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Both hypomethylation and hypermethylation of DNA associated with arsenite exposure in cultures of human cells identified by methylation-sensitive arbitrarily-primed PCR

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Abstract

In a previous study we reported that methylation within the promoter region of *p53* was altered in human lung A549 cells exposed to arsenite over a 2-week period in culture. In the present study the methylation status of the 5′ control region of the tumor suppressor gene, *von Hippel Lindau* syndrome (*VHL*), a gene known to be silenced transcriptionally by CpG methylation was assessed. No changes in DNA methylation in *VHL* in human kidney UOK cell lines exposed to arsenite were seen after 4 weeks in culture, assessed by simple *Hpa*II digestion followed by PCR amplification. Using methylation-sensitive arbitrarily-primed PCR we identified eight differentially methylated regions of genomic DNA of ~300–500 bp from three UOK cell lines and from human lung A549 cells after arsenite exposure in culture. Six fragments were hypermethylated, and two were hypomethylated, relative to untreated controls. Sequence analysis revealed two DNA fragments contained repeat sequences of mammalian-apparent LTR retrotransposons, five contained promoter-like sequences, and 13 CpG islands were identified. Three fragments had 99–100% homology to regions on human chromosomes 6, 9, and 15 but these genes have not yet been identified. Our findings are consistent with a potential role for *both* hypermethylation and hypomethylation of DNA that coexist after exposure to arsenite. The results, in total, could support the existence of a state of DNA methylation imbalance that could conceivably disrupt appropriate gene expression in arsenite exposed cells.

Keywords: Arsenic; Arsenite; DNA methylation; Arbitrarily-primed PCR

Abbreviations: AP-PCR, arbitrarily-primed PCR; MTase, methyltransferase; SAM, *S*-adenosylmethionine; MaLRs, mammalian apparent LTR-retrotransposons; *VHL*, von Hippel-Lindau syndrome gene; IC_{xy}, concentration of arsenite in nM that reduces the survival of cells to *xy* percentage as compared to untreated controls; dNTPs, deoxynucleotide triphosphates.

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1. Introduction

The inorganic salts of arsenic (subsequently called inorganic arsenic) are naturally occurring, environmentally ubiquitous substances considered to be human carcinogens (IARC, 1987; ATSDR, 1997). Chronic exposure of individuals to high concentrations of inorganic arsenic via drinking water has been associated with increased risk of skin, lung, liver, kidney, bladder, and prostate cancers (USEPA, 1988; Chen et al., 1992; Smith et al., 1992; Hopenhayn-Rich et al., 1996; Lewis et al., 1999). However, the mechanism of arsenic carcinogenesis is poorly understood.

Inorganic arsenic is biotransformed to methylated species by a methyltransferase (MTase), using *S*-adenosylmethionine (SAM) as a methyl donor (Zakharyan et al., 1995). Because DNA MTases also require SAM as a methyl donor, it has been proposed that carcinogenesis by arsenic could be mediated as a result of interference with a common set of pathways affected by DNA methylation that encompass DNA damage/repair, cell cycle and differentiation (Mass, 1992; Mass and Wang, 1997; Zhao et al., 1997). Alteration of DNA methylation has the potential to generate mutation (Yang et al., 1996) and alter gene expression (Herman et al., 1994; Gonzalez-Zulueta et al., 1995). Perturbation of the machinery of DNA methylation is among the most common of changes associated with neoplasia (Yang et al., 1996).

The hypothesis that alteration of DNA methylation is associated with arsenic exposure has been investigated in our laboratory. Previously we showed that hypermethylation of a fragment of the *p53* promoter region containing major transcription start sites was associated with exposure of human lung A549 cells to arsenite and arsenate after approximately 2 weeks in culture (Mass and Wang, 1997; Schroeder and Mass, 1997). Others have shown that arsenite exposure is associated with global reduction of DNA methylation in a rat hepatocyte cell line (Zhao et al., 1997).

In order to determine if arsenic exposure also induced alterations of DNA methylation in promoter regions of other tumor suppressor genes, we chose to look at the tumor suppressor gene of the hereditary von Hippel-Lindau (VHL) syndrome utilizing human kidney UOK cell lines (Shuin et al., 1994; Kondo and Kaelin, 2001). The VHL syndrome is associated with human clear cell carcinoma of the kidney among other neoplasms and this gene is altered in cases of renal cell carcinoma even without inherited VHL syndrome. The methylation of the 5′ regions of the *VHL* gene is closely linked to expression of this gene and results in transcriptional silencing (Herman et al., 1994). Because of this known linkage between *VHL* and methylation of the 5′ control region of this gene and because the kidney is a target tissue in humans exposed to arsenic, we chose to determine if the methylation status of the *VHL* gene could be modified by exposure to arsenite in culture. We observed no alteration of methylation of the *VHL* promoter/exon 1 region after arsenite exposure for 4 weeks using *Hpa*II-digestion followed by PCR. Therefore, we utilized the methylation-sensitive variant of the technique arbitrary-primed (AP)-PCR that is very sensitive to DNA methylation alterations (Gonzalgo et al., 1997) to identify potential differentially methylated regions of genomic DNA in both human kidney UOK cell lines and human lung A549 cells, as an adjunct to our previous work with A549 (Mass and Wang, 1997). We found examples of both under- and over-methylation of DNA sequences associated with arsenite exposure.

