenic PAH benzo[a]pyrene (BP) to explore inter-individual variability in BP–DNA adduct formation and to compare BP–DNA adduct formation with expression of the metabolic enzymes. An earlier study has documented the metabolism of PAHs in cells of similar derivation [18], but advanced methods to compare gene expression of the metabolic activation enzymes with DNA adduct formation (the toxicogenomic aspects of PAH metabolism) were not available at that time. In a previous study [4], we used oligonucleotide arrays to investigate the complete pattern of gene expression changes in BP-exposed cultured NHMECs obtained from four different donors, and those studies showed that some of the most consistently and extensively induced genes were *CYP1A1* and *CYP1B1*. Here we compare the formation of BP–DNA adducts with the extent of *CYP1A1* and *CYP1B1* induction in cell strains from 22 individuals exposed to BP. Values for *CYP1A1* and *CYP1B1* induction determined by oligonucleotide arrays were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and stratified

by *GSTM1* status for comparison with BP–DNA adduct levels.

2. Materials and methods

2.1. Human tissues and cells

Reduction mammoplasty provides a valuable source of normal human mammary epithelial cells. Cells were isolated from discarded tissues of female donors by a process of mechanical and enzymatic disruption that has been well documented [19]. Cell strains were cultured for several passages, frozen and re-cultured in order to expose multiple strains simultaneously at the same passage. Tissues for this study were obtained through the Cooperative Human Tissue Network, which is sponsored by the National Cancer Institute and the National Disease Research Interchange. Human Studies Review Board approval was sought at NIOSH, and a waiver was granted as no unique identifiers accompanied the tissues. The information collected included age and race, and anonymous pathology reports indicating that the tissues were normal.

2.2. Cell exposure, DNA and RNA preparation

Twenty-three NHMEC strains were all exposed at passage 6 to BP (4 μM) in serum-free media (Clonetics™, Walkersville, MD). Cells were incubated for 12 h in the presence of either BP or vehicle (acetone:ethanol 1:22.5, final concentration 0.1%) alone. Four replicate exposures were performed for each of the 23 cell strains. The total RNA was isolated from two of the replicates using RNeasy (Qiagen, Valencia, CA), while DNA was extracted from the remaining duplicate samples. For DNA preparation, media was removed and cells were washed first with PBS, then with lysis buffer (5 ml: 100 mM Tris, pH 8.5; 5 mM EDTA; 0.2% SDS; 200 mM NaCl). Cells were incubated (3 h, 37 °C) with RNase A (250 μg) and proteinase K (500 μg), and an equal volume of isopropanol was added. The DNA precipitate was transferred to an eppendorf tube and washed twice with ethanol (70%) and dissolved in 200 μl of molecular biology grade water [20].

2.3. Determination of BP–DNA adducts

Adducts of BP, formed in DNA after metabolic activation, were measured using a chemiluminescence immunoassay (CIA) [21]. Each assay was performed in triplicate for duplicate cell treatments, where each individual unexposed culture served as the control for its own BP-exposed cell strain. Briefly, opaque 96-well high binding plates (Greiner Bio-one, Longwood, FL) were coated with 100 pg of sonicated BPDE–DNA (modified to 0.33%) or calf thymus DNA, in 0.1 ml of Reacti-Bind DNA coating solution (Pierce Biotechnology, Inc., Rockford, IL) at room temperature for 48 h. Plates were stored at −20 °C until further use. For assay, plates were thawed to room temperature and washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) using an automated plate washer (Ultrawash Plus, Dynex Technologies, Guernsey, UK). Plates were first incubated with casein (0.25%, 37 °C, 90 min) (PE Applied Biosystems, Foster City, CA) in PBST to reduce non-specific binding and washed with PBST. Sample DNA or standard BPDE–DNA sonicated (20 s at 20% amplitude using an Ultrasonic Processor, Sonics & Materials, Inc., Newtown, CT), denatured (4 min at 95 °C) and cooled (10 min on ice), was mixed with an equal volume of PAH–DNA antiserum (rabbit # 31, bleed # 08/16/78), diluted 1:3,000,000 in PBST containing casein (0.25%) and added to the wells. Serial dilutions of the standard BPDE–DNA (modified to 1.0 BPdG/10^6 nucleotides) in carrier calf-thymus DNA were prepared such that each well contained an equal quantity of DNA but varying amounts of BPdG adduct (0–16 fmol/well). Plates were incubated (90 min at 37 °C), washed and incubated with biotinylated anti-rabbit antibody (1:2500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in casein solution (0.25%, room temperature, 90 min). After washing, plates were incubated with streptavidin alkaline phosphatase (Avidix-AP: 1:5000; PE Applied Biosystems, Foster City, CA) in casein solution (0.25% at room temperature/60 min). Plates were subsequently washed with PBST, distilled water, and Tris buffer (20 mM Tris-1 mM $MgCl_2$, pH 9.5), before adding CDP star with Emerald II solution and incubating at 4 °C (overnight). Plates were warmed to room temperature and luminescence was measured using

