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Application of Oligonucleotide Microarray Technology to Toxic Occupational Exposures

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Microarray technology has advanced toward analysis of toxic occupational exposures in biological systems. Microarray analysis is an ideal way to search for biomarkers of exposure, even if no specific gene or pathway has been identified. Analysis may now be performed on thousands of genes simultaneously, as opposed to small numbers of genes as in the past. This ability has been put to use to analyze gene expression profiles of a variety of occupational toxins in animal models to classify toxins into specific categories based on response. Analysis of normal human cell strains allows an extension of this analysis to investigate the role of interindividual variation in response to various toxins. This methodology was used to analyze four occupationally related toxins in our lab: oxythioquinox (OTQ), a quinoxaline pesticide; malathion, an organophosphate pesticide; di-*n*-butyl phthalate (DBP), a chemical commonly found in personal care and cosmetic items; and benzo[a]pyrene (BaP), an environmental and occupational carcinogen. The results for each exposure highlighted signaling pathways involved in response to these occupational exposures. Both pesticides showed increase in metabolic enzymes, while DBP showed alterations in genes related to fertility. BaP exposure showed alterations in two cytochrome P450s related to carcinogenicity. When used with occupational exposure information, these data may be used to augment risk assessment to make the workplace safer for a greater proportion of the workforce, including individuals susceptible to disease related to exposures.

The advancement of microarray technology has opened up an entirely new area of biomarker analysis for occupational diseases. Microarrays allow for the analysis of thousands of genes at one time, without compromising sensitivity and specificity. Analysis of gene expression changes following occupational exposures to chemicals may be used to learn more about various occupationally related illnesses. In particular, gene expression variation, related to the presence of genetic polymorphisms, may be used to learn more about illnesses that appear only in susceptible workers. This general approach is a hallmark of "anticipation" as it relates to industrial hygiene.

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The findings and conclusions in this report are those of the authors and do not necessarily represent those of the National Institute for Occupational Safety and Health.

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MICROARRAY TECHNOLOGY

Changes in gene and protein expression levels have long been a source of information related to disease. Before microarray technology, it was only possible to analyze few genes simultaneously. Selection of genes for analysis, although important, was difficult because valuable information could be missed and may lack the required context; for example, analysis of only the part of a signaling pathway upstream from where any changes may have occurred. The first arrays were described in the mid-1990s and in the past 10 yr the analytical and data management technology evolved rapidly. Many changes occurred related to microarray format, with both the original complementary DNA (cDNA) arrays as well as the newer oligonucleotide array formats, both still in use. The following sections address the differences between these formats and the analytical methods currently in use today.

Microarray Formats

The first microarrays described were produced by spotting cDNA onto glass slides (Schena et al., 1995). These arrays were then hybridized to RNA from either treated or untreated samples that had been processed to allow differential gene expression levels to be measured. The processing of the RNA for these arrays was described in detail (Schena et al., 1995). Briefly, RNA is isolated from both treated and untreated samples. Following isolation, the RNA is reverse transcribed to cDNA with either Cy3 or Cy5 dye incorporation. Generally,

Cy3 (red) is incorporated into the treated sample and Cy5 (green) is then incorporated into the untreated sample. Both samples are then mixed, and hybridized to the glass slides spotted with the cDNA probes. If gene expression is increased in the treated sample, the spot on the array shows an increased specific signal in the red part of the spectrum. In turn, if the gene expression is decreased in the treated, the Cy5, or green spectrum, is stronger. If no change occurred between the treated and untreated samples, the spot appears yellow. Some early issues with microarray technology arose when it became apparent that the Cy3 and Cy5 dyes bound to the cDNA samples with different efficiencies. To overcome this problem it became standard to perform a complementary reaction by labeling the treated sample with Cy5 and the control with Cy3. Two arrays were analyzed in tandem with each sample hybridized to Cy3 and Cy5. This was generally called a reciprocal labeling experiment, due to the use of both dyes for each sample.

An enhanced high-density oligonucleotide array-based method was introduced by Hacia et al. (1998) to analyze multiple genes of interest in a single experiment. These were designed with much shorter probes, ranging from 20 to 50 oligonucleotides that were sequence matched to the expression of selected genes. The use of oligonucleotides changed the format from the two-color arrays described already to a one-color format that used two arrays, from which data were then compared by analytic software (Figure 1). The one-color system worked by incorporating biotin into cRNA (complementary RNA produced from cDNA) and then a streptavidin-linked fluorescent molecule, which binds at a level proportional to the level of gene expression (Figure 1, lower panel). This allowed for a measurement of gene expression levels for any sample, which were then in turn compared to a control sample to measure expression changes. This system also allowed for the simultaneous analysis of multiple genes.

The most important characteristic of all microarrays, regardless of the format, is that the cDNA or oligonucleotide probe used must be sequence-specific to the gene in question. High-technology companies that create arrays generally use complex algorithms to determine this specificity, but with large tracts of the genome that still have unknown function, this information needs to be updated constantly.

