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Transcriptional profiles of benzo(a)pyrene exposure in normal human mammary epithelial cells in the absence or presence of chlorophyllin☆

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Abstract

Benzo(a)pyrene (BP) exposure causes alterations in gene expression in normal human mammary epithelial cells (NHMECs). This study used Affymetrix Hu-Gene133A arrays, with 14,500 genes represented, to evaluate modulation of BP-induced gene expression by chlorophyllin in six NHMEC strains derived from different donors. A major goal was to seek potential biomarkers of carcinogen exposure and how they behave in the presence of a chemopreventive agent. NHMECs (passage 6 and 70% confluence) were exposed for 24 h to either vehicle control, or BP, or chlorophyllin followed by BP and chlorophyllin together. BP exposure resulted in approximately 3-fold altered expression of 49 genes in at least one of the six NHMEC strains. When cells were exposed to chlorophyllin pre-treatment followed by BP plus chlorophyllin, expression of 125 genes was similarly altered. Genes in the functional categories of xenobiotic metabolism, cell signaling, cell motility, cell proliferation, cellular transcription, metabolism, cell cycle control, apoptosis and DNA repair were identified. Only *CYP1B1* and *ALDH1A3* were consistently up-regulated by ~3-fold in most of the cell strains (at least 4) when exposed to BP. Cluster analysis identified a suite of 13 genes induced by BP where induction was mitigated in the presence of chlorophyllin. Additionally, cluster analysis identified a suite of 16 genes down-regulated by BP where induction was partially restored in the presence of chlorophyllin.
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Keywords: Carcinogens; Chemoprevention; Chlorophyllin; Gene expression; Polycyclic aromatic hydrocarbons

1. Introduction

Exposures to xenobiotics such as a polycyclic aromatic hydrocarbons (PAHs), halogenated aromatic hydrocarbons, aromatic amines, nitrosamines, mycotoxins and other industrial pollutants are unavoidable given their ubiquitous presence in the environment [1,2]. Some PAHs, like benzo(a)pyrene (BP), are known animal carcinogens and have been declared by the International Agency for Research on Cancer to be probable human carcinogens (group 2A) [3].

Cytochrome P450s belong to a super-family of enzymes some of which are induced by exposure to PAHs and are involved in the metabolic activation of BP to reactive metabolites that cause genetic damage through the formation of DNA-adducts [4]. We propose the hypothesis that the metabolic, toxic and mutagenic effects of BP exposure causes extensive changes in gene expression patterns [5,6]. We further propose that mitigation of DNA damage from exposure to BP by chemopreventive agents, like the water soluble metalloporphoryn chlorophyllin, will result in modulation of BP-induced changes in gene expression.

To test these hypotheses, and to potentially develop gene expression matrices as surrogate biomarkers of environmental exposures and efficacy in their mitigation through parallel exposures to chemo-protective agents, we examined genome-wide changes in gene expression following exposure of a panel of six

Abbreviations: BP, benzo(a)pyrene; CYP, cytochrome P450; NHMEC, normal human mammary epithelial cell; PAH, polycyclic aromatic hydrocarbon.

☆ *Disclaimer*: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institutes for Occupational Safety and Health.

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NHMECs to BP in the absence or presence of chlorophyllin. Given the wide inter-individual variation in human response to carcinogen exposure, it seems likely that identification of gene expression-based biomarkers of carcinogen exposure and the efficacy of intervention strategies will be complementary to measures of DNA-adducts.

2. Materials and methods

2.1. Chemicals and reagents

BP (CAS no. 50-32-8) was purchased from the NCI Chemical Carcinogen Reference Standard Repository (Kansas City, MO). Chlorophyllin (CAS no. 11006-34-1), molecular biology grade water, MES (2-[*N*-morpholino]-ethane sulphonic acid) free acid monohydrate (CAS no. 145224-94-8) and MES sodium salt (CAS no. 71119-23-8) were procured from Sigma–Aldrich (St. Louis, MO). Herring sperm DNA was obtained from Promega (Madison, WI), Tween-20 (CAS no. 9005-64-5) was purchased from Pierce Biotechnology (Rockford, IL); SSPE (NaCl, 3M; NaH_2PO_4, 0.2M; EDTA, 0.02M) and culture media (minimum essential basal medium [MEBM], Clonetics™) were from Cambrex Biosciences (Walkersville, MD). Biotinylated anti-streptavidin antibody was bought from Vector Laboratories (Burlingame, CA); acetylated bovine serum albumin, phosphate buffered saline (pH 7.2 and 7.4) and R-phycoerythrin streptavidin were purchased from Invitrogen Life Technologies (Carlsbad, CA). Phenol:chloroform:isoamyl alcohol and RNAse free NaCl solution were from Ambion (Austin, TX). RNeasy kits were from Qiagen (Valencia, CA), T7-oligo(dT) promoter primer kits, GeneChip IVT Labeling Kits, Hu-Gene 133A arrays, a GeneChip Fluidics Station 400, and data analysis software were from Affymetrix (Santa Clara, CA); SuperScript Double-Stranded cDNA Synthesis kits were from Invitrogen Life Technologies (Carlsbad, CA), single stranded cDNA Advantage RT-PCR kits were bought from BD Biosciences (Palo Alto, CA), Phase Lock Gels were from Brinkmann Instrument (Westbury, NY), and an HP GeneArray 2500 Scanner was procured from Hewlett Packard (Palo Alto, CA). Primer Express v1.5 software and SYBR Green were obtained from PE Applied Biosystems (Foster City, CA), and PCR primers were synthesized by Sigma–Genosys (Woodlands, TX).