2. Materials and methods

2.1. Cell culture

Human kidney carcinoma cell lines UOK123, UOK109 and UOK121 were kindly provided by Dr Robert Worrell (NCI, Bethesda, MD). The promoter/exon 1 region of the *VHL* gene is not methylated in UOK123 and UOK109, but is methylated in UOK121. These cell lines were cultured in DMEM (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum. The A549 human lung carcinoma cell line obtained from the American Type Culture Collection (Rockville, MD) was grown in RPMI 1640 medium with 10% fetal bovine serum. The culture medium was replaced

twice weekly in all studies. A clonal cytotoxicity assay was used to define concentrations of arsenite under which UOK cells could grow. The cells were seeded into six-well plates (35 mm wells) at 800 cells/well and exposed to sodium arsenite 24 h later when cells attached. Cells were stained with 10% Giemsa and colonies were counted after growing in the presence of sodium arsenite for 10 days. All determinations were performed in triplicate. For A549 cells, arsenite cytotoxicity had been determined previously by Mass and Wang, 1997.

2.2. Sodium arsenite treatment

Cells were exposed to sodium arsenite as described by Mass and Wang (1997). For UOK cells, sodium arsenite was used at concentrations that yielded IC_{30}, IC_{50} and IC_{80}. For A549 cells, IC_{20}, IC_{50} and IC_{80} (Mass and Wang, 1997) were used. In brief, 20 000 cells from each cell line were seeded in one 75 cm^2 culture flask. Sodium arsenite was added 24 h later. This summarizes the growth protocol: when grown to 75% confluence, the cells were split equally into three 75 cm^2 culture flasks. After the cells reached 75% confluence, the cells were treated with trypsin, detached from the plastic substrate, and seeded in equal number into three 150 cm^2 culture flasks. The cells were collected for analysis when they were 75% confluent. For A549 cells this passage regimen required approximately 2 weeks. For the UOK cell lines this passage level was achieved in about 4 weeks due to their slow growth rate.

2.3. Methylation status of the promoter/exon 1 region of VHL gene in UOK cell lines

Total genomic DNA (5 μg) was isolated (Mass and Wang, 1997), and digested with 100 units of *Hpa*II and *Msp*I (Boehringer Mannheim), respectively at 37 °C overnight. Since the *VHL* gene would be expected to be unmethylated in placental DNA (Sigma, St. Louis, MO), it was used as a control to confirm that *Hpa*II/*Msp*I digestion was complete. Digested DNA then was amplified by PCR using primers VHLPF1 and VHLE1R (Table 1). PCR reactions contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each primer, 100 ng of digested DNA and 1 unit of *Taq* polymerase. The PCR protocol was 1 cycle of 94 °C for 5 min (prior to addition of *Taq* polymerase), and 35 cycles of 94 °C for 1 min, 63 °C for 1 min, 72 °C for 2 min, followed finally by 1 cycle of 10 min at 72 °C.

2.4. Restriction enzyme digestion for AP-PCR

DNA was isolated per above. Before restriction enzyme digestion, genomic DNA was dialyzed at 4 °C overnight. Five micrograms of total genomic DNA were digested with 50 units *Rsa*I alone, with 50 units of *Rsa*I and 100 units of *Hpa*II, or with 50 units *Rsa*I and 100 units *Msp*I (Boehringer Mannheim, Indianapolis, IN) at 37 °C overnight.

2.5. Methylation-sensitive AP-PCR

The methylation sensitive AP-PCR technique was used to find specific regions of the genome that have altered methylation patterns relative to untreated controls. This technique can be thought of as the methylation sensitive variant of 'differential display' (Liang and Pardee, 1992) but using genomic DNA. Methylation-sensitive AP-PCR uses random primers that target CG-rich contain-

Table 1
Primers used in this study

Name	Sequence
For AP-PCR	
MGE2	5′-AAC CCT CAC CCT AAC CGG CC-3′
MGF2	5′-AAC CCT CAC CCT AAC CCG CG-3′
MLG2	5′-AAC CCT CAC CCT AAC CCC GG-3′
OP-JHind 12	5′-AGC TTG TTC ATG-3′
OP-NHind 12	5′-AGC TTC TCC CTC-3′
OP-RHind 12	5′-AGC TTC CGG TGA-3′
For PCR	
VHLPF1	5′-CAG TAA CGA GTT GGC CTA GC-3′
VHLE1R	5′-GTC GAA GTT GAG CCA TAC GG-3′