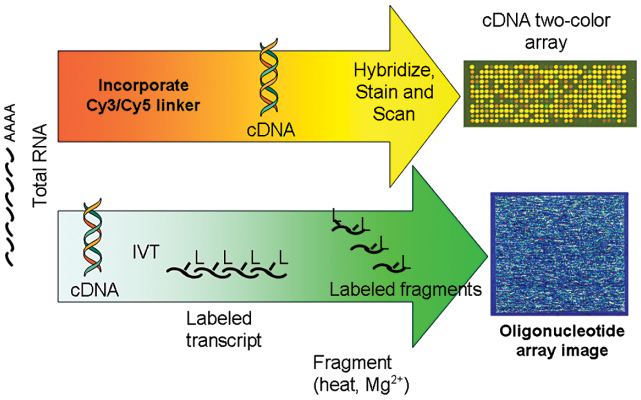

FIG. 1. Microarray methods in use. The two main types of microarray are cDNA and oligonucleotide. Starting with total RNA for both cDNA (orange arrow) and oligonucleotide (green arrow), both are processed to cDNA. For the cDNA arrays, linkers (conventionally Cy3 and Cy5) are incorporated for both treated and untreated samples at this point. These labeled transcripts are then pooled and hybridized to the array to result in an image like shown. For the oligonucleotide arrays, the cDNA is used in an *in vitro* transcription reaction to produce cRNA. At this step, biotin is incorporated into the sample. These samples are then fragmented and hybridized to the array. Following hybridization for both systems, the arrays are washed, stained, and scanned for viewing.

Analytic Methods

Accurate measures of expression changes become challenging when hundreds and thousands of genes are analyzed at one time. Statistical analysis of gene expression changes needs to be more sophisticated compared to those used when few genes were monitored. As microarray technology has grown, so has analytical methodology. Microarrays have given rise to an entirely new area of statistics, one that allows for the analysis of thousands of experiments at one time. The first data analytical methods were not described until 1998 (Eisen et al., 1998). As the field has advanced, the use of clustering has become an efficient way to group array data for analysis. Various clustering methods may be used, including self-organizing maps, K-means, and hierarchical methods. In the studies described here, various unsupervised clustering methods were used to establish potential biomarkers related to occupational exposures to toxic chemicals, or diseases resulting from these exposures. Unsupervised clustering of microarray data allows for pattern recognition among genes and samples, and is often used as a starting point for studying unknown genes or responses. The three forms of clustering described here show the data in various forms. Self-organizing map (SOM) clustering allows one to analyze microarray data based on a selected number of nodes, or neurons, in a neural network. The benefit of this is that depending on how many clusters one selects, one can refine the data to as few genes as possible. One of the drawbacks of this is that all nodes have initial random values, so that the same clustering analysis with the same data will generally not give the exact same response. K-means clustering analysis works with k random points, or centroids, being selected, and the remaining data set is assigned to the closest centroid. This is an iterative process, until clustering is found. This again has the drawback of random starting points, making it difficult to repeat the same analysis (Jain et al., 1999). However, both of these methods allow for a quick analysis of large data sets, from which smaller subsets of data can be further analyzed. The last clustering analysis described in this article is hierarchical analysis. Hierarchical clustering is often chosen to

display gene array because of the ability to show patterns of expression as well as expression levels. In general, each of these three clustering analyses was utilized for all of the chemicals described here. For this report, each chemical was used to highlight the various options in clustering analysis, and the results of only one analysis per chemical are described.

Variability Between Methods

Studies were performed that compared not only the array types used but also the software analysis methodology. Early microarray analysis soon became suspect due to changes related to format differences, probe sequence selection, dye incorporation, and data analysis methods (Bassett et al., 1999; Brown & Botstein, 1999; Cheung et al., 1999; Debouck & Goodfellow, 1999; Duggan et al., 1999; Lipshutz et al., 1999). Analysis using the same RNA sample on different formats (Gwinn et al., 2005a; Tan et al., 2003), or taking data from the same format and using different analysis packages (Jenssen et al., 2002; Kane et al., 2000; Kothapalli et al., 2002; Kuo et al., 2002), showed variance unrelated to the samples and treatments under investigation. It became clear that microarray investigations, while uncovering much usable data, needed a careful analytic approach in order to report meaningful results. Most microarray results at this time are verified by at least one other method, generally real-time polymerase chain reaction (RT-PCR).

OCCUPATIONALLY RELATED TOXIN EXPOSURES

Low-level exposures to toxic chemicals may yield adverse effects in the general population, but also lead to severe health issues when exposures are increased in the occupational environment. Occupations at high risk are constantly under surveillance to confirm that exposures are decreased through various measures, whether it is through the use of personal protective equipment or engineering controls (Mastrangelo et al., 2005). Ideally, susceptible workers are placed in different areas where the exposure to the particular toxin or chemical is decreased (Mastrangelo et al., 2005; McCanlies et al., 2003).