a TR717 Microplate Luminometer (PE Applied Biosystems, Foster City, CA) (542 nm). For each CIA, samples were assayed in triplicate experimental wells and one control well. All samples were assayed in two separate CIAs. The standard curve 50% inhibition was at 1.40 ± 0.36 fmol BPdG and the lower limit of detection using 10 μg DNA was 0.33 adducts/10^8 nucleotides.

2.4. DNA-oligonucleotide microarrays

Affymetrix™ HuGeneFL DNA-microarrays were used (Affymetrix, Santa Clara, CA). cRNA was prepared from total RNA exactly according to the DNA-microarray manufacturer's instructions. An oligo $(dT)_{24}$ primer with 5′ T7 polymerase promoter sequences was used to generate double stranded cDNA. Enzo BioArray high yield RNA transcript labeling (Enzo Diagnostics, Inc., Farmingdale, NY) was used to generate the fluorescent-labeled cRNA transcript. Labeled antisense RNA was purified (RNeasy) and 20 μg was fragmented [$Mg(CH_3CO_2)_2$, 30 mM; KCH_3CO_2, 100 mM; $H_2NC(CH_2OH)_3C \cdot H_3CO_2$, 40 mM, pH 8.1; 94 °C, 35 min], 15 μg of the fragmented cRNA sample was mixed with eukaryotic oligonucleotide hybridization controls (20×; *BioB, BioC, BioD, cre* at 1.5, 5, 25, 100 pM, respectively), control oligonucleotide B2, herring sperm DNA (10 mg/ml), acetylated BSA (50 mg/ml) and hybridization buffer (2×) were also added. The hybridization mixture was heated (99 °C for 5 min and 45 °C for 5 min) prior to introduction into the HuGeneFL cassette. The cassettes were incubated (16 h, 45 °C) with rotation (60 rpm), following which they were purged with two buffers, non-stringent buffer 25 °C (6× SSPE [NaCl, 3 M; NaH_2PO_4, 200 mM; EDTA, 20 mM]; Tween 20, 0.01%; anti-foam, 0.005%) and stringent buffer at 50 °C (MES, 100 mM; Na^+, 100 mM; Tween 20, 0.01%). Microarrays were scanned using a GeneArray® 2500 Scanner (Affymetrix) and data were analyzed using Microarray Suite 4.0 software (Affymetrix).

2.5. Quantitative real-time polymerase chain reaction (RT-PCR)

Relative quantitation was performed by RT-PCR using Taq-Man™ technology (Applied Biosystems). The cDNA obtained from the total RNA for microarray analysis was used as a template to perform RT-PCR after dilution (1:50). One microliter was used as template to perform PCR in a 50 μl reaction mixture containing 2× Taq-Man Universal PCR Master Mix and 20× Assays-on-Demand Gene Expression Primers and Probes for both *CYP1A1* (Hs00153120_m1/X02612), *CYP1B1* (Hs00164383_m1/U03688) and *GAPDH* (Hs99999905_m1/NM002046) (Applied Biosystems/GenBank). This two-step PCR reaction was performed on a ABI 7700 Sequence Detection System. Each sample was assayed in duplicate and the Cycle Threshold (C_T) values were normalized to the housekeeping gene (*GAPDH*) and the fold change was calculated using the $2^{-\Delta\Delta C_T}$ method [22].