2.2. Cell strains and cell culture

Six NHMEC strains from different donors (M98035, M99005, M98015, M98025, M99025 and M99016) were used in this study. They were developed in our laboratory from healthy breast tissue discarded at elective reduction mammoplasty (Cooperative Human Tissue Network sponsored by the National Cancer Institute and National Disease Research Interchange) through a process involving mechanical disruption and enzymatic digestion of breast tissues [5,6] according to previously published methods [7]. The National Institute for Occupational Safety and Health–Human Studies Review Board was consulted regarding approval for use of human tissues, and since no identifying information was received with the discarded breast tissues a waiver of human studies review was granted.

2.3. Cell treatments

A uniform population of cells at passage 6 (~70% confluence) was then subjected to one of the following treatment protocols: T1-control vehicle (acetone:ethanol, 2:3 at 1:1000 dilution in culture media) for 24 h; T2-BP (4 μM) alone for 24 h; T3) 24 h pre-treatment with chlorophyllin (5 μM) followed by co-treatment with BP (4 μM) and chlorophyllin (5 μM) together for 24 h. Control vehicle and BP were diluted in culture media (MEBM) and chlorophyllin in nuclease free water. All treatments were carried out in duplicate.

2.4. Target generation for microarray

Total RNA was isolated from the above three treatment groups (T1, T2 and T3) using an RNeasy kit according to manufacturer's protocol. Total RNA (12 μg; 260:280 ratio >1.9) was used for synthesis of double stranded cDNA using a T7-oligo(dT) promoter primer kit and SuperScript Double-Stranded cDNA Synthesis kit according to the manufacturer's instructions. The resulting cDNA was purified by phenol/chloroform extraction using Phase Lock Gels, ethanol precipitated and finally resuspended in nuclease free water (12 μl). A portion (6 μl) of the purified cDNA was used for the generation of biotinylated complementary RNA (cRNA) using the GeneChip IVT labeling kit. Following purification (RNeasy kit), cRNA was reduced to 35–200 base fragments by metal-induced hydrolysis (94 °C, 35 min). Fragmented cRNA (15 μg) was hybridized (45 °C, 16 h) on to Hu-Gene 133A arrays (representing 14,500 genes with 22,283 probe sets). The efficiency of target hybridization was assessed by incorporating pre-mixed, staggered concentrations of biotin labeled cRNA transcripts for three bacterial genes of the biotin synthesis pathway (*bioB*, *bioC*, *bioD*) and one bacteriophage gene for recombinase (*cre*) at staggered concentrations (1.5, 5, 25 and 100 pM, respectively) in a hybridization cocktail. The hybridized arrays were subsequently washed and stained using the GeneChip Fluidics Station 400 and scanned (HP GeneArray 2500 Scanner).

2.5. Data analysis

The scanned images (.dat files) were analyzed using Microarray Suite (MAS) 5.0. Initially, an 'absolute analysis' was conducted to compute absolute signal intensities of the control (T1) and treated groups (T2 and T3). Subsequently, 'comparison analyses' comparing the fold change (FC) of the BP treated groups (T2 and T3) over the control vehicle (T1) was also conducted. 'Absolute analysis' provides a measure of the presence or absence of particular transcripts (establishing a 'detection' call), whereas their abundance is determined through 'comparison analysis' (establishing a 'signal' value), these analyses also provide a '*p*-value' for the 'detection' call. 'Comparison analysis' enables comparison of gene expression patterns of treated cells versus those of the control vehicle baseline. Chiefly, two algorithms are used by MAS to define changes in expression patterns. A 'change algorithm' defines a change (increase, decrease or no change) in expression patterns and also assigns a '*p*-value' to the same while a second algorithm provides a quantitative measure of the magnitude of change in terms of signal log ratio (SLR). SLRs are calculated by comparing the mean log ratios of probe pair intensities across the treated and control probe arrays being compared [8]. The log scale employed is to base 2 and a SLR of 1 corresponds to a FC of 2. Only genes having a present call (P) and a FC ~3.0 or greater (SLR ≥ 1.5) in both replicates in at least one of the six NHMEC strains were considered altered. Graphical representation of all the altered genes was generated using Cluster and Tree View software [9] using the average-linkage method [10].