To determine biomarkers of exposure across a population, gene expression patterns need to be compared among individuals to account for inherent variation. Hitherto, gene expression signatures resulting from chemical exposures were created from analysis of exposures in rat, mouse, or other genetically comparable animal models, excluding information about interindividual variations (de Longueville et al., 2003; Ellinger-Ziegelbauer et al., 2004; Hamadeh et al., 2002a, 2002b; Naciff et al., 2002, 2003; Shan et al., 2002). In the work reported herein, primary normal human mammary epithelial cells (NHMECs) were used derived from different women undergoing reduction mammoplasty [Cooperative Human Tissue Network (National Cancer Institute and National Disease Research Interchange)] (Figure 2). Limited information is available on these strains due to conditions of anonymity. However, for the strains used here, age and race of the donors were as follows: strain one, 26-yr-old Caucasian; strain two, 26-yr-old Caucasian; strain three, 25-yr-old women of unknown race; and strain four, 36-yr-old Caucasian. The use of these primary cell strains allowed analysis of the potential role of interindividual variations in the response to various occupational toxins.

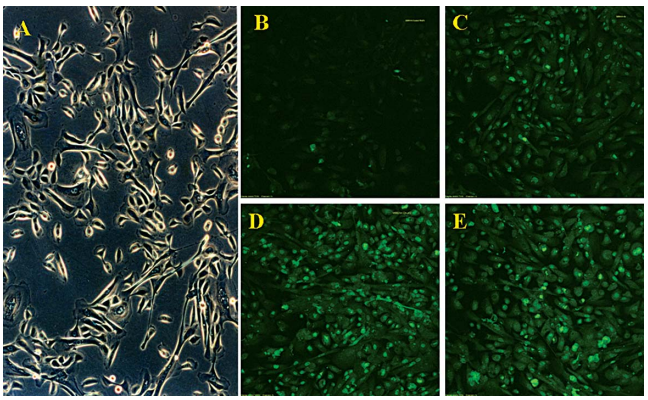

FIG. 2. Primary normal human mammary epithelial cell (NHMEC) with p53 staining. (A) Image of NHMEC strain at stage used in treatment for all assays (passage 6, 70% confluence). Cells are derived form tissue obtained through the Cooperative Human Tissue Network (NCI, NDRI) from normal human donors following reduction mammoplasty. Various protein baseline levels characterized for each strain (vimentin, alpha actin, cytokeratins) to determine homogeneity. Genotype analysis for all cell strains has also been determined. Cells under confocal microscopy following staining with p53-FITC antibody: (B) zero time, no exposure; (C) 6 h BaP exposure; (D) 12 h BaP exposure; and (E) 24 h BaP exposure.

Normal cell strains harbor wide variations between individuals, making it difficult if not impossible to analyze all differences and determine which genetic variant will play a significant role in the response to various toxins (Moggs et al., 2004). Genotyping of the cell strains derived in our laboratory allowed for selection of strains with specific genotype related to genes of interest. In the studies described here, genotyping related to cell cycle control and/or chemical metabolism was performed to determine if known polymorphisms may be linked to susceptibility in the response. Given the large amount of unknown variability attributable to the background genetics in these strains, follow-up studies with transgenic mice or isogenic strains of cells would be required to definitively determine the role of one specific gene in the responses described here. However, analyzing a few select genetic polymorphisms may reveal information of interest relating to specific pathways.

All studies described here used at least 4 cell strains, two of which expressed a *p53* major variant with respect to codon 72 and two of which expressed a *p53* minor variant, as defined by Sjalander et al. (1996) (Figure 3). The use of genotyping of polymorphisms of interest may be used in concert with gene expression profiling to determine if genetic variation plays a key role in altering the signaling response to specific exposures.

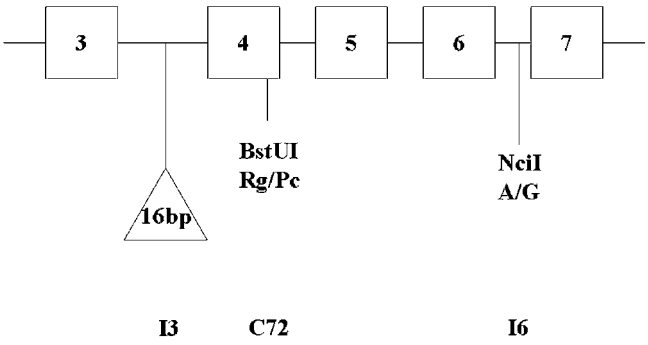

FIG. 3. Polymorphisms in p53. Schematic diagram of p53 polymorphisms used to define variant status in cell strains used for analysis. There is a 16bp insertion/deletion polymorphism in intron 3 that has been associated with ovarian cancer. There is a G>C SNP in exon 4, codon 72 that can code for either arginine or proline respectively. There is a G>C SNP in intron 6. It was reported by Sjalander et al. (1996) that haplotypes of these three polymorphisms were associated with breast cancer. This latter finding was corroborated using a physical haplotyping method (Keshava et al., 2002a; Weston et al., 1997).