ing genomic DNA sequences. The primers we used were among primers suggested by the publications of the Jones' laboratory (Gonzalgo et al., 1997; Liang et al., 1998). The only constraint in the use of these primers is that they should not produce too many bands when the PCR product is separated on a polyacrylamide gel, which would make the analysis uninterpretable due to overlap, (>200 bands), nor should they produce too few bands making the detection of changes in methylation unlikely (<20 bands). Specific restriction enzyme-digested DNA was amplified using AP-PCR with a single primer or a combination of two primers (Table 1). Primers MGE2, MGF2 and MGL2 (Gonzalgo et al., 1997) contain CG sequences at the 3′-end region that presumably increase the annealing between primers and CG-rich regions. Primers OP-Jhind 12, OP-Nhind 12 and OP-Rhind 12 were random primers and were purchased from Operon, Inc. (Alameda, CA). Use of more primers yields more candidate differentially methylated DNA sequences.

2.6. 20-Mer primers

PCR reactions contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 μM primer (or 0.6 μM each primer if the two primer combination was used), 200 ng of digested DNA, 2 μCi α-^{33}P dATP and 1 unit of *Taq* polymerase. The PCR protocol was initial denaturation of DNA at 94 °C for 5 min, 5 cycles of 94 °C for 30 s, low stringency annealing at 40 °C for 60 s, and 72 °C for 90 s, then 30 cycles of 94 °C for 15 s, 55 °C for 15 s, 72 °C for 60 s, followed by 4 min at 72 °C.

2.7. 12-Mer primers

PCR reactions were the same as performed with 20-mer primers except using 0.5 μM primer (or 0.3 μM each primer if the two primer combination was used). The PCR protocol was initial denaturation of DNA at 94 °C for 5 min; then 35 cycles at 94 °C for 1 min, low stringency conditions of 40 °C for 1 min and 72 °C for 2 min, followed by 10 min at 72 °C. Each PCR reaction was loaded and resolved on a 5% polyacrylamide sequencing gel under denaturing conditions (7 M urea). The gels were dried, and exposed to autoradiographic film.

2.8. Isolation and sequencing of DNA fragments generated by AP-PCR

Candidate bands that were differentially methylated were identified visually and were excised carefully from dried polyacrylamide gels using a clean razor blade or scalpel and re-amplified by PCR using the same PCR reaction mix and amplification parameters as described for AP-PCR in the above section. PCR products were cloned using a TA Cloning Kit (Invitrogen, San Diego, CA). Positive clones were screened for an insert by PCR using primers M13 and T7, and sequenced using the automated DNA Sequencer, ABI 377 (PE Applied Biosystems, Foster City, CA).

For homology searches to identify gene sequences, we used the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/). Repetitive sequences were identified using the REPEATMASKER program of University of Washington (http://repeatmasker.genome.washington.edu/repeatmasker). Putative promoter regions were predicted using the TSSG and TSSW programs of Baylor College of Medicine (http://searchlauncher.bcm.tmc.edu/gene-finder/gf.html) and the NNPP program of the UC/Berk-eley (http://www.fruitfly.org/seq_tools/promoter.html).

3. Results

3.1. Cytotoxicity of sodium arsenite to UOK cell lines

The cytotoxicity of sodium arsenite was measured by a colony-formation efficiency assay. The three UOK cell lines were very sensitive to sodium arsenite compared to other human cell lines (Mass and Wang, 1997; Salazar et al., 1997; Hamadeh et al., 1999). The IC_{30}, IC_{50} and IC_{80} were determined to be 10, 20 and 50 nM for UOK123; 7, 21

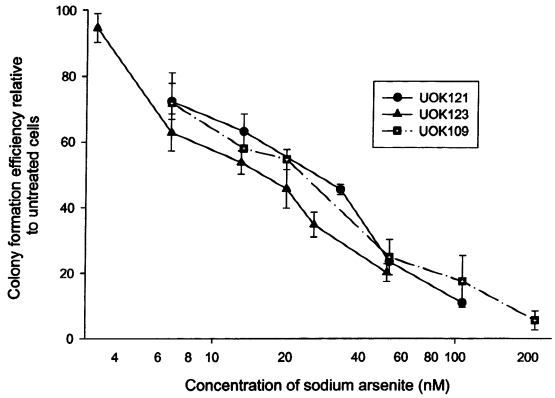

Fig. 1. Concentration-response curves for the effect of arsenite on the colony forming efficiency of UOK cell lines. Cells were seeded at clonal density and allowed to attach for 24 hrs. Sodium arsenite was then added to the culture media and the cells allowed to grow for 7 days. Cells were stained with 10% Giemsa and then colonies enumerated.

and 93 nM for UOK109; and 9, 20 and 74 nM for UOK121 (Fig. 1). In subsequent studies, UOK cell lines were exposed to sodium arsenite at the IC_{30}, IC_{50} and IC_{80}. For A549 cells, sodium arsenite at IC_{20} (80 nM), IC_{50} (400 nM) and IC_{80} (2000 nM) was used per our previous studies with this cell line (Mass and Wang, 1997).