To identify common patterns of gene expression patterns across cell strains following different treatments 'coefficient of correlation clustering' was conducted using Micro DB 3.0 and Data Mining Tool (DMT) 3.0. In the 'correlation of coefficient' clustering, 'seeding', a pre-clustering process involving an initial grouping of a small subset of genes with similar expression patterns occurs to generate a 'seed' which is based on a user specified correlation coefficient threshold. The higher the threshold the greater is the similarity of the probe sets used in defining the respective seed. Subsequently, a correlation coefficient threshold to assign probe sets to a particular 'seed' in order to create a 'cluster' is also defined [11]. In this study, the correlation threshold of forming 'seeds' and assignment of genes to respective clusters were both set at 0.90. The correlation coefficient can range between +1 and −1 with +1 representing complete correspondence. Clustering was carried out to first find genes common across all six cell strains on comparison of treated groups versus control vehicle (T2 versus T1; and T3 versus T1). Only genes with a present call (P) in both the replicates were used for the preliminary clustering. Subsequently, an unpaired, one-sided *T*-test was carried out on the above set of genes common to all six-cell strains to find genes significantly altered due to respective treatment ($p < 0.05$) and also determine the direction of change ('up' or 'down' regulated). The final analysis step was the generation of gene expression 'cluster maps' from subsets of statistically significantly altered genes.

2.6. Real-time polymerase chain reaction (RT-PCR)

Validation of gene expression on gene-chip(s) (GC) was carried out through RT-PCR. Single stranded cDNA was generated from reverse transcription of

total RNA (1 μg) using Advantage RT-PCR and RNeasy kits. Template from each treatment group (T1, T2, T3) was used to quantitate the expression of a subset of 17 genes altered by SLR ≥ 1.5 as determined by microarray analysis. Primers designed using Primer Express v1.5 and synthesized by Sigma–Genosys were used at a concentration of 50 pmol of each primer in a reaction mix with SYBR Green Universal PCR Master Mix. Quantitation was carried out using a standard 96-well block on 7900HT Fast Real-Time PCR System preloaded SDS software v2.2.2. Each sample was assayed in duplicate and their expression normalized to that of GAPDH using the $2^{-\Delta\Delta CT}$ method [12]. The specificity of the generated product was confirmed by looking for a single specific product on 'Amplify' software v1.2β (University of Wisconsin, Genetics, Madison) prior to RT-PCR as well as producing dissociation curves at the end of the analysis reaction.

3. Results

The expression patterns of 22,283 probe sets, representing 14,500 genes, in each of six NHMEC strains in response to BP exposure alone (T1 versus T2) or BP exposure in the presence of chlorophyllin (T1 versus T3) were determined using Hu-Gene 133A arrays. Control signals for *bioB*, *bioC*, *bioD* and *cre* were all present and detected in increasing order, as expected indicating, that the gene-arrays had performed according to the manufacturer's specifications.

Data for most of the 14,500 genes indicated no changes in gene expression in response to the carcinogen or the carcinogen together with the chemopreventive agent exposures. However, we considered all genes induced or repressed by a signal exhibiting an SLR ≥ 1.5 and a present call (P) in both the replicates. A complete list of all the genes meeting these criteria on comparison of BP exposed cells as well as chlorophyllin and BP exposed cells versus control vehicle exposed cells (T1 versus T2, T1 versus T3) can be found on the Web [13] in Tables A–D.

A total of 49 genes (0.34%) were altered in at least one of the six NHMEC strains on comparison of BP treated cells with control vehicle (T1 versus T2). Of these, 43 were up-regulated and six were down-regulated. Only *CYP1B1* showed consistent up-regulation by SLR ≥ 1.5 in all six cell strains. The next most consistently up-regulated gene was *ALDH1A3*, which showed up-regulation in four cell strains. Other consistently induced genes included genes which were primarily in the following categories: xenobiotic metabolism (Phase I and Phase II, e.g., *CYP1A1*, *ALDH3A1* and *AKR1D1*), cell signaling (e.g., *RYR3*), cell motility (e.g., *KRT23*), cellular transcription (e.g., *ZBED2*), and cell proliferation and angiogenesis (e.g., *SECTM1*). No gene was consistently down-regulated by SLR ≥ 1.5 in all cell strains, although one cell strain (M99016) did show marked down-regulation in *DOC1*, *GPNMB*, *BBOX1*, *PPP1R3C*, *CTGP* and *SGK*, and most cell-strains showed down-regulation of these genes indicated by a negative SLR ≤ 1.5. The data for these changes in gene expression are displayed graphically (Fig. 1, Panel A), where red tiles represent up-regulation and green tiles down-regulation. These data also reflect considerable inter-individual variation in response of NHMECs from different donors to exposure to BP.

A total of 125 genes (0.86%) were altered in at least one of the six NHMEC strains on comparison of BP and chlorophyllin treated cells with control vehicle (T1 versus T3). Of these 103 were up-regulated and 22 down-regulated. Again, only *CYP1B1*