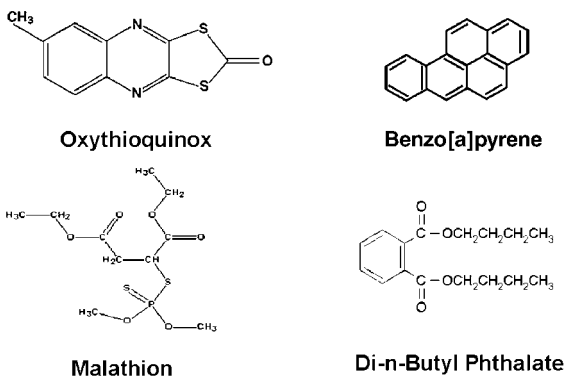

FIG. 4. Chemical structures of occupational toxins studied. Schematic structures of the 4 chemicals analyzed.

Gene transcription in each study was analyzed using high-density oligonucleotide DNA microarrays (HuGeneFL or U133A, Affymetrix), and changes in the expression of selected genes were verified by RT-PCR at extended times. Cells were exposed to one of four different occupational toxins as detailed in Figure 4, followed by RNA isolation and microarray analysis. Table 1 shows a list of several of the genes altered following each exposure.

Oxythioquinox

Oxythioquinox (OTQ) is a pesticide originally sprayed on citrus crops. OTQ has been classified by the U.S. Environmental Protection Agency (EPA) as a probable human carcinogen (B2) despite limited research (Carlson & DuBois, 1970; Carrera et al., 1979; Gaillard et al., 1977) and as such was listed as a priority toxin by the National Toxicology Program in 2000. The use of OTQ, though at that time limited, was still believed to be potentially hazardous to those who were occupationally exposed. Due to some inconclusive results from early animal studies, the use of OTQ was limited in the late 1990s and voluntarily canceled in 2001 (U.S. EPA, 1999); however, related compounds are still in use today.

In order to look at acute exposures similar to those encountered by pesticide applicators, DNA microarrays were hybridized to materials prepared from total RNA that was collected after OTQ treatment for 15, 60, and 120 min. Times selected were related to the short-term exposures found in occupational exposures. RNA was harvested from the vehicle control (dimethyl sulfoxide, DMSO) at 120 min after exposure (Figure 5). The gene expression profile included all genes altered by at least a signal log ratio (SLR) of $\pm$ 0.6 and p value $\leq$ 0.05 in 3 of 4 cell strains analyzed. This is equivalent to a 1.5-fold change in gene expression, an empirical cutoff value for these microarray experiments. The use of a 1.5-fold change value was based on the number of statistically significant genes found to be altered at this level. Given that the analysis was examining subtle changes in toxicity, this lower fold change cutoff value allowed for analysis of a larger number of genes across all exposures. RNA species were clustered in various patterns of expression highlighting genes with altered expression in one or more of the cell strains, including metabolic enzymes and transcription factors (Table 1).

Self-organizing map (SOM) clustering (Figure 6) was performed to further analyze the gene expression profile. This type of clustering is often used for temporal studies to visually determine differences between treatments and times. SOM clustering groups all genes to the cluster point that closest resembles the same expression pattern. With SOM clustering, all neighboring clusters are closely related. Clustering was performed on genes that were found to be present on at least one array. This clustering method focused our study on 36 genes altered in similar patterns for at least 3 of 4 cell strains (13 upregulated, 23 downregulated) (Gwinn et al., 2004). Of these genes, studies focused on genes that may show statistically significant changes or that are related to the metabolism of this pesticide. Either of these metrics might then be used to infer exposure to this pesticide had occurred. Further, alterations in these genes of interest may also lead to information about individuals susceptible to pesticide related diseases.

Genetic alterations related to cancer susceptibility are a key area of study. Polymorphic variants that may in some manner affect the function of a gene, in particular genes related to cell cycle regulation, DNA repair, and metabolism of known environmental carcinogens, are under study to determine the role they may play in carcinogenesis (Bigler et al., 2005; Cascorbi, 2005; Zhang et al., 2005). *p53* is the most commonly mutated gene in human cancers, and specific alterations to this gene are associated with cancer in human studies (Keshava et al., 2002a; Peller et al., 1999; Sjalander et al., 1996; Wang-Gohrke

TABLE 1
Genes Altered Following Chemical Exposure

	Chemical/gene							
	Oxythioquinox[a]		Malathion[b]		Di-*n*-butyl phthalate[c]		Benzo[a]pyrene[d]	
Cell strain	AKR1C1	CYP2A13	AKR1C1	CDC20	AKR1C1	Inhibin A	CYP1A1	CYP1B1
1	5.7	1.8	18.6	–5.9[e]	5.5	13.4	8.7	6.0
2	2.7	3.4	60.9	–3.8	1.9	12.1	1.0[f]	1.8
3	1.0	1.8[g]	32.2	–11.3	1.0	NA[h]	1.7	3.6
4	1.9	2.4	15.1	–1.5	2.9	NA	10.9	7.5

Note. Gene expression variations between four cell strains (1–4) for six genes (*AKR1C1, inhibin, CDC20, CYP2A13, CYP1A1, CYP1B1*). Some genes were increased in all strains depending on treatment (*AKR1C1, CYP1B1*) while others were increased only in the cells expressing the major p53 variant (inhibin). Values shown are fold change as compared to vehicle exposure. Exposures: OTQ (*a*); malathion (*b*); DBP (*c*); BaP (*d*).