3.2. Methylation pattern of the promoter/exon 1 of the VHL gene

Primers VHLPF1 and VHLE1R were designed to amplify the promoter/exon 1 region of the *VHL* gene. In this region, there is a large CpG island of 10 CCGG sequences (Latif et al., 1993). As expected, placental DNA, and DNA from UOK123 and UOK109 grown in the absence of sodium arsenite yielded a 0.5 kb PCR product using primers for the 5′ region of the *VHL* gene. No PCR product was obtained with *Hpa*II or *Msp*I (Fig. 2) digestion prior to PCR amplification since this region is unmethylated in the cell lines mentioned above and in placental DNA. No PCR products resulted from *Hpa*II digestion of DNA isolated from cells grown in the presence of the IC_{30}, IC_{50} and IC_{80} of sodium arsenite for 1 month, indicating that there was no hypermethylation detected in this region by this technique. For UOK121 which normally has a methylated *VHL* promoter/exon 1, PCR products were obtained from uncut DNA as well as DNA treated with *Hpa*II prior to PCR amplification regardless of sodium arsenite treatment for 1 month (Fig. 2), suggesting that CCGG sequences in *VHL* promoter/exon 1 were not undermethylated. Thus, exposure of UOK cell lines to arsenite in culture for 4 weeks did not change the methylation status of the *VHL* gene in any way that we could detect using simple *Hpa*II digestion followed by PCR amplification.

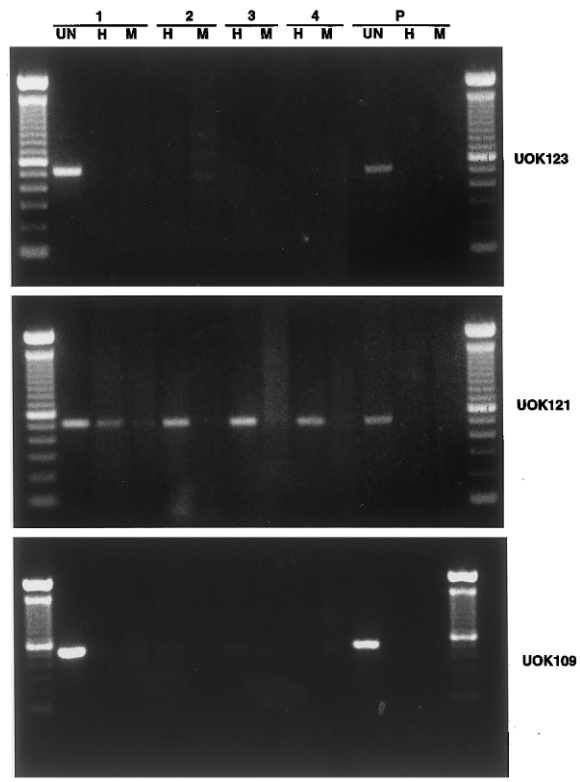

Fig. 2. Methylation of the promoter/exon 1 region of VHL gene. DNA from UOK123, UOK121 and UOK109 digested by HpaII (H) and MspI (M) was amplified by PCR, respectively, using primers that flanked the promoter/exon 1 region. 1): DNA from untreated cells; 2), 3), and 4): DNA from cells treated with sodium arsenite at IC_{30}, IC_{50} and IC_{80}, respectively. UN: uncut DNA; P: placental DNA.

3.3. Identification of differentially methylated DNA fragments using methylation-sensitive AP-PCR

The lack of methylation changes seen in UOK cells exposed to arsenite for 1 month lead us to ask the question whether arsenite exposure was producing any methylation changes in the genomes of the exposed cell lines? We relied on methylation-sensitive AP-PCR to answer this question. Methylation-sensitive AP-PCR was performed on *Rsa*I/*Hpa*II/*Msp*I digested DNA using a single primer or a combination of two primers under low-stringency annealing conditions. DNA digested with *Rsa*I only and with *Rsa*I + *Msp*I served as controls for determining if bands observed in the AP-PCR of *Rsa*I + *Hpa*II-digested DNA were due to differential methylation of CCGG sequences within the region of amplification (Fig. 3A). In other words, the *Rsa*I digestion produced a fragment of particular size, and *Hpa*II digestion determined whether that fragment contained methylated CCGG sequences. *Msp*I is used as methylation insensitive isoschizomer of *Hpa*II digestion for methylation status of the internal cytosine of the CCGG sequence. Fig. 3B shows that a band was present in all four samples from UOK123 digested with *Rsa*I, but absent in all four samples digested with *Rsa*I + *Msp*I. The same size band was only observed in the AP-PCR of *Rsa*I + *Hpa*II-digested DNA from cells treated with sodium arsenite at IC_{50} and IC_{80}, but not from untreated cells and cells treated with sodium arsenite at IC_{30}. This result indicates that one or more CCGG sequences within the region amplified by the primer(s) were differentially hypermethylated in dose-dependent fashion after sodium arsenite treatment. Fig. 3C shows that a band was present in all four samples from UOK121 digested with *Rsa*I, but absent in all four samples digested with *Rsa*I + *Msp*I. The same size band only appeared in the AP-PCR of *Rsa*I + *Hpa*II-digested DNA from untreated cells, but not from cells treated with sodium arsenite. This result suggests that at least one of CCGG sequences within this region was differentially hypomethylated after sodium arsenite exposure. Using this technique, we identified a total of eight