2.6. GSTM1 genotyping

Genotyping for *GSTM1* (AC000032, GenBank) was performed using a published method [23]. Briefly, constitutive DNA samples (100 ng) were mixed with primers (GSTM1-F and GSTM1-R specific for *GSTM1* (25 mM, forward-5′CTG CCC TAC TTG ATT GAT GGG 3′ fragment, reverse-5′CTG GAT TGT AGC AGA TCA TGC 3′)) in a reaction mixture (50 μl) containing buffer (Tris, HC1, 10 mM, pH 9.0; $MgCl_2$, 2 mM; KC1, 50 mM; Triton X100, 0.1%; dNTPs, 1 mM) and Taq polymerase (1 U). The initial melting temperature (94 °C) was held for 5 min, and was followed by 30 cycles of melting (94 °C for 30 s), annealing (56 °C for 1 min), extension (72 °C for 1 min), and a final extension (72 °C for 6 min). Reaction products were visualized under UV light after electrophoresis on agarose gels (2%) containing ethidium bromide (100 μg/l).

2.7. Statistical analysis

Correlation coefficients were determined according to the method of Pearson using Excel™ [24]. Pearson's correlation coefficients were calculated to determine a point estimate of the strength of the association between RT-PCR and GC for both *CYP1A1* and *CYP1B1*, as well as the induction of *CYP1A1*, *CYP1B1* and adduct formation. Also, correlation coefficients were determined to estimate the strength of the relationship between the induction

of *CYP1A1* and *CYP1B1* in the presence of BP–DNA adducts (samples categorized as ND were assigned a value of half the level of detection for the purpose of statistical analysis). The correlation coefficient value can range between −1 to +1. Positive correlation coefficients indicate that as the value of the independent variable increases, the value of the dependent variable also increases.

3. Results

3.1. Origins of mammary epithelial cells

Normal human mammary epithelial cells were obtained from tissues salvaged at reduction mammoplasty. The 23 donors in this study were between 18 and 51 years old (28±7 years, mean±SD) and most were Caucasian (Table 1). There was no correlation between age and *CYP1A1* or *CYP1B1* induction or BP–DNA adduct levels ($r=-0.032$, −0.117 and 0.177, respectively).

3.2. Expression of CYP1A1 and CYP1B1 by oligonucleotide arrays and RT-PCR

RNA samples from 22 cells strains were examined for a battery of gene expression changes in BP-exposed cells (4 μM BP, 12 h), compared to unexposed cells, using the Affymetrix oligonucleotide array system (Fig. 1A). The majority of the observed changes in four cell strains have been reported elsewhere [4], however, among the most striking changes were the induction of *CYP1A1* and *CYP1B1*. As these enzymes are well-known to participate in the activation of PAHs the result was not unexpected, however, it was considered necessary to confirm the oligonucleotide array observations using RT-PCR (Fig. 1B).

Induced *CYP1A1* transcription, as measured by DNA-oligonucleotide arrays and RT-PCR, is presented in Table 2. The correlation between the data obtained by DNA-oligonucleotide microarrays and by RT-PCR is shown in Fig. 2A. Although the results of both assays for increased *CYP1A1* transcription were highly correlated (Fig. 2A; $r=0.73$, $P<0.001$), it was clear that RT-PCR was the more sensitive method (Table 2). Cell strain M98014 had virtually no *CYP1A1* increased transcription by RT-PCR, while 10 strains were 10- to 48-fold induced by RT-PCR and one strain was 100-fold induced by RT-PCR.

A similar pattern emerged for *CYP1B1* (Fig. 2B and Table 3) where there was a high degree of correlation between RT-PCR and DNA-oligonucleotide microarray measures of increased transcription (Fig. 2B; $r=0.70$, $P<0.001$). By RT-PCR, levels of *CYP1B1* transcription were not increased in three cell strains (M98013, M98014 and M98016, Table 3), while seven strains showed 7- to 20-fold induction and two strains (M98026 and M00012) showed the highest, 23- and 39-fold induction (Table 3).

A comparison of *CYP1A1* induction with *CYP1B1* induction for the 22 individuals was performed using both DNA-oligonucleotide arrays and RT-PCR. The induction in transcription by BP was highly correlated for the two enzymes when measured by both RT-PCR (Fig. 3, $r=0.88$, $P<0.001$) and DNA-oligonucleotide arrays (data not shown, $r=0.70$, $P<0.001$). These associations remained strong even when the data for cell strain M98026, a highly inducible outlier, were removed from the analysis. In addition, these