[a]Duration of exposure (DoE) 1 h.
[b]DoE 24 h.
[c]DoE 10 h.
[d]DoE 6 h.
[e]Exposure exception (6 h).
[f]At later time points CYP1A1 was induced, e.g., 2.3-fold at 24 h.
[g]Exposure exception (2 h).
[h]No data available.

Exposure	Concentration	Time Points	Cell Strains
Oxythioquinox	6.25 µM	15, 60 and 120 min	1, 2, 3, 4
Malathion	50 µg/ml	6 and 24 hrs	1, 2, 3, 4
Di-n-Butyl-Phthalate	1 µM	5 and 10 hrs	1, 2, 3, 4
Benzo[a]pyrene	4 µM	2, 6, 12, 24 and 36 hrs	1, 2, 3, 4

FIG. 5. Four chemical exposure parameters. Diagrammatic view of chemical exposures analyzed (toxin, concentration, exposure time, and cell strains used). Cell strains are labeled with an internal numbering system, with strains 1 and 2 both expressing the major *p53* variant while strains 3 and 4 express the minor variant *p53* gene.

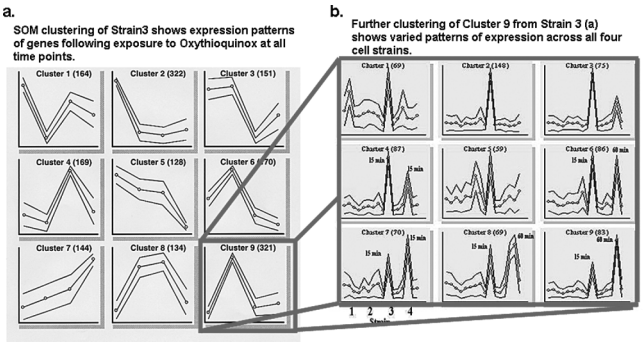

FIG. 6. SOM clustering. (a) Panel summarizes the behavior of the patterns of gene expression for a particular cell strain (3). Each point represents a treatment time, with zero, 15, 60, and 120 min, respectively. Center line shows mean and the outer lines show standard deviation at each time point. As an example, cluster 9 was selected for further follow-up in all four strains. (b) Further SOM clustering of cluster 9 from (a) shows genes increased at 15 min for one minor variant strain (3) follow similar pattern in second minor variant strain (4). All four cell strains are shown (as labeled on bottom left cluster), with all times of exposure (zero, 15, 60, and 12 min). For ease of viewing, only those points with peak expression are labeled in the figure. Some genes in similar clusters are often of similar function. Follow-up analysis of these genes may aid in determining the functional role of these polymorphic variants.

et al., 1999; Weston et al., 1997; Wu et al., 2002). These data were further clustered to examine the effects of OTQ on the cells with specific *p53* polymorphisms. The two strains expressing the major variant of *p53* had 83 common genes altered (35 increased, 48 decreased) at one or more time by at least ± 0.6 SLR. The minor variant strains showed 105 common genes altered (80 increased, 25 decreased) in both strains. Differential changes in expression of these genes may yield biomarkers that provide insight into inter-individual variation in cancer risk. Further clustering based on genes of interest, for example, genotypic variants of metabolic genes like cytochrome P-450s or aldo-keto reductases, may shed more light on the variability of response to pesticide exposures.

MALATHION

Malathion is an organophosphate pesticide, and this class of pesticides represents a major occupational exposure in the United States. Organophosphates are used largely because they

are cheap and considered to produce low toxicity. Malathion has been sprayed over major urban populations in an effort to control mosquitoes carrying West Nile virus, thereby potentially increasing environmental exposures to this pesticide. Symptoms of pesticide-related illness are very similar to those of common illnesses like influenza or upper respiratory infection, making it difficult to diagnose pesticide related illnesses and decreasing the number of treated individuals. Biomarkers of exposure might be used to help identify adverse health outcomes in susceptible subpopulations.

Previous research on the genotoxicity and carcinogenicity of malathion was inconclusive, although malathion is a known endocrine disruptor Choi et al. (2004). Gene expression profiles may be used to compare this pesticide with other chemicals of known carcinogenicity to determine the effects of exposure (de Longueville et al., 2003; Ellinger-Ziegelbauer et al., 2004; Hamadeh et al., 2002a, 2002b; Naciff et al., 2002, 2003; Shan et al., 2002). Further, increased information on the response to malathion exposure in human cells might aid in determining the biologic activity of this pesticide. Here interindividual variations and commonality of gene expression signatures were studied in normal human mammary epithelial cells following exposure as described in Figure 5. The concentration used is equivalent to 0.2 mg/0.1 m^3, which is 10-fold less than the no-observed-adverse-effect level (NOAEL) for acute exposures in humans at which early signs of malathion-related illness have been reported (ATSDR, 2001).