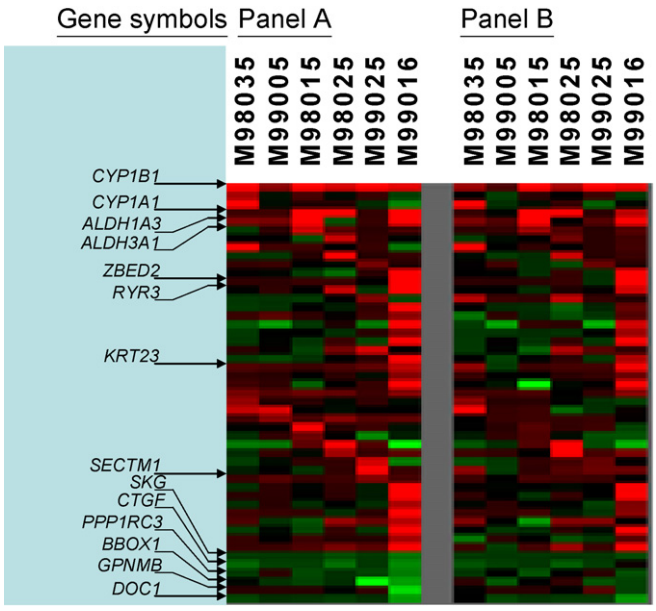

Fig. 1. Idiogram of gene expression in six normal human mammary epithelial cell strains (M98035, M99005, M98015, M98025, M99025, M99016) where red tiles represent gene induction and green tiles gene down-regulation for benzo[a]pyrene exposed (Panel A) or benzo[a]pyrene exposed in the presence of chlorophyllin (Panel B) relative to the vehicle control. These data show that *CYP1B1* is consistently induced in all six cell strains, and that several other genes are relatively consistently induced or down-regulated as indicated. Further, that the effects of chlorophyllin broadly mitigate those of benzo[a]pyrene, and that there is significant inter-individual variation in response.

was consistently up-regulated by SLR ≥ 1.5 in all but one cell strain. No gene was consistently down-regulated by SLR ≥ 1.5 in any of the cell strains. Genes modulated were found primarily in those categories altered by BP treatment, in addition to genes involved in cell cycle control, apoptosis, DNA repair and various other cellular processes such as neurogenesis, gene regulation, and hormonal metabolism.

Modulation of the effects of BP exposure by the presence of chlorophyllin can be seen when data for altered gene expression are compared between exposure to BP alone (T1 versus T3) with exposure to BP plus chlorophyllin (Fig. 1: Panel A versus Panel B). In general, there are two obvious patterns: first, genes are induced by BP exposure but the level of induction is mitigated in the presence chlorophyllin, as in the case of *CYP1A1* and *CYP1B1* where induction is reduced by approximately 10–20% in the presence of chlorophyllin; second, genes are down-regulated and again this down-regulation is mitigated by the presence of chlorophyllin, as in the case of *CTGF*. To explore the modulating effects of chlorophyllin on the effects of BP exposure in NHMECs a cluster analysis was performed.

Expression data for all genes exhibiting a present call for at least 1 treatment (T1, T2 or T3) and altered significantly ($p < 0.05$) were subjected to cluster analysis by treatment. This analysis revealed a total of 5 different expression clusters, with essentially 3 pattern types (Table 1 and Fig. 2). One cluster each was generated for genes significantly up-regulated and down-regulated (SLR > 1.5) by BP (clusters A and C). One cluster was generated for genes significantly down-regulated by BP plus

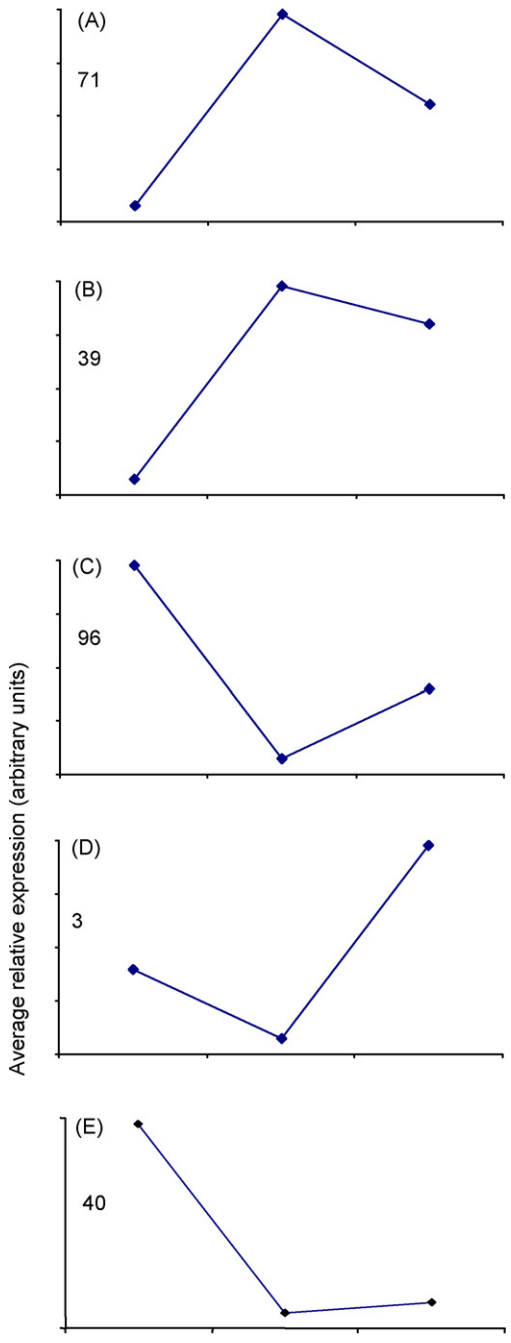

Fig. 2. Cluster analysis based on the premise that gene expression is either induced or down-regulated by each treatment. This analysis yielded five clusters: A and B where 71 and 39 genes, respectively, were induced by benzo[a]pyrene, but this induction was mitigated in the presence of chlorophyllin (overall 28 of the genes were common to both clusters, see Table 2); C and E where 96 and 40 genes, respectively, were down-regulated by benzo[a]pyrene, but this down-regulation was mitigated in the presence of chlorophyllin (overall 25 of the genes were common to both clusters, see Table 2); D where 3 genes down-regulated by benzo[a]pyrene were induced in the presence of chlorophyllin. The bars in this histogram are qualitative, only within a cluster do they have any relative significance.