Table 1
Origins of mammary epithelial cells

Cell strain designation	Age of donor (years)	Donor race
M98011	45	Caucasian
M98013	26	Caucasian
M98014	26	Caucasian
M98015	51	Caucasian
M98016	25	Unknown
M98018	36	Caucasian
M98019	33	Caucasian
M98021	21	Caucasian
M98025	27	Caucasian
M98026	21	Caucasian
M98030	30	Caucasian
M98035	19	American–Indian
M98040	20	Caucasian
M99003	42	Caucasian
M99004	40	Caucasian
M99005	22	Caucasian
M99006	27	Caucasian
M99016	27	Caucasian
M99021	26	Caucasian
M99025	31	Caucasian
M00004	23	African–American
M00012	18	Caucasian
M00015	27	Caucasian

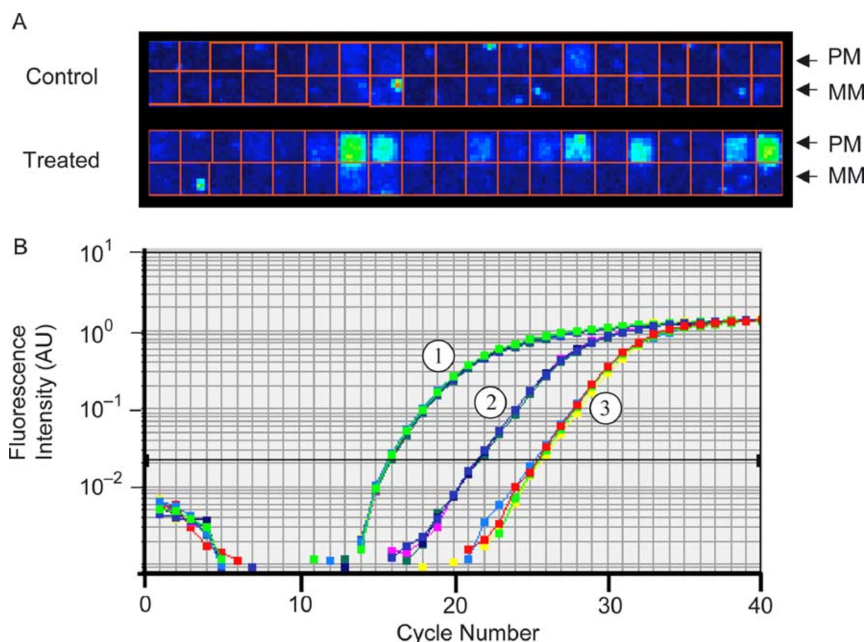

Fig. 1. Detection of benzo[a]pyrene-induced *CYP1B1* transcription in NHMECs (the example of cell strain M98026) using (A) DNA-oligonucleotide microarrays and (B) reverse transcription and polymerase chain reaction (RT-PCR by TaqMan™ assay). Panel A shows four rows of 20 probe tiles, where each tile represents a *CYP1B1* DNA-sequence that was introduced onto the Affymetrix™ microarray by a photolithographic process. The top row and second row are 20 perfect match (PM, top) and corresponding 20 mismatch (MM, second) hybridized to fluorescence-labeled products of RNA isolated from untreated NHMECs. The third and fourth rows are identical tiles hybridized to fluorescence-labeled products of RNA isolated from BP-treated NHMECs. Panel B shows the relative accumulation of PCR products with increasing PCR cycle number for RNA encoding: (1) *GAPDH* from BP-treated and untreated NHMECs, (2) *CYP1B1* from NHMECs treated with BP, and (3) *CYP1B1* from untreated NHMECs.

Table 2
Induction of *CYP1A1*, stratified by *GSTM1* status, in human mammary cells treated with benzo[a]pyrene

Cell strains positive for *GSTM1*			*GSTM1*-null cell strains		
Strain	Induction of *CYP1A1*[a]		Strain	Induction of *CYP1A1*[a]	
	OA	RT-PCR		OA	RT-PCR
M98011	8.7±0.4	20.5±1.9	M98013	4.3±2.7	2.9±1.0
M98014	−0.5±2.1	1.0±0.2	M98021	5.5±0.5	5.1±1.0
M98015	11.2±0.3	7.7±1.6	M98025	7.0±1.5	47.7±12.1
M98016	0.3±2.3	3.0±1.0	M98030	2.9±0.7	15.5±3.9
M98018	4.1±0.4	12.5±2.5	M98035	−1.0±3.8	4.1±2.1
M98019	0.3±2.4	4.9±2.0	M99003	3.0±0.4	13.8±2.2
M98026	16.6±0.4	99.5±49.4	M99004	9.9±2.8	28.5±3.9
M98040	−1.2±0.1	9.0±1.3	M99021	0.2±2.6	3.6±0.7
M99005	2.1±0.0	10.2±0.4	M00012	6.3±0.8	15.2±4.5
M99006	1.9±0.4	7.7±1.7	M00015	4.1±0.9	21.5±3.6
M99016	4.4±1.6	6.3±3.3			
M99025	−1.9±0.1	10.9±3.5			
M00004	NM	NM			

NM, Not measured; OA, oligonucleotide microarray.
[a] Fold increase mean ± SD ($n=2$) of duplicate measurements on duplicate samples.