Interindividual variation of gene expression patterns in response to malathion was observed in various clustering patterns for the four cell strains. Genes were clustered by using the Affymetrix Data Mining Tool query process. These analyses allowed for sorting based on "Present" call status on at least one array, with further K-means clustering narrowing the list of genes down to those of interest. K-means clustering is similar to SOM clustering but the neighboring clusters do not have any relationship to each other. Results are shown as a line graph, or as a gene tree in Figure 7. The number of nodes (*K*) was chosen at the beginning of the analysis, and genes are grouped to the node that most closely resembles the expression pattern. Further clustering identified three genes with increased expression after treatment in all four cell strains. These genes were two aldo-keto reductases (*AKR1C1* and *AKR1C2*) and an estrogen-responsive gene (*EBBP*). Decreased expression of six RNA species was seen at various times in all cell strains analyzed; these were plasminogen activator (*PLAT*), centromere protein F (*CPF*), replication factor C (*RFC3*), thymidylate synthetase (*TYMS*), a putative mitotic checkpoint kinase (*BUB1*), and a gene of unknown function (GenBank accession number AI859865) (Table 1). Expression changes in all these genes, detected by DNA microarrays, was verified by real-time PCR (Gwinn et al., 2005b). Differential changes in expression of these genes may yield biomarkers that provide insight into interindividual variation in malathion toxicity.

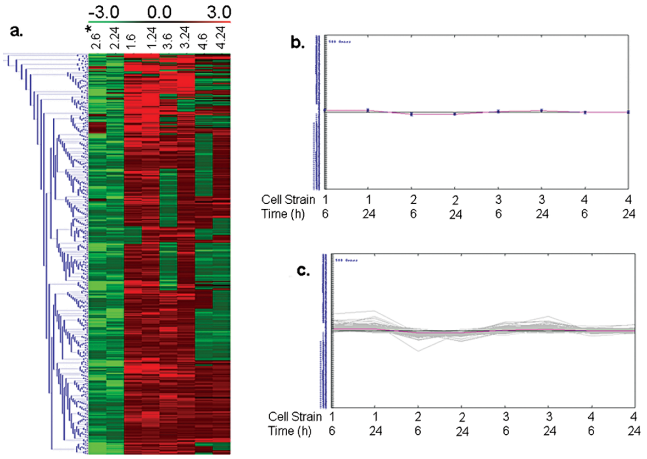

FIG. 7. K-means clustering. Malathion gene expression patterns as measured by K-means clustering. TMEV software (TIGR) allows for the clustering to be viewed in three different manners. Hierarchical clustering of K-means results (a) shows the gene expression changes for each of the treatments, with those in green showing decreased expression and those in red showing increased expression as compared to control. K-means is most often viewed as an average of all genes in that cluster in a centroid graph (b) or as a line graph (c) showing the pattern of expression for all genes at all times. The results shown here are for one cluster of all gene expression patterns for all four cell strains at both 6 h and 24 h.

DI-*n*-BUTYL PHTHALATE (DBP)

Chemicals found in cosmetics have recently come under close scrutiny. One of the major concerns related to DBP exposure is related to fertility issues found in exposed populations (Pollan & Gustavsson, 1999; Koo & Lee, 2004). Studies show that female workers in the personal care industry have an increased risk of developing cancer, believed to be the result of enhanced exposure to toxic and/or carcinogenic chemicals found in cosmetics, hair dyes, and nail polish. These chemicals include nitrosamines, formaldehyde, and various phthalates. One chemical found in multiple personal care products, DBP, is a known endocrine disruptor and reproductive toxin (Kim et al., 2005; Kang & Lee, 2004) and is found in increased levels in women of child-bearing age.

The goal of this study was to elucidate mechanisms underlying DBP toxicity in normal human breast cells to provide information concerning interindividual variation and gene–environment interactions. DNA microarrays were hybridized with total RNA that was collected after DBP treatment for 5- or 10h exposure times, selected based on potential workplace exposure times for cosmetologists. RNA was harvested from the vehicle control (acetone) at 10h (Figure 5).

Data Mining Tool software (Affymetrix) was used to separate genes in clusters based on their expression patterns over time. Further hierarchical clustering analysis was performed using TMEV v 3.0 software (TIGR) (Figure 8). Hierarchical clustering has a benefit over both SOM and K-means clustering

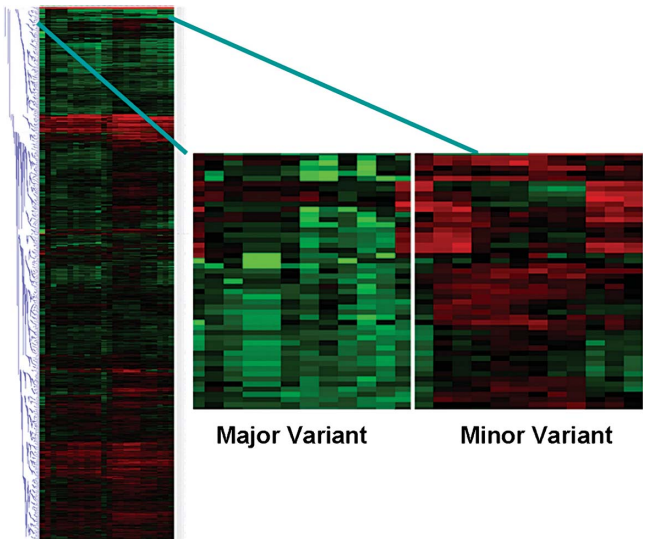

FIG. 8. Hierarchical clustering. Cells treated with DBP show interindividual variation in response to DBP exposure. Results shown here are cell strains 1 and 2 treated with DBP on the left, with cell strains 3 and 4 on the right. While some genes show a similar response, this snapshot shows a group of genes decreased in the major variant strains (left) and increased in the minor variant strains (right).