Table 1
Cluster analysis

Clusters*	Number probes $p<0.05$	Significantly altered $p \leq 0.001$	Number of genes in common by Pattern Type ($p \leq 0.001$)	
A	71 (0.99†)	22	Pattern Type1‡	
B	39 (0.99)	12	Pattern Type1‡	10
C	96 (0.93)	13	Pattern Type 2§	
D	3 (0.99)	3	Pattern Type 3	
E	40 (0.99)	10	Pattern Type 2§	9

*A: Genes induced by BP where induction was partly mitigated by chlorophyllin based on initial premise of induction after exposure to BP in the absence of chlorophyllin (‡Pattern 1). B: Genes induced by BP where induction was partly mitigated by chlorophyllin based on initial premise of induction after exposure to BP in the presence of chlorophyllin (‡Pattern 1). C: Genes down-regulated by BP where down-regulation was partly mitigated by chlorophyllin based on initial premise of induction after exposure to BP in the absence of chlorophyllin (§Pattern 2). D: Genes down-regulated by BP where down-regulation was reversed by chlorophyllin based on initial premise of induction after exposure to BP in the presence of chlorophyllin (Pattern 3). E: Genes down-regulated by BP where down-regulation was partly mitigated by chlorophyllin based on initial premise of induction after exposure to BP in the presence of chlorophyllin (§Pattern 2). †Correlation coefficient.

chlorophyllin treatment (cluster E). However, 2 different expression clusters were generated for genes significantly up-regulated by BP plus chlorophyllin treatment (clusters B and D). Because of the commonality between clusters A and B and between clusters C and E, three different "Pattern Types" emerged (Table 1 and Fig. 2). In the case of all clusters the correlation of the altered genes in each cluster was 0.99. A complete list of all the genes in each cluster can be found at the Web site [13] [Tables G–J].

In order to identify those genes for which altered expression to BP was mitigated by chlorophyllin, we looked for commonality between clusters exhibiting similar expression patterns. Overall there were 28 genes common to clusters A and B (that is for which expression was induced by BP where induction was partially mitigated in the presence of chlorophyllin) that showed significantly altered expression ($p<0.05$). Of these there were 13 genes that showed highly significant alterations in expression ($p \leq 0.01$ for 4 genes and $p \leq 0.001$ for 9 genes; Table 2, also see the Web site [13] Tables G–J]). Similarly, there were 25 genes common to clusters C and E for which expression was down-regulated by BP where down-regulation was partially mitigated in the presence of chlorophyllin ($p<0.05$). Of these there were 16 genes that showed highly significant alterations in expression ($p \leq 0.01$ for 7 genes and $p \leq 0.001$ for 9 genes; Table 2, also see the Web site [13] Tables G–J]). A third pattern that was observed was for three genes down-regulated by BP but where expression was more than fully restored in the presence of chlorophyllin (Fig. 2).

Altered expression of a subset of 17 genes detected by the Hu-Gene 133A microarrays was validated by RT-PCR (Tables 3A and 3B). A more extensive version of this table can be found at [13] [Tables E and F]. For genes altered by exposure to BP alone, in general good correlations in fold change were observed between the gene microarray method and RT-PCR. However, there were several exceptions, for example *CALB1* and *SECTM1* which exhibited only a moderate correlation and

Table 2
Altered expression of genes defining BP exposure with chlorophyllin present

Genes common to clusters A and B*	Genes common to clusters C and E†
$p \leq 0.001$	$p \leq 0.001$
CYP1B1: cytochrome p450	KIAA0992: palladin
TRIOBP: actin binding protein	PLS3: plastin 3
ALDH1A3: aldehyde dehydrogenase	WDR1: WD repeat domain 1
S100A9: calcium binding protein	ADAM10: metallopeptidase
HPCAL1: hippocalcin-like 1	SGK: serum glucocorticoid receptor
NQO1: quinone dehydrogenase	TFCP2: transcription factor
TSC22D1: TSC22 domain	RRAS2: vRAS related protein
SSH1: slingshot homologue (Drosophila)	APBB2: amyloid β precursor
IER3: immediate early response 3	PDLIM2: PDZ and LIM domain 2
$p \leq 0.01$	$p \leq 0.01$
S100A8: calcium binding protein	CRIM1: cys-rich tansmembrane
TIPARP: poly(ADP-ribose) polymerase (TCDD inducible)	SRPK2: SFRS protein kinase 2
ARHGEF4: Rho guanine nucleotide exchange factor 4	SEMA3F: sema domain, immuno
	CLN5: ceroid-lipofuscinosis
	HNRPK: heterogenous nuclear
	LIMK2: LIM domain GRB2-like
	HADHSC: L-3-hydroxyacyl-coenzy

* Clusters exhibiting Pattern 1 (genes induced by BP where induction was partly mitigated by chlorophyllin, see Table 1).
† Clusters exhibiting Pattern 2 (genes down-regulated by BP where down-regulation was partly mitigated by chlorophyllin, see Table 1).

