Chemical Research in Toxicology

RAPID REPORT

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Epigenetic Changes in Individuals with Arsenicosis

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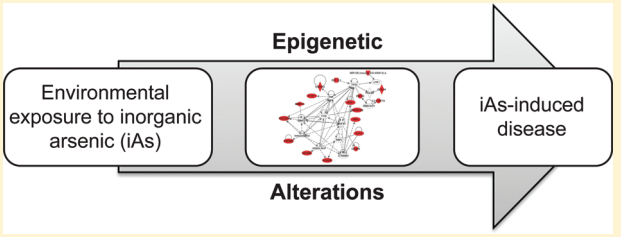
Supporting Information

ABSTRACT: Inorganic arsenic (iAs) is an environmental toxicant currently poisoning millions of people worldwide, and chronically exposed individuals are susceptible to arsenicosis or arsenic poisoning. Using a state-of-the-art technique to map the methylomes of our study subjects, we identified a large interactome of hypermethylated genes that are enriched for their involvement in arsenic-associated diseases, such as cancer, heart disease, and diabetes. Notably, we have uncovered an arsenic-induced tumor suppressorome, a complex of 17 tumor suppressors known to be silenced in human cancers. This finding represents a pivotal clue in unraveling a possible epigenetic mode of arsenic-induced disease.



Inorganic arsenic (iAs) is an environmental toxicant currently poisoning tens of millions of people worldwide. Individuals chronically exposed to iAs are susceptible to arsenicosis or chronic arsenic poisoning. Heavily suffering areas such as West Bengal and Bangladesh saw a rise in incidents of arsenicosis when government officials and international aid agencies, in the hopes of mitigating waterborne diseases, introduced tube wells fed from arsenic-contaminated aquifers.[1] Other regions, such as Mexico, are affected by both naturally occurring arsenic as well as anthropogenic sources such as smelters and ore mining operations. Chronic exposure to iAs is associated with the development of various diseases including heart disease, diabetes, and cancer, and exposed individuals often present with hallmark skin lesions.[2] Premalignant skin lesions may indicate increased risk for arsenic-related cancer.[3] While the precise mode of action in arsenic-induced disease is unknown, one of the proposed mechanisms is altered gene regulation via epigenetic modes of action such as DNA methylation.[4] Supporting this is the finding that early life exposure can result in long-term health consequences,[5,6] suggesting that there are heritable changes to the genome.

Previous studies highlight the association of arsenicosis with altered gene expression patterns in humans displaying the hallmark skin lesions.[7] Moreover, gene-specific analyses suggest the role of altered DNA methylation at target sites such as tumor protein p53 (p53), cyclin-dependent kinase inhibitor 2A (CDKN2A/p16), and Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A).[8,9] However, it remains to be shown whether multiple genes and pathways are affected by epigenetic processes in individuals with signs of arsenicosis. Therefore, we set out to identify differentially methylated genomic regions associated with arsenicosis in humans from Zimapan, Hidalgo State, Mexico who were exposed to varying levels of iAs via their drinking water as assessed by urinary arsenic (Supporting Information, Table 1).

To our knowledge, this is the first study to examine genome-wide site-specific DNA methylation alterations due to arsenic-induced toxicity in a population with ongoing exposure. Peripheral blood lymphocyte DNA of 16 of the individuals, half with established elevated levels of iAs exposure and showing signs of arsenicosis (skin lesions), was analyzed using a methylated CpG island recovery (MIRA)-chip assay. In addition to the difference in skin lesion status, the two groups showed different levels of iAs exposure as assessed in urine ($p < 0.001$). CpG-methylated DNA was isolated using a methyl binding domain protein complex and hybridized to Affymetrix Human Promoter arrays, assessing over 4.6 million sites tiled through human promoter regions. Our analysis enabled a comprehensive examination of DNA methylation levels within CpG islands for over 14,000 genes.

We employed a comparative analytical approach to identify differentially methylated CpG islands and found 183 genes with differential patterns, of which 182 were hypermethylated in

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individuals with signs of arsenicosis (Figure 1). Specifically, the identified genes showed a statistically significant (false discovery rate (FDR) q-value <0.05) difference in average DNA methylation for each CpG island. This assay allowed for interrogation of one the three genes previously identified as being hypermethylated in individuals with signs of arsenicosis, e.g., p16. While the fold change of p16 did not meet the statistical threshold for this study, here we also observed increased promoter methylation in individuals with signs of arsenicosis (fold change of 1.24).

Using a systems level approach, the 183 genes were analyzed for known molecular interactions, and a large interactome of hypermethylated genes was identified (Figure 2A). These are enriched for their involvement in cancer-associated pathways mediated by genes such as p53 (Figure 2B). Interestingly, we found that many of the proteins encoded by genes with differentially methylated CpG islands are known players in arsenic-associated disease, such as heart disease, diabetes, and cancer (Supporting Information, Table 5).

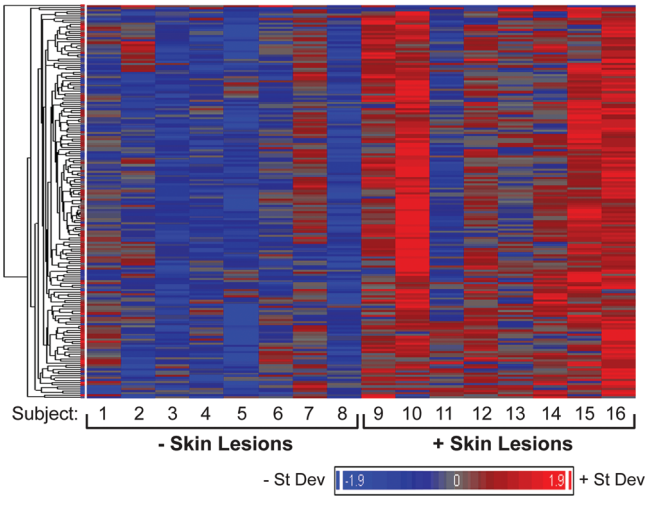

Figure 1. Arsenicosis-associated patterns of DNA methylation. The heat map illustrates the average DNA methylation levels in promoter regions of 183 genes. Data are z-score normalized for each gene. Red represents a relative