in that it links genes together based on their expression pattern. This hierarchical clustering approach may aid with pathway analysis as well as biomarker discovery. Only 57 genes were found to be altered in all 4 cell strains following exposure to DBP. These included genes involved in fertility (inhibin, placental growth factor), immune response (tumor necrosis factor-induced protein), and antioxidant status (glutathione peroxidase) (Table 1) (Gwinn et al., 2007). The results described here lend further support to previous studies described suggesting a role for DBP in reproductive toxicity. Future studies on DBP might now focus on biomarkers related to this result, including inhibin and PlGF, which is already analyzed clinically in studies on preeclampsia (Bersinger & Odegard, 2004; Bersinger et al., 2003). Data from this study may help to clarify the role of DBP in reproductive toxicity. For example, studies suggested a role for increased inhibin in reproductive effects in male rats exposure to DBP (Kobayashi et al., 2003). Pathway analysis of the microarray data presented here may assist in determine specific mechanistic information related to this reproductive toxicity.

BENZO[a]PYRENE (BaP)

Benzo[a]pyrene is a polycyclic aromatic hydrocarbon (PAH), discovered as one of the first purified carcinogens in 1933 (Osborne & Crosby, 1987; Phillips, 1983). PAHs are ubiquitous environmental and occupational contaminants that form from the incomplete combustion of vegetable matter (Osborne & Crosby, 1987). In the workplace, exposures occur in coke ovens and in foundries, and also result from roofing, asphalting, and work as bartenders and wait-staff, resulting from exposure to secondhand tobacco smoke (IARC, 1973, 2004).

Because PAHs are chemically inert, they must be metabolized to reactive electrophiles to exert their biological effects (Miller 1951; Sims et al., 1974). The ultimate carcinogens are considered to be diol-epoxide metabolites (especially occurring in bay regions of the chemical structure) that are formed through the action of dioxin inducible cytochrome P-450s (e.g., CYP1A1, CYP1B1, and others) and epoxide hydrolase (Keshava et al., 2004; Weston & Harris, 2005).

In order to identify potential biomarkers of exposure to PAH, data from gene-expression studies in the normal human mammary epithelial cell model are provided. It was found that a number of genes were consistently induced with a substantial statistical significance by exposure to BaP when monitored by gene arrays as described (Figure 5); these included interleukin-1 beta (IL1β), matrix metalloproteinase 1 (MMP1), and cytochrome P-450 1A1 and 1B1 (CYP1A1 and CYP1B1) (Keshava et al., 2005a, 2005b) (Table 1). When induction of CYP1A1 and CYP1B1 was confirmed by RT-PCR it was clear that there was an excellent correlation between the two methods ($r > 0.7$), but RT-PCR was more sensitive than gene arrays for detection of gene expression (Figure 9). These data are to a certain extent consistent with those reported by others (Lampe et al., 2004) who used gene arrays to develop a battery of induced genes as a biomarker of tobacco smoking.

It was noted earlier in this article that PAHs require metabolic activation to exert their biological effects (genotoxicity; DNA-adduct formation). The question of whether interindividual induction of gene expression will correlate with DNA-adduct formation is an important one because DNA adducts are used as biomarkers, but they are a lagging indicator because damage has already been done. Correlations between cytochrome P-450 induction and DNA adduct formation were also sought in these initial studies, but they were weak (Keshava et al., 2005a). More sophisticated studies looking critically at additional factors like phase II metabolism and DNA repair will be needed. However, even though there was wide interindividual variation in induction between different tissue donors, induction of *CYP1A1* was closely correlated with *CYP1B1*.

One limitation of detection of RNA as a measure of gene induction is that it does not account for increased stability of RNA species resulting in protein accumulation without detectable RNA induction. When NHMECs are exposed to BaP, both p53 and p21 proteins accumulate (Figure 2). This is likely due to response to DNA damage through BaP-dG adduct formation. However, there was no detectable increase in p53 transcription. When DNA damage is detected by the cell (through the BRCA system,) p53 accumulates due to stabilization of transcripts already present. This causes either G1