MERTK which exhibited a very poor correlation. Again, generally good correlation in fold change was observed between the gene microarray method and RT-PCR for expression of most genes altered on treatment with BP plus chlorophyllin, exceptions being *AKR1C2*, *DHRS3* and *MCM5* which exhibited only a moderate correlation.

4. Discussion

The principal changes observed in NHMECs exposed to BP were, as expected, induction of Phase I and Phase II metabolism genes known to be involved in carcinogen metabolism. The most consistently up-regulated of all genes was *CYP1B1* probably suggesting its role as the predominant cytochrome P450 isoform involved in the metabolism of BP to DNA binding electrophiles in most individuals. In addition to involvement in BP and BP-dihydrodiol metabolism and activation, CYP1B1 is implicated in hormone metabolism. As such its induction by xenobiotics may affect estrogen responsive tissues and consequently hormonally related cancer risk [14].

The results from this study are in agreement with previous findings from our laboratory [5,6]. In addition to *CYP1B1*, various members of the detoxication battery, *AKR1D1*, *ALDH1A3* and *ALDH3A1* known to be involved in the metabolism of steroids, various endogenous substrates and toxicants were up-regulated by BP and may suggest their probable role in the scavenging of any free radicals generated by catalytic cycling of BP [15]. Our results are also in broad agreement with a study that investigated expression signatures of tobacco smoke in peripheral leukocytes that also found induction of *CYP1B1* and *IL-1B* [16] (also see Web site Table G).

Various other cellular targets altered in different cancers may typically include different candidates involved in processes as diverse as transcriptional regulation, cellular signaling, inter- and intra-cellular transport, cell adhesion and other developmental and intermediary metabolic processes. BP was shown to alter expression levels and patterns of various members belonging to the above functional classes in NHMECs. Genes coding for *ZBED2*: a zinc finger protein, *EREG*: an epidermal growth factor, *KRT23*: a histone deacetylase inducible acidic keratin, *SECTM1*: a secreted transmembrane protein, *BEX1*: a presently uncharacterized hypothalamus protein, and *HSD17B2*: hydroxysteroid dehydrogenase 2, were all consistently up-regulated in all 6 donors though not all to the extent of SLR $\geq$ 1.5 in all 6 donors. Of these, *EREG* (epiregulin) [17], *KRT23* [18] and *BEX1* are reported to be involved in different cancers or different aspects of carcinogenesis with *BEX1* also having a role in neurogenesis [19].

Surprisingly, genes involved in DNA damage and repair were not altered. This is in agreement with some previous studies [20,21] but not with others [22,23]. Apparently, the time and duration of exposure, nature of the carcinogen used (active metabolite or parent compound), dose, toxicity and model system employed could all impact response patterns. Bartosiewicz et al. [20] and Hockley et al. [21] used the parent compound, BP, and observed no significant changes in the expression of DNA repair or stress response genes as opposed to Akerman et al. [22] and Luo et al. [23] who employed the active metabolite, benzo(a)pyrene diol epoxide and observed an elevation in the expression of DNA damage/repair response genes such as *Gadd45*, *Ref*-1 and *RAD* [22] and various genes involved in all major DNA repair pathways [23]. Probably, depending on the nature and magnitude of damage involved, cellular damage processing may be mediated by alterations in the expression of genes coding for various xenobiotic metabolizing enzymes, detoxication enzymes, proteins involved in stress response, cell cycle control and apoptosis, all of which we observed in our study, or in some

Table 3A
Correlation of fold change (FC) on Hu-Gene 133A arrays and RT-PCR for partial list of genes altered by ∼3-fold change (SLR ≥ 1.5) in at lest one of the six NHMECs when treated with BP alone

