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DNA methylation analysis using CpG microarrays is impaired in benzopyrene exposed cells

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Abstract

Epigenetic alterations have emerged as a key mechanism involved in tumorigenesis. These disruptions are partly due to environmental factors that change normal DNA methylation patterns necessary for transcriptional regulation and chromatin compaction. Microarray technologies are allowing environmentally susceptible epigenetic patterns to be mapped and the precise targets of environmentally induced alterations to be identified. Previously, we observed BaP-induced epigenetic events and cell cycle disruptions in breast cancer cell lines that included time- and concentration-dependent loss of proliferation as well as sequence-specific hypo- and hypermethylation events. In this present report, we further characterized epigenetic changes in BaP-exposed MCF-7 cells. We analyzed DNA methylation on a CpG island microarray platform with over 5400 unique genomic regions. Depleted and enriched microarray targets, representative of putative DNA methylation changes, were identified across the genome; however, subsequent sodium bisulfite analyses revealed no changes in DNA methylation at a number of these loci. Instead, we found that the identification of DNA methylation changes using this restriction enzyme-based microarray approach corresponded with the regions of DNA bound by the BaP derived DNA adducts. This DNA adduct formation occurs at both methylated and unmethylated CpG dinucleotides and affects PCR amplification during sample preparation. Our data suggest that caution should be exercised when interpreting data from comparative microarray experiments that rely on enzymatic reactions. These results are relevant to genome screening approaches involving environmental exposures in which DNA adduct formation at specific nucleotide sites may bias target acquisition and compromise the correct identification of epigenetically responsive genes.

Keywords: DNA methylation; Benzopyrene; Microarrays; Epigenetic; Environmental

Introduction

Epigenetic mechanisms play an essential role in the cellular processes of transcriptional regulation, chromatin compaction, imprinting and X-chromosome inactivation (reviewed in Bernstein et al., 2007). As well, the disruption of epigenetic mechanisms has emerged as a key mechanism in the process of tumorigenesis with global genomic hypomethylation, as well as gene-specific hypermethylation being observed in nearly all cancers (reviewed by Esteller et al., 2001; Ehrlich, 2002; Esteller, 2007). Various environmental and dietary agents and lifestyles are suspected to be implicated in cancer causation, although the precise targets of environmentally induced epigenetic alterations during cancer development have not been fully identified (Herceg, 2007). While much of our current understanding of epigenetics is based on gene-specific approaches, the development of technologies that allow genome-wide epigenetic profiling (epigenomics; Esteller, 2007) holds great promise in mapping epigenetic patterns susceptible to environmental exposures.

Abbreviations: BaP, benzo(a)pyrene; BPDE, benzo(a)pyrene diolepoxide; CpG, cytosine/guanine dinucleotide pair; PFT, pifithrin-α; PAH, polycyclic aromatic hydrocarbons.

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Epigenetic instructions act through precise, intricate patterns of DNA methylation and histone modifications that establish and maintain the chromatin template on which transcription can be initiated or suppressed (Rodenhiser and Mann, 2006). DNA methylation occurs primarily at cytosines within CpG dinucleotides clustered within gene regulatory regions. CpGs are usually unmethylated in actively transcribed genes and methylated in silenced DNA. Together with histone modifications, these reversible epigenetic 'tags' can be placed on DNA to ensure proper patterns of gene expression and chromosomal integrity, thereby ensuring that specific genes can be expressed (or repressed) in response to changes in hormone levels, diet or drugs.

Environmental exposures to a wide range of contaminants, including polycyclic aromatic hydrocarbons (PAHs) and heavy metals can have genome-wide effects (Rosenkranz, 1996; Ciganek et al., 2004; Mahadevan et al., 2005). Exposures to these environmental chemicals can result in genetic and epigenetic alterations that can modify endogenous cellular pathways, destabilize the genome, and alter patterns of gene expression in target cells (Nyce, 1989, 1997; Lee et al., 1995; Davis et al., 2000; Cooney, 2001). PAHs can affect epigenetic profiles in cells by forming DNA adducts (Denissenko et al., 1996; Hu et al., 2003), inducing cell cycle arrest (Jeffy et al., 2002), activating multiple signal transduction pathways and directly affecting transcription (Wang et al., 1993; Jeffy et al., 1999; Safe and McDougal, 2002). Benzopyrene (BaP) is a common PAH present in cigarette smoke, in certain foods and due to occupational exposures (Lijinsky, 1991; Rosenkranz, 1996; Scherer et al., 2000). DNA adducts involving the BaP metabolite benzo (a)pyrene diolepoxide (BPDE) have been identified in breast epithelial cells and human breast milk, providing evidence that BaP can reach ductal breast epithelial cells from which most breast cancers are thought to arise (Li et al., 1996, 2002; Gorlewska-Roberts et al., 2002; Thompson et al., 2002).