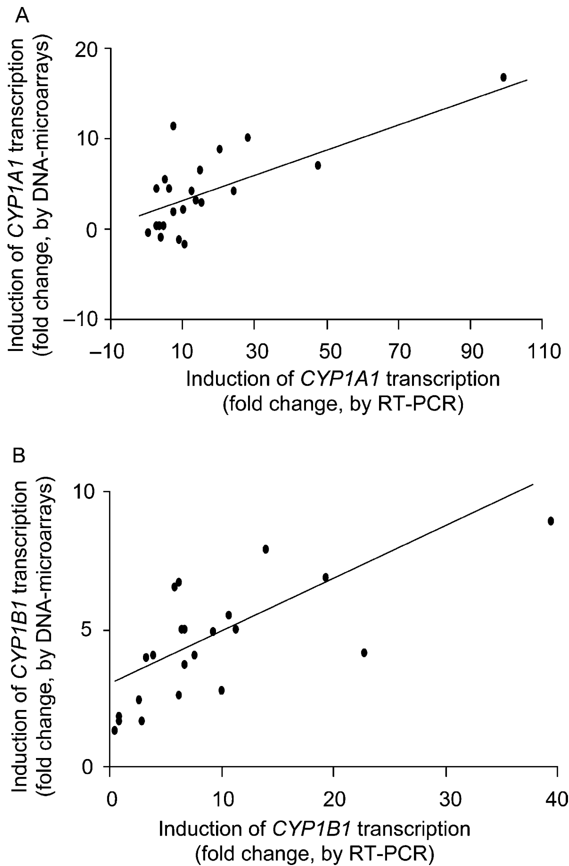

Fig. 2. Comparison of the detection of BP-induced *CYP1A1* and *CYP1B1* transcription in 22 NHMEC strains by RT-PCR with detection of *CYP1A1* and *CYP1B1* induction by DNA-microarrays. Panel A shows that for *CYP1A1*, detection of induction by RT-PCR was well-correlated with that detected by DNA-microarrays ($r=0.73$, $t=4.80$, $P<0.001$, d.f.$=20$). Similarly, panel B shows that for *CYP1B1*, detection of induction by RT-PCR was well-correlated with that detected by DNA-microarrays ($r=0.70$, $t=4.38$, $P<0.001$, d.f.$=20$).

associations also remained strong when the data were log-transformed (log base 2, data not shown). The inducibility range for *CYP1A1* was approximately twice that of *CYP1B1*, and there was an eight-fold variation in the *CYP1A1*:*CYP1B1* ratio.

3.3. BP–DNA adduct formation and comparison with CYP1A1 and CYP1B1 induction

A total of 23 different NHMEC strains, seeded at passage 6 and grown to semi-confluence, were exposed to 4 μM BP for 12 h. DNA samples were prepared from two independently exposed cultures and each was assayed once. BP–DNA adduct levels were measured using an anti-BPDE–DNA CIA and are presented as adducts/10^8 nucleotides (mean$\pm$range) in Table 4. From the total of 23 NHMEC strains, only three gave non-detectable values while the positive samples ranged from 0.84 ± 0.25 to 15.8 ± 0.4 adducts/10^8 nucleotides (one was lost).

Comparison of BP–DNA adduct levels with *CYP1A1* and *CYP1B1* induction revealed a weak correlation between formation of BP–DNA adducts and level of *CYP1A1* induction (Fig. 4A; $r=0.40$, $P<0.10$). Also there was a weak correlation between formation of BP–DNA adducts and level of *CYP1B1* induction (Fig. 4B; $r=0.50$, $P<0.05$).