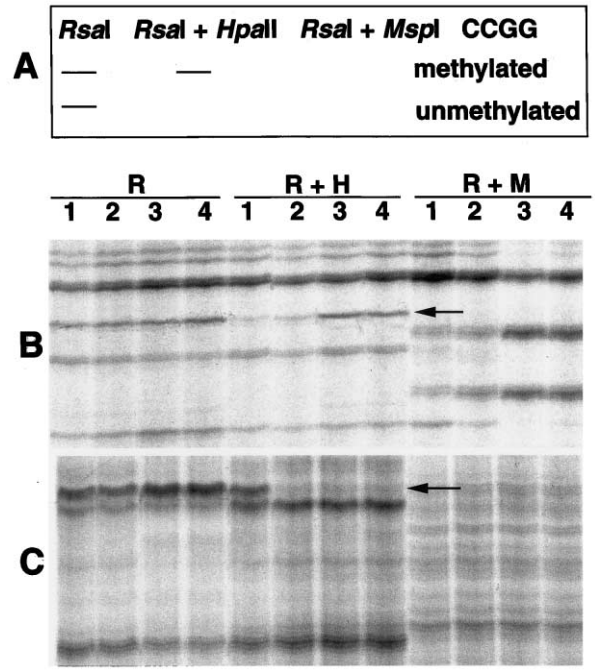

Fig. 3. Methylation-sensitive AP-PCR fingerprints of UOK123 and UOK121 cell lines with and without sodium arsenite exposure. A: the principle of methylation-sensitive AP-PCR, B: typical methylation-sensitive AP-PCR gels demonstrating hypermethylated bands in UOK123 and C: a hypomethylated band in UOK121 after sodium arsenite exposure, respectively. Differentially methylated bands are indicated by arrows. R: RsaI digestion; R + H: RsaI + HpaII digestion; R + M: RsaI + MspI digestion. Lanes 1: DNA from control cells; 2, 3, and 4: DNA from cells treated with sodium arsenite at IC_{30}, IC_{50} and IC_{80}, respectively

differentially methylated DNA fragments, i.e. one hypermethylated and one hypomethylated DNA fragment in A549, one hypermethylated DNA fragment in UOK123, four hypermethylated DNA fragments in UOK109, and one hypomethylated DNA fragment in UOK121 induced by culture in the presence of sodium arsenite (Table 2).

All differentially methylated DNA fragments were cloned and sequenced (Table 2). DNA sequence analysis revealed that three of the eight DNA fragments had significant homology matches (99–100% homology) to sequences in the GENBANK database after BLAST search and were found to reside on human chromosomes

Table 2
Differentially methylated DNA fragments identified by methylation-sensitive AP-PCR from cell lines exposed to arsenite in culture

Cell line	Primer	Methylation status relative to untreated control	Size (bp)	Sequence designation	Homology and promoter prediction parameter	%GC	CpG/GpC	CG/Frag	# CpG Islands	GB[h] #
UOK123	OP-JHind 12	Hypermethylated	293	MaLRs element[d]	Homology from 79 to 289 bp	46	0.1	1	0	AQ936562
UOK109	OP-NHind 12	Hypermethylated	405	Putative promoter region	nn[a]: 0.93; tssw[b]: 5.65	53	1.6	37	2	AQ936563
		Hypermethylated	330	Putative promoter region	nn: 0.83; tssw: 4.26	56	1.5	42	3	AQ936564
		Hypermethylated	339	Putative promoter region	nn: 0.99; tssg[c]: 5.47; tssw: 8.36	50	1.2	23	2	AQ936565
	OP-RHind 12	Hypermethylated	316	Putative promoter region	nn: 0.98; tssw: 5.65	52	1.4	22	3	AQ936566
UOK121	MGE2+MGF2	Hypomethylated	373	Putative promoter region	nn: 0.81; tssg: 9.91	50	1.3	21	3	AQ936567
A549	MGF2	Hypermethylated	508	Not identified[e]		44	0.1	13	0	AQ936568
	MGF2+MLG2	Hypomethylated	287	MaLRs element[f]	Homology from 131 to 226 bp	53	0.4	1	0	AQ936569

The presence of CpG islands was determined based on criteria described by Gardiner-Garden and Frommer (1987): minimum length, 200 bp; GC content, >50%; CpG/GpC, >0.5.