Gene	Probe ID	Accession no.	BP alone vs. vehicle control (avg. FC ± S.D.)												Correlation (r) GC-RT BP alone
			1 (M98035)		2 (M99005)		3 (M98015)		4 (M98025)		5 (M99025)		6 (M99016)		
			GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	
CYP1B1	202437_s_at	NM_000104	6.76 ± 0.99	10.14 ± 0.46	3.62 ± 0.53	7.61 ± 0.32	11.72 ± 0.57	32.10 ± 0.85	5.55 ± 1.35	7.52 ± 2.41	13.46 ± 0.66	21.58 ± 2.18	5.89 ± 0.86	9.28 ± 2.35	0.87
CYP1A1	205749_at	NM_000499	1.68 ± 0.87	10.48 ± 1.47	1.65 ± 0.92	14.50 ± 0.79	11.34 ± 1.11	47.62 ± 2.64	8.59 ± 0.84	15.92 ± 2.62	1.32 ± 0.13	9.83 ± 0.99	9.21 ± 0.90	95.29 ± 5.83	0.67
ALDH1A3	222168_at	NM_000693	3.23 ± 1.09	4.54 ± 0.44	3.03 ± 0.00	3.44 ± 0.53	14.43 ± 0.71	7.74 ± 0.59	−1.96 ± 5.80	3.14 ± 0.67	1.44 ± 0.42	3.84 ± 2.98	16.65 ± 2.44	14.42 ± 2.49	0.9
MERTK	206028_s_at	NM_006343	7.25 ± 1.06	0.67 ± 0.47	1.57 ± 0.60	1.59 ± 0.64	1.57 ± 0.60	2.06 ± 0.33	0.27 ± 2.25	1.94 ± 1.88	1.42 ± 0.14	NA ± −	−2.82 ± 5.86	0.92 ± 0.27	−0.30[†]
CALB1	205625_s_at	NM_004929	−1.37 ± 3.35	0.48 ± 0.09	−1.27 ± 4.26	0.90 ± 0.04	−1.42 ± 0.14	0.53 ± 0.05	0.06 ± 2.55	0.38 ± 0.09	3.26 ± 0.32	1.68 ± 0.62	−1.80 ± 4.42	0.83 ± 1.17	0.20
SECTM1	231716_s_at	NM_003004	3.06 ± 0.60	3.07 ± 0.31	2.17 ± 0.93	4.28 ± 0.45	2.65 ± 0.26	7.48 ± 2.33	2.38 ± 0.12	6.55 ± 0.51	1.52 ± 0.15	5.97 ± 0.86	3.41 ± 0.83	10.94 ± 0.83	0.39
SGK	201739_at	NM_005627	−1.46 ± 0.07	0.61 ± 0.12	−1.41 ± 0.00	0.78 ± 0.16	−1.41 ± 0.00	0.62 ± 0.11	−1.94 ± .028	0.54 ± 0.02	−1.43 ± 0.28	0.86 ± 0.05	−2.93 ± 0.14	0.37 ± 0.04	0.83
CTGF	209101_at	NM_001901	−2.64 ± 0.00	0.48 ± 0.09	−1.63 ± 0.16	0.90 ± 0.04	−1.63 ± 0.16	0.53 ± 0.05	−2.81 ± 0.95	0.38 ± 0.09	−1.43 ± 0.28	1.05 ± 0.02	−3.56 ± 1.03	0.30 ± 0.13	0.85
PPP1R3C	204284_at	NM_005398	−1.47 ± 0.22	0.64 ± 0.08	1.11 ± 0.05	0.82 ± 0.08	−1.87 ± 0.00	0.52 ± 0.01	−1.28 ± 0.19	0.61 ± 0.14	−1.83 ± 0.44	0.33 ± 0.03	−3.26 ± 0.32	0.24 ± 0.06	0.89

A more extensive version of this table can be found at [13] [Table E]. NA, no amplification by RT-PCR.

[†] Only 5 donors with amplification considered for correlation analysis.

Table 3B
Correlation of fold change (FC) on Hu-Gene 133A arrays and RT-PCR for partial list of genes altered by ∼3-fold change (SLR ≥ 1.5) in at lest one of the six NHMECs when treated with pre-chlorophyllin followed by co-treatment

Gene	Probe ID	Accession no.	Pre CHL + co-treatment vs. vehicle control (average FC ± S.D.)												Correlation (r) GC-RT PreCHL + co
			1 (M98035)		2 (M99005)		3 (M98015)		4 (M98025)		5 (M99025)		6 (M99016)		
			GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	GC	RT-PCR	
AKR1C2	211653_x_at	NM_001354	3.04 ± 0.30	1.62 ± 0.13	1.74 ± 0.00	1.82 ± 0.06	2.07 ± 0.10	2.00 ± 0.16	5.10 ± 0.25	2.39 ± 0.30	2.08 ± 0.30	2.25 ± 0.22	2.22 ± 0.11	1.76 ± 0.48	0.50
CLDN4	201428_at	NM_001305	7.03 ± 1.37	4.87 ± 0.39	−6.06 ± 0.00	0.13 ± 0.01	1.88 ± 0.37	1.43 ± 0.08	15.13 ± 6.46	3.71 ± 0.14	−0.31 ± 2.59	0.46 ± 0.04	3.62 ± 0.53	0.35 ± 0.04	0.76
DHRS3	202481_at	NM_004753	1.80 ± 0.09	1.52 ± 0.17	−1.19 ± 0.06	0.96 ± 0.03	1.76 ± 0.34	2.17 ± 0.94	3.26 ± 0.32	1.53 ± 0.16	2.30 ± 0.00	3.35 ± 0.28	1.15 ± 0.00	1.52 ± 0.16	0.50
SOD2	216841_s_at	NM_000636	−1.15 ± 0.00	0.66 ± 0.02	−3.14 ± 0.15	0.38 ± 0.06	1.15 ± 0.00	1.28 ± 0.07	−1.33 ± 0.26	0.51 ± 0.05	−1.15 ± 0.11	0.87 ± 0.08	−1.81 ± 0.69	0.11 ± 0.02	0.83
CYR61	201289_at	NM_001554	−2.83 ± 0.00	0.23 ± 0.01	−1.11 ± 0.05	1.00 ± 0.05	−1.62 ± 0.00	0.53 ± 0.02	−2.30 ± 0.00	0.27 ± 0.02	−1.80 ± 0.09	0.62 ± 0.05	−0.22 ± 1.83	0.97 ± 0.15	0.92
SERPINB2	204614_at	NM_002575	9.52 ± 0.47	10.18 ± 1.55	−3.14 ± 0.15	0.25 ± 0.01	1.46 ± 0.07	1.75 ± 0.14	3.49 ± 0.34	2.20 ± 0.28	−4.00 ± 0.00	0.25 ± 0.01	4.46 ± 0.65	0.58 ± 0.03	0.81
MCM5	216237_s_at	NM_06739	−1.87 ± 0.18	0.33 ± 0.02	1.69 ± 0.25	1.84 ± 0.52	−1.52 ± 0.15	0.49 ± 0.02	−3.26 ± 0.32	0.23 ± 0.02	−0.07 ± 1.52	1.34 ± 0.32	−2.35 ± 0.68	1.08 ± 0.22	0.37
GADD45B	207574_s_at	NM_015675	−1.87 ± 0.18	0.30 ± 0.03	−1.23 ± 0.12	0.69 ± 0.07	−0.28 ± 1.91	0.79 ± 0.09	−3.38 ± 0.50	0.16 ± 0.03	−1.57 ± 0.08	0.83 ± 0.15	−1.37 ± 0.20	0.27 ± 0.02	0.71