3.4. Correlation of BP–DNA and CYP1A1 and CYP1B1 induction with GSTM1

In agreement with the published literature, approximately half of the donors were found to be *GSTM1*-null when DNA samples were genotyped by PCR [23]. We therefore stratified BP–DNA adduct levels with the extent of *CYP1A1* and *CYP1B1* induction, in strains from *GSTM1*-null donors, and found no correlation. Similarly, the correlation between BP–DNA-adduct formation and *CYP1A1* and *CYP1B1* induction was examined in cell strains derived from *GSTM1*-positive donors. In 11 of 12 donors positive for *GSTM1*, BP–DNA adduct levels appeared to be highly correlated with inducibility of both *CYP1A1* ($r=0.84$, $P<0.001$) and *CYP1B1* ($r=0.77$, $P<0.001$). However, when the data from the highly inducible outlier (cell strain M98026) were removed the correlation was poor and not statistically significant.

It was possible to examine expression patterns of several other metabolism genes potentially involved in the activation and/or detoxication of BP from data derived from the oligonucleotide arrays. These genes included: epoxide hydrolase, dihydrodiol dehydrogenase, and glutathione transferases (M3, M4 and M5), however, no correlations with their expression patterns and formation of BP–DNA adducts were observed (data not shown).

Table 3
Induction of *CYP1B1*, stratified by *GSTM1* status, in human mammary cells treated with benzo[a]pyrene

Cell strains positive for *GSTM1*			*GSTM1*-null cell strains		
Strain	Induction of *CYP1B1*[a]		Strain	Induction of *CYP1B1*[a]	
	OA	RT-PCR		OA	RT-PCR
M98011	6.7±0.1	6.3±1.4	M98013	1.6±0.2	1.0±0.1
M98014	1.8±0.1	1.0±0.2	M98021	2.4±0.6	2.7±0.5
M98015	5.0±0.4	6.8±1.1	M98025	7.9±0.6	14.0±0.6
M98016	1.3±0.1	1.0±1.0	M98030	4.0±0.7	7.7±0.7
M98018	6.5±0.6	5.8±0.4	M98035	3.9±0.7	3.3±0.6
M98019	4.0±0.1	4.0±0.5	M99003	4.9±0.1	9.3±0.5
M98026	8.9±0.4	39.5±6.8	M99004	6.8±1.3	19.4±1.7
M98040	5.0±0.6	11.3±1.4	M99021	1.6±0.0	2.9±0.6
M99005	3.7±1.3	6.7±0.7	M00012	4.1±0.6	22.8±4.6
M99006	2.6±0.4	6.3±0.8	M00015	5.5±0.6	10.7±1.3
M99016	5.0±1.4	6.5±2.6			
M99025	2.7±0.1	10.0±1.0			
M00004	NM	NM			

NM, Not measured; OA, oligonucleotide microarray.
[a] Fold increase mean±SD ($n=2$) of duplicate measurements on duplicate samples.

4. Discussion

This report shows that measurement of induction of cytochrome P450 genes, at the transcription level, by TaqMan™-based RT-PCR is highly correlated with DNA-oligonucleotide microarray data. However, RT-PCR is considerably more sensitive than DNA-microarrays for the determination of *CYP1A1* and *CYP1B1* transcription. These studies were originally designed to identify key gene expression biomarkers of BP exposure in normal human epithelial cells, and use those biomarkers to define inter-individual variation in response to BP exposure. Our preliminary studies, presented elsewhere [4] revealed that *CYP1A1* and *CYP1B1* were the genes that were most consistently induced among the four NHMEC strains studied at that time. Here we have demonstrated use of DNA-microarrays as an investigative tool to identify key genes that can be further investigated to reveal human inter-individual variation in such biomarkers by studying a broader panel of donors with a robust and relatively inexpensive method (RT-PCR). This approach may be useful in the context of toxicogenomic studies that use DNA-microarray technology to study the effects of carcinogens and other toxins in laboratory animal models that have limited host variability parameters [25–29]. Moreover, the current approach has the advantage that the cells are normal and diploid, compared to studies using human cell lines, including: HepG2, MCF-7, MCF-10A, T-47D, TSU-Pr1 and CL5, that are partially or completely transformed or have limited and abnormal phenotypes [30–34].