[a] nn, cutoff score for promoter prediction by neural network is 0.80.

[b] tssw, threshold for LDF (linear discriminant function) is 4.00.

[c] tssg, threshold for LDF (linear discriminant function) is 4.00.

[d] 100% homology to human genome sequence NT_019491.1 on chromosome 9.

[e] 99% homology to human genome sequence NT_010216.1 on chromosome 15.

[f] 99% homology to human genome sequence NT_007203.1 on chromosome 6.

[h] Genbank accession number.

6, 9, and 15 although no named genes are presently identified in these regions. Two MaLRs, one from a differentially hypomethylated fragment from A549 cells and the other from a differentially hypermethylated fragment from UOK123 were identified by using the REPEATMASKER program (Table 2). Five out of eight sequences were predicted as putative promoters by using the TSSG, TSSW and NNPP computer programs (Table 2). In addition five of the eight DNA fragments contained two or more CpG islands (Gardiner-Garden and Frommer, 1987), resulting in a total of 13 CpG islands among the eight differentially methylated fragments.

4. Discussion

Previously, our laboratory reported that dose-dependent hypermethylation of the p53 promoter region in the human lung cancer A549 cell line was observed after approximately 2 weeks of exposure to arsenite in culture (Mass and Wang, 1997). In order to determine if arsenic exposure also induced alterations of DNA methylation in the promoter regions of other tumor suppressor genes that might be important cancer target genes, we looked at the *VHL* gene in UOK cell lines. The kidney has been cited as a human target organ in arsenic epidemiology studies (as has the lung), and the expression of the *VHL* gene is known to be regulated by methylation of its 5′ control regions. However, after arsenic exposure for up to 4 weeks in culture, we did not observe changes of methylation pattern in the promoter/exon 1 of *VHL* gene in the UOK cell lines. This determination was made using simple *Hpa*II-digestion followed by PCR amplification of the promoter region of interest. We suspected this could indicate that the DNA methylation changes we observed previously in the *p53* gene promoter might be restricted to that gene due to its particular chromatin conformation, characteristics of the cell line used, induction of selection for pre-existing cells with methylation changes in the *p53* promoter, or that the simple *Hpa*II digestion/PCR amplification technique was not sensitive enough to detect these changes in the *VHL* gene. We therefore used a more sensitive approach. Methylation-sensitive AP-PCR (Gonzalgo et al., 1997) is used to assess alterations of DNA methylation status randomly at multiple regions in the genome and those affected regions can be isolated and sequenced.

Using methylation sensitive AP-PCR we examined arsenic exposed UOK cell lines as well as the A549 cell line that we previously reported was associated with hypermethylation of the *p53* promoter in response to arsenic exposure. AP-PCR was performed on restriction enzyme digested DNA using arbitrary primers under low-stringency conditions. The ability to detect methylation changes by AP-PCR requires the selection of appropriate random primers. The selection of primers is done empirically to fit within the constraints of measurement. A primer set that gives hundreds of overlapping bands cannot be analyzed. A primer set yielding too few bands makes it unlikely that methylation alterations will be detected since the methylation alterations are not common in a random set of sequences. Using AP-PCR in the present study, we identified six differentially hypermethylated regions and two differentially hypomethylated regions of genomic DNA in three UOK cell lines, and we also detected DNA methylation changes in A549 cells after growth in the presence of sodium arsenite. Five out of eight differentially methylated DNA fragments we isolated met the criteria of containing CpG islands (Gardiner-Garden and Frommer, 1987), indicating that we may have identified new genes that have methylation sensitive control regions that are altered by arsenic exposure. In the future these sequences could be explored as potential biomarkers that signal exposure to arsenic.

Our findings are consistent with previous report from our lab (Mass and Wang, 1997), i.e. arsenite can induce hypermethylation. We found that arsenite can also induce hypomethylation as was reported in rat cultured hepatocytes exposed to arsenite (Zhao et al., 1997). We have demonstrated that hypermethylation and hypomethylation coexist in A549 cells grown in the presence of sodium arsenite. Liang et al. (1998) and Gonzalgo et al. (1997) reported similar findings in tumor tissues and cell lines compared to normal tissues

using methylation-sensitive AP-PCR: the coexistence of hypomethylation and hypermethylation. These observations support the view that it is the particular alterations of DNA methylation patterns that may be better assessments of DNA methylation changes rather than the absolute level of DNA methylation, or measurements of global DNA methylation status (Jones, 1996). Our results have the potential to reconcile apparently conflicting observations regarding DNA methylation changes seen after arsenite exposure in culture since we have now observed both hypermethylation and hypomethylation in the genome after growth in the presence of arsenite.