A more extensive version of this table can be found at [13] [Table F]. A negative sign for FC by gene-chip (GC) refers to down-regulation of gene-expression by the corresponding value.

cases perhaps by elevated expression of DNA damage or repair genes.

Simple comparison of gene expression profiles in different NHMEC strains from different donors exposed to either BP alone or BP in the presence of chlorophyllin showed alterations in the expression of a relatively small percentage of genes in response to carcinogen exposure that were largely mitigated by the presence of a chemopreventive agent. In addition, it was clear that wide inter-individual variations in response to both agents occurred. This latter observation may be important in the selection of chemopreventive agents, since it may be possible in future for individuals to select only those agents that will be specifically effective.

A more sophisticated cluster analysis was useful for two reasons, first it confirmed modulation in the expression of certain genes that were observed to be consistently altered across most or all cell strains, like *CYP1B1* and *NQO1*. Second, it identified a group of genes for which expression was not so obviously altered in a consistent manner that may be useful in future for assessing response to carcinogens and chemopreventive agents.

Alterations in expression profiles brought about by BP appear to be chiefly mediated through up-regulation of genes rather than down-regulation given that only one cell strain exhibited down-regulation of genes. Most of the down-regulated genes appear to bear positive or negative relationship with various cancers and included genes involved in the processes of cell signaling, proliferation and metabolism. SGK, a serum and glucocorticoid-regulated kinase was found to be over-expressed in various cancers [24–26], CTGF, a connective tissue growth factor, shown to act like a 'tumor suppressor' in some studies [27,28], BBOX1, a hydroxylase and DOC1 a gene down-regulated in ovarian cancer [29] were genes for which down-regulation was a consistent feature in all 6 donors.

Chlorophyllin has been documented to modulate various aspects of carcinogenesis and mutagenesis either through non-specific inhibition of cytochrome P450s [30], up-regulation of various members of the detoxification battery [31,32], enhancement of apoptosis [33], modulation of carcinogen transport [34], mitigation of oxidative stress [35] or various combinations of these. Alteration in the expression profiles of genes belonging to various functional categories that were observed here may point to additional targets for chlorophyllin. However, being present along with BP in a pre-chlorophyllin plus co-treatment may suggest some that of the altered genes could be due to the individual effects of BP alone or chlorophyllin alone, yet others may be due a synergistic interaction of BP and chlorophyllin.

Overall, some genes exhibited a good correlation between expression levels as measured on GC and by RT-PCR. However, there were others which correlated poorly. In general, RT-PCR was more sensitive than GC in measuring gene expression levels [5,6]. The lack of good correlation for some genes may be due to the comparatively lower sensitivity of GC in relation to RT-PCR in addition to differences in the exact regions being probed by GC and RT-PCR.

In summary, this study has revealed previously unknown potential biomarkers of exposure some of which could be potential targets for intervention strategies. Clearly, *CYP1B1* is one such target as seen from this study and previous studies. Expression of various other genes such as *ALDH1A3*, *ALDH3A1*, *ZBED2*, *RYR3*, *KRT23*, *SECTM1*, *BEX4* (up-regulated by BP treatment), *SGK*, *CTGF*, *BBOX1*, *DOC1* (down-regulated by BP), *AKR1C1*, *ZEB2*, *CALB1*, *FGFBP1*, *HMOX1*, *HS3ST2*, *SPRR1B*, *SGNE1*, *OUTB2* (up-regulated in the presence of chlorophyllin) and *SOD2*, *CRY61*, *GADD45B*, *NRG1* (down-regulated in the presence of chlrophyllin) were altered in a consistent manner. These could serve as biomarkers following further validation. Additionally, wide inter-individual variations need to be accounted for in designing intervention strategies.

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