Previously, we observed BaP-induced epigenetic events and cell cycle disruptions in breast cancer cell lines (Sadikovic and Rodenhiser, 2006). These effects included robust time- and concentration-dependent loss of proliferation, S/G2M accumulation and apoptosis in p53 positive MCF-7 and T47-D cells. As well, DNA methylation profiling analyses showed dynamic, sequence-specific hypo- and hypermethylation events that reinforce the link between environmental exposures, DNA methylation and breast cancer (Sadikovic and Rodenhiser, 2006). In this report, we follow up our initial studies with microarray experiments to identify BaP-induced epigenetic changes in methylatable CpG regions. We labeled and hybridized DNA from BaP treated and control MCF-7 cells to human CpG-island 12k arrays to identify gene-specific targets and genomic changes in DNA methylation patterns. Depleted and enhanced methylation patterns were identified across the genome; however, subsequent sodium bisulfite analyses revealed no changes in DNA methylation at a number of these loci. Instead, DNA adduct formation appears to be occurring at both methylated and unmethylated CpG dinucleotides and affecting target amplification during sample preparation. These results are relevant to genome screening approaches involving environmental exposures in which DNA adduct formation at specific nucleotide sites may bias target acquisition and compromise the correct identification of epigenetically responsive genes.

Materials and methods

Cell treatments. Breast carcinoma MCF-7 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 20 mM HEPES Buffer Solution (Gibco), and Penicillin/Streptomycin in humidified tissue culture incubators at 37 °C and 5% CO_2. 2×10^6 cells were plated on 15 cm tissue culture plates in 20 ml growth media. After 24 h, triplicate plates of cells (in 30 ml media) were exposed to 0.05% DMSO (as a control) or to 0.5 μM Benzo (a)Pyrene (BaP; Sigma) or [^{3}H] BaP (GE Healthcare) suspended in 0.05% DMSO and exposed for up to 4 days.

CpG island DNA methylation microarrays. Methylation profiling was undertaken to identify benzopyrene-induced gene-specific methylation changes using CpG island (CGI) microarrays, purchased from the Microarray Centre at the University Health Network in Toronto (http://data.microarrays.ca/cpg). These arrays consist of approximately 12,000 (12K) methylation-sensitive DNA regions representing 5400 specific gene promoter-associated sequences. Previous studies have used these arrays to identify altered DNA methylation patterns in cancer, tumor-specific epigenetic signatures, and to address the mechanisms of chromatin organization (Yan et al., 2002; Heisler et al., 2005). Protocols established by the Microarray Centre at the University Health Network in Toronto were used that involved methylation sensitive endonucleases to discriminate between the methylation profiles of benzopyrene treated and matched untreated controls. Three arrays for each of three biological replicates were probed. Briefly, genomic DNA was isolated using GenElute mammalian genomic DNA miniprep kit (Sigma), and 2 μg of genomic DNA was first digested with 10U of *Mse*I, which targets (TTAA) sequences that rarely reside in CpG islands. DNA was purified with the QIAquick PCR Purification Kit (Qiagen) and adaptors (H24: 5′-AGG CAA CTG TGC TAT CCG AGG GAT-3′; H12: 5′-TAA TCC CTC GGA-3′; Sigma-Genosys) were ligated to the *Mse*I ends.

Next, DNA was digested with the methylation-sensitive endonuclease *Hha*I which targets GCGC sequences that are often found in CpG islands. These DNA fragments were then amplified for 25 cycles (94 °C (1 min); 55 °C (1 min); 72 °C (1 min)) with an initial incubation at 72 °C for 2 min to fill in the 3′ overhang in the annealed adaptors, and with a final 5 min extension at 72 °C, in the presence of 10 μM H-24 primer. PCR products were again purified using the QIAquick PCR Purification Kit (Qiagen). Control and treated samples were then labeled with Cy3/Cy5 (Mirus) respectively, and purified with the CyScribe GFX purification kit prior to hybridization to the CpG microarrays. The remaining preparations were performed at the London Regional Genomic Centre. The Cy3/Cy5 combined sample was mixed with hybridization solution containing DIG Easy Hyb, sonicated calf thymus DNA and yeast tRNA as hybridization controls, and loaded onto the microarray slides and incubated overnight at 37 °C. Slides were then washed 3 times in 1× SSC/0.1% SDS (at 50 °C) and once in 1× SSC at room temperature, rinsed with MilliQ water, dried and scanned for analysis.

Data analysis. False-color fluorescent images obtained for each channel were saved as 16 bit .tiff images and imported into the Arrayvision 6.0 software (Imaging Research, Waterloo, Canada) with the Cy3 as the control channel, and the Cy5 as the data channel. The raw spot intensity was calculated for each channel using the MTM Density algorithm of the software. Background for each spot was calculated using the average pixel intensity of four (2×2) pixel regions surrounding each spot. This background intensity was subtracted from the raw spot intensity to give the background subtracted spot intensity in both channels. These results were saved as tab-delimited .txt files, which were then imported into GeneSpring v7.2 (Agilent) for downstream analyses. The arrays were normalized using the LOWESS intensity-dependent algorithm of GeneSpring v7.2 at the default settings, and then for each biological replicate, significantly increased or decreased data/control ratios were calculated using the Student's *t*-test, with the *p*-value cut-off set at 0.05. The lists of significant "Increasers" or "Decreasers" obtained from each biological replicate were compared using the VENN tool of GeneSpring v7.2, which allowed for detection of regions of interest for further analysis (significant regions appearing in at least 2 of 3 biological replicates).