It would have been desirable to work with normal human epithelial cells in this study. However, the length of time we needed to observe DNA methylation changes was at least 2–4 weeks which is too long to maintain primary human epithelial cells without senescence (a form of programmed cell aging) that is also associated with DNA methylation alterations (Machwe et al., 2000). It would be difficult to dissect out methylation changes that could be due to senescence and those due to arsenic exposure. Instead, we chose immortalized cell lines derived from tumors due to their stability in culture. Ultimately, what would be most important would be to perform these analyses (methylation sensitive AP-PCR) with human tumors that have likely arisen through environmental or occupational arsenic exposure, as these tumors may still contain signature changes in gene structure, gene expression, or DNA methylation patterns that may be peculiar to arsenic induced cancers. Among eight differentially methylated DNA fragments that we found, two contained MaLR elements, and five contained putative promoter-like sequences as identified by the TSSG, TSSW and NNPP computer programs. Changes of methylation patterns of repetitive regions are known to alter genome organization and chromatin structure (Kundu and Rao, 1999). Four other putative promoter regions in UOK109 cells were hypermethylated, and one putative promoter region in the UOK121 cell line was hypomethylated after arsenite treatment. Hypermethylation and hypomethylation in promoter regions can directly affect gene expression (Greger et al., 1994; Herman et al., 1994; Gonzalgo et al., 1997). This would be consistent with the proposal that changes of DNA methylation can presumably activate some genes and repress others in response to arsenite exposure. Cells with altered methylation patterns that do not sustain lethal DNA damage may retain a selective advantage for survival and proliferation because they can still replicate, hence propagating changes in gene expression. This might be a mechanism for development of a heritable resistance to arsenic toxicity that does not rely on direct DNA damage or mutation.

It is unclear at this time what the genesis of DNA methylation changes are in response to arsenite exposure but that does not lessen the impact of the observations. The arsenite concentrations used in culture of UOK cells are very low (nanomolar range; near levels found in drinking water) for the UOK cell lines, and it would not be high enough to directly deplete the cellular SAM appreciably considering that twice weekly replenishment of culture medium provides SAM precursors. SAM levels in cells have been reported to be present in the low micromolar range (Wise et al., 1997). It is clear, however, that changes in DNA methylation may have more than one mechanism of occurrence. Arsenite is a reactive molecule capable of reacting with vicinal dithiols present in proteins and inactivating their active sites. The trivalent methylated arsenic metabolites have been shown to be most potent enzyme inhibitors among arsenicals (Styblo and Thomas, 1995; Lin et al., 1999). Such metabolites recently have been shown to be direct-acting DNA damaging agents (Mass et al., 2001) and they could conceivably alter the ability of DNA methyltransferases to act upon regions of DNA with arsenic-associated DNA damage. Both UOK cell lines and the A549 cell line can methylate arsenite (R. Sharma and M.J. Mass, unpublished observations). It is possible that direct inhibition or stimulation of the enzymes of the SAM synthesis pathways is also involved, as might be alterations of activities of DNA methyltransferases due to interaction of their active sites with arsenite or its metabolites.

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Appendix A

DNA sequences isolated from human cell lines identified by AP-PCR to undergo cytosine methylation changes after exposure to arsenite;

AQ936562 from UOK123: 293
bpAGCTTCTCCCTCCAGACACAAGTCTTTAAAATGCTGATCAGAGCATTGAAAACTGAAAC-
AGACACAATACCGACCTCCTGTGGTAGACTGACTCATGGTCCCACAGATGTCCATCCACAT-
CCTAAACCCCAGAACCTATTACCTTACATGGTTCAAAGGATTTTGCAGCTGTGACTAAGCT-
ATGGACCTTAAAATGGAGAGATTACCCTAGATTATCCAGGTGGGTTTGGTGTAATCACAAG
GGTCCTCCTGGGAGGGAAGTGAGAGGATCAGAGTCAGAAGAGGGAGAAGCT

AQ936563; 405 bp from
UOK109TCTTTCTTAAACACCCCCATACCGGCGACGGTATTATGCCTTCCGGCGCCCCTTAGC
TCTAAGTTACGTACCTCCATCGTGGAGGTTCGGTAGGGCTTCGGTCCTCTTAAGGCTATGA-
AGATGAGGGGGTTCTGCTTAACTAGTTCTACAGTTAGGTGAGTTTAAGTTGCGGGCGGAG-
GCGGTTGAGAGGACGTCGCCTCGAACCCACGTTCCCACTTTCGATTTTAGCAGGTTTTTGG-
GACTTCCTTCAGTACCGATCGTCCCGAGCCCGCCGACGACGCCGGCCAACGTTCGCTCTTG-
TCATTGGGTTATCTCTCGAGAGGTAGAGATCTCCGTCCCCGCTATCGTCGCGCTTTTAAGCA
CGCGGTATAAGCGCTCGGTATACGGCAGATTACTAAACAAAAA