These data showed wide inter-individual variation in both *CYP1A1* and *CYP1B1* induction, up to 100-and 40-fold, respectively, as measured by RT-PCR, for NHMECs exposed to BP in a 12-h period. This is consistent with many other literature reports that have

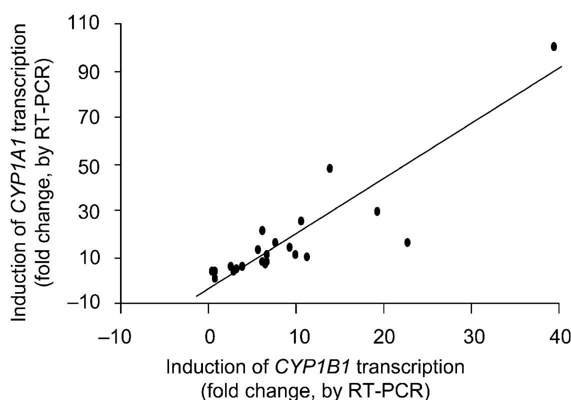

Fig. 3. Comparison of induction of *CYP1A1* and *CYP1B1* transcription by induced BP exposure (4 μM) in 22 NHMEC strains, measured by RT-PCR. Induction of *CYP1A1* was highly correlated with that of *CYP1B1* ($r=0.88$, $t=8.29$, $P<0.001$, d.f.$=20$).

Table 4
Formation of BP–DNA adducts (per 10^8 nucleotides), stratified by *GSTM1* status, in NHMEC strains exposed to 4 μM benzo[a]pyrene for 12 h

Cell strains positive for *GSTM1*		*GSTM1*-null cell strains	
Strain	BP–DNA adducts/ 10^8 nucleotides	Strain	BP–DNA adducts/ 10^8 nucleotides
M98011	1.95±0.29[a]	M98013	10.76±1.12
M98014	1.98±0.33	M98021	0.85±0.25
M98015	NM	M98025	ND
M98016	3.08±0.55	M98030	2.56±0.08
M98018	4.94±1.00	M98035	1.16±0.49
M98019	3.14±0.50	M99003	1.28[b]
M98026	10.57±0.34	M99004	15.80±0.37
M98040	0.99±0	M99021	0.98±0.12
M99005	ND	M00012	5.94±0.66
M99006	1.77±0.20	M00015	4.28±2.31
M99016	4.09±1.27		
M99025	0.91±0.51		
M00004	ND		

ND, Not detectable; NM, not measured.
[a] Mean±range of two assays.
[b] Single assay.

long documented human inter-individual variation in drug and xenobiotic metabolism [2,35–37]. The origins of such variations are the subject of a recent review, which concludes that genetic variation in a range of factors, from the cytochrome P450s themselves to regulatory elements (AhR and Arnt), as well as chaperones (hsp90), contribute to this phenomenon [38].

The levels of induction for both *CYP1A1* and *CYP1B1*, in NHMECs exposed to BP, were highly correlated in the 22 cell strains studied, suggesting that they are under the control of the same dioxin responsive element. A similar correlation was demonstrated recently in cultivated human lymphocytes, from 32 male and female Chinese donors, that were exposed to benz[a]anthracene in vitro. It was noted that cells derived from female donors displayed a significantly higher response, compared to males [39]. Despite these and other reports, which conclude that CYP1A1 and CYP1B1 are AhR-dependent enzymes [40,41], some studies suggest that control of these pathways is more complex. For example, in AhR null mice, *CYP1A1*, but not *CYP1B1*, was induced by BP [42]. These findings were corroborated for extra-hepatic tissues, including heart, kidney, thymus, testis, ovary and brain [43]. In human tissues and cells evidence for differential expression of these genes has also been found [32,34,44].

Formation of BP–DNA adducts was only weakly correlated with *CYP1A1* and *CYP1B1* induction. The enzymes CYP1A1 and CYP1B1 are important in the metabolic activation of BP, causing transformation from the parent compound to simple arene oxides, primarily across the 3,4, 7,8 and 9,10 positions. These enzymes are further involved in the oxidation of BP-7,8-dihydro-diol across the olefinic bond, following conversion of BP-7,8-oxide to BP-7,8-dihydrodiol.