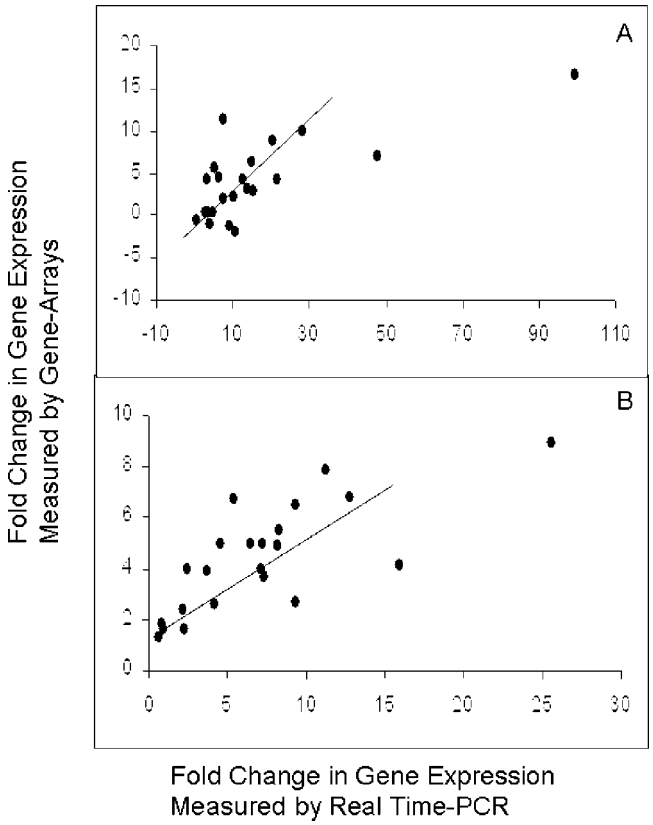

FIG. 9. Correlation between gene expression measured by RT-PCR and gene arrays following exposure to benzo[a]pyrene. Correlation between fold induction of CYP1A1 (A) and CYP1B1 (B) measured by real-time polymerase chain reaction compared to gene arrays. For each gene, measurement of fold induction was highly correlated between RT-PCR and gene arrays ($r = .735$, $p < .001$ for CYP1A1 and $r = .746$, $p < .001$ for CYP1B1). However, RT-PCR was a more sensitive technique than gene array detection.

arrest, whereupon the damage may be repaired, or apoptosis in the event that the damage is too severe to be repaired (Balint & Vousden, 2001).

PAHs were used for many years to induce liver enzymes of lab rodents (Sims, 1972). Selection of sampling times was loosely based on this time frame (6, 12, 18, 24, or 36 h), and limited by the cost of sampling multiple times. However, data are presented for a 6-h exposure, which is comparable to duration of exposure in a single shift.

SUSCEPTIBLE WORKER PROFILES

Genetically susceptible individuals are those that are more vulnerable to cancer and other diseases because of their genetic makeup. These diseases, however, occur only as a result of certain exposures to chemicals in the workplace, the environment, or foods and other products. To fully protect the workforce, it is important to take the response of susceptible individuals into account when determining safe levels of exposure in the workplace. Polymorphisms were studied to examine their role in response to various exposures in the environment and the workplace (Angelini et al., 2005; Bartsch et al., 1999; Brescia et al., 1999; Donaldson et al., 2002; Glenn et al., 2001; Gwinn et al., 2004, 2005b; Hirvonen, 2005; Keshava et al., 2002b; Koh et al., 1999; Leng et al., 2004; McCanlies et al., 2003; Pavanello & Clonfero, 2004; Pavanello et al., 2005; Wan et al., 2002; Weston et al., 2002; Wong et al., 2002, 2003; Zhang et al., 2004). One example is the analysis of genetic variation in the *HLA-DP* gene in workers exposed to beryllium and the link to chronic beryllium disease or beryllium sensitization (McCanlies et al., 2004). The 2004 report describes increased susceptibility to chronic beryllium disease in workers with the HLA-DP1 Glu69 alleles. Further, workers who were homozygous for this allele were more apt to have CBD over sensitization to beryllium. The role that genetic polymorphisms play in response to various toxins, in occupational or environmental exposures, highlights the need for a better understanding of interindividual variations in the workplace. Incorporating this information into workplace safety and regulation may help to ensure the safety and health of more members of the workforce.

CONCLUSIONS

Microarray technology has allowed researchers to quickly analyze thousands of gene expression changes at one time, for multiple time points and doses. As with many new areas in technology, as the technology bounds forward the analytic abilities fall behind. The past decade has shown an amazing increase in the use of microarray analysis for a variety of purposes. Microarray profiles following exposure to various chemicals was compared to profiles of different chemical classes (carcinogenic, toxins, etc.) to learn more about novel chemicals (http://dir.niehs.nih.gov/microarray/backgrnd.htm), susceptible populations (Angerer et al., 2006), biomarkers of exposure to toxins (Forrest et al., 2005), and diagnostics (http://www.nature.com/nbt/journal/v21/n9/full/nbt0903–959.html). To date, there is still a lack of standardization in the field of microarray technology and analysis. An excellent review of comparative microarray studies (Yauk et al., 2004) demonstrates areas in this field that still need improvement, and key steps to optimal comparability between labs and microarray platforms.

Microarray technology was applied to determine alterations related to disease and to search for biomarkers of disease and of exposure to chemicals. Further, this methodology can be used to determine information about as yet unknown chemicals in the workplace. The near future use of microarray technology may expand to include the ability to diagnose disease, as well as susceptibility to disease.

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