AQ936564 from UOK109: 330
bpTGAACCTCGTAAGACCAAGTCCTCTTTCTTCGGGACGCTCTATAGTATTCGGTACATAG-
GCTCTTATATAAGTAAGCCTCTCGCGCGCCGCGGTAAGCGGCGCTAAGACCCGGACGCCC-
GCGAAATCATAAAGATCGTAGGACCTGGTAACATTATCCGCGGGTACGCGCGCGTGCGCG-
CGCGCTCGCGAACGACTAAGCCGGAAACTCGGGATACCCTCCCCGAACCACCCCGAGAAT-
CTCGCCCGTAAGCAAAAGCGAGTCTCCAGGTGTAATCGCTACCCTTCGATTGCGTCGCTA-
ACGCGAGGGAGAAGTCAACTTACTATATCGG

AQ936565 from UOK 109: 339
bpGTCACTCGTAGAAACATAGTCCAAATTTCTAATAAAACGTCCATTAAGCCCAACAAAAA-
GAAGACCTACCGAAGACCCTCCAAGCTAAGACGAAGAACTCAAACGCCGCCCTACGAACA-
CATTGCGGTAAGTAAGCGTGCTAATCAAACCCGAGCGGATAAGAAGTCGCAACCAGGGA-
GATAAAGCTAGAAGGTATCCGTAATCTAGATTCGTCAAGTTAGCACATCCGTAGCGGCGA-
GGGAGAAGTCAAGTTGAACCTTAGTATATCGGGTGGGAGCCATCAAGCGGACTTGAGTC-
TGGCATTAAGTCCGGTGCAACTACGGCTACAGTCGCCCTTCG

AQ936566 from UOK 109: 316 bp
GACACTCGTAGAACTGGTCCAATCCGTGGCGACTACCCCATAAAGCACACGCACACTAAA-
ATACTACTCCGCATATTCCAAACACACATAGCCCCCACCCGTTAACTGTATCCTAATAAAG-
TCGGGAGAAAAATCTGCCTTCCCCTCACATTACTTCTCCACCGCCCTACAGAGACAACGCC-
CCACTCTCACGAAGAAAACATCGATCCACCGCCCGACCGCCCGCCCCGAGATTAAGCTCT-
GTCTCGCTGTTTAGGTCGGCGCGTAGCGGTGTGACTCTGGTCTATTGTAAGTCAAGTGAA-
CCTTAGTATATCGG

AQ936567 from UOK 121 373
bpTATTAGGGGGATAGAAATTATACGGCACGTCCAAATAAGGGAAGCCCAGTACAAGAACC
ACCAATCACCCATAGGGAAGCCGATCAAGAGGGAATAAAAGAGAAACTTATAGAATACTTC
AGAGCAAAGGGAGGCAATCAAGGGAAGGGACCTAGATTCAGGAGAGGGCGCGACTAAAA
GTATGTATCAGACGACGATGCTGATCGGGTCGCTTTAGGATAGAATCGAGGGTTAAGAATT
ATGCGTGAGGATGTCAATATAGCGAAGTCGGAAGGTCGTCCGAGGAGCGTCGGAAAGTTG
TGGGCCAGGCGAGGGCCAACTCGTAGAACCTGGTCAAGTTGAACCTTAGTATATCGGTGGT
TGCCATCACCGG

AQ936538 from A549: 508
bpAACCCTCACCCTAACCCGCGGCCATTCTTGAAAGCATTAAGTTGCCCTACAGTGGGCCACA
TCCCCTTCTTGAGAGGTACCAAAGAACTTGCTCCGCTCTCTAGTGATGGCCCGCTGACCCCA
GACAACAGCCTTTCTGAGACGATGGCTTTTATCAGTGGCTTGTCTCAGGAAGCTGGAGCAAT
GTTAGGTAAAACTACACCCAATGCACCCAGAGAACTCAACAGAGCCAAGAATGTCAAAGGA
AATCAGCCAGGATAGCTGCTGGTTACTGGTGTCACCACTCCCACCCACGCAAGAATGACTCT
CAGCCACGGTGAAGACAAACTTTGTCTCTTAAACTTTGACTCTCAGCCATGGTGAAGACAAA
CCTCACGTGAGCTTTTGCCAAGCCCATTCAGCAGAATCTCGGGCCGCTTGGGACGCATCTTC
TCCCTTGGGACACCGTCTTCCCCTCAGTAGAGGCACCACTATACCAGCCTCTGTGCCCGGGG
TTAGGGTGAGGGTT

AQ936569 from A549: 287
bpTCTCCCTCATGAAGACAGACAGTAAATATCACAACGGACTCCCAACATCCCTTCTGCCTTT
CCTCTGCCAAACAGCAGCAGCACAATGTGAGGAATGTTCTTCTGCTCAAGGAGTGGGTCAG
CCTGGTTGATGGTAACCCTATTCTCCCTTGCCAATAGTTTTTTGAAGAAAGGGGCTTATGAC
CCAATTCTGGACAATTACATGAAAGGAAATTTCTGCTGGAAGCCAATAAAGGTATAGGAAA
CTAATTTATCTTTCCTGTTGGACAAGGAGGAGGGAGAAGCTA

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