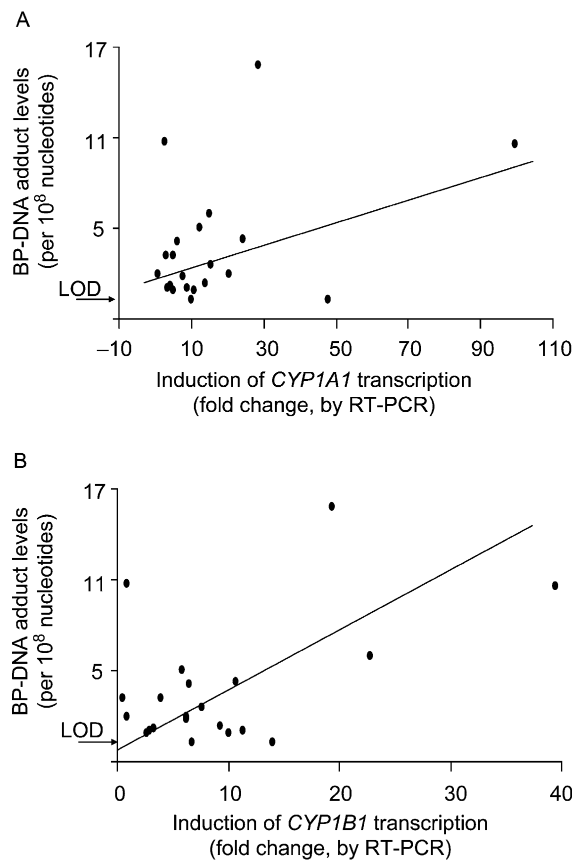

Fig. 4. Comparison of the relationships between BP–DNA adduct formation and BP induction of *CYP1A1* and *CYP1B1* transcription in NHMECs, as measured by RT-PCR, with BP–DNA adduct formation, as measured by chemiluminescence immunoassay. Panel A shows that induction of *CYP1A1* was poorly correlated with BP–DNA adducts in BP-treated NHMECs ($n=21$, $r=0.40$, $t=1.92$, $P=0.071$, d.f.$=19$). LOD, limit of assay detection (0.33 adducts/10^8 nucleotides). Panel B shows that *CYP1B1* was also poorly, but significantly, correlated with BP–DNA adducts in BP-treated NHMECs ($r=0.50$, $t=2.54$, $P=0.021$, d.f.$=19$).

The resulting ultimate carcinogenic metabolite, BP-7,8-diol-9,10-epoxide, can then bind to DNA [2]. However, additional enzymes are involved, specifically epoxide hydrolase, which is polymorphic and converts the arene oxide into a dihydrodiol [45, 46], and other cytochrome p450s (for example, CYP3A4), are known to catalyze the oxidation steps [6]. Levels of the resultant DNA adducts, often collectively referred to as the biologically effective dose, are also impacted by a variety of enzyme systems that are involved in detoxication, including UDP-glucuronosyl transferases, sulphatases and glutathione-*S* transferases, as well as DNA repair enzyme systems [2]. Therefore, the weak correlation between induction of these two cytochrome p450s and BP–DNA adduct formation, as well as the lack of correlation between BP–DNA adduct formation and GSTM1 status, are probably reflections of this biological complexity.

When the cell strain donors were stratified by *GSTM1* genotype, it appeared that there might be a relatively strong correlation between BP-DNA adduct levels and cytochrome P450 induction, but these associations were primarily driven by one highly inducible outlier (cell strain M98026). Further studies with greater numbers of cell strains will be required to address this question more rigorously. Overall, the weak correlation between DNA adduct formation and induction of the enzymes studied may reflect the relative capacity of different polymorphic variants of these cytochrome p450 genes to catalyze specific reactions, such as the conversion of BP to its 7,8-arene-oxide, which has been recently reported [6]. In one study of 76 cases and 60 controls, when normal breast tissues were treated with BP (4 μM), BP–DNA adduct formation (in the range of $6–14/10^8$) was found to be correlated with *CYP1B1* genotype [7]. Moreover, several molecular epidemiologic studies have suggested that *CYP1B1* polymorphisms may be implicated in breast cancer [47,48], but other studies find no association [49–51]. In the current study, the small numbers do not allow us to correlate *CYP1A1* and *CYP1B1* polymorphisms with BP–DNA adduct formation, however, all new cell strains are routinely genotyped for these and other polymorphisms of interest. Expression patterns, measured by oligonucleotide arrays, for epoxide hydrolase, dihydrodiol dehydrogenase, and glutathione transferases (M3, M4 and M5) were also examined for correlation with BP–DNA adduct formation, but there was none.

In summary, development of a panel of NHMECs has enabled the study of human inter-individual variation in response to carcinogen exposure. It has been demonstrated that induction of *CYP1A1* and *CYP1B1* is highly variable, but in spite of the critical role of these two genes in the metabolic activation of BP, the transcription of these genes is not a good predictor of BP–DNA adduct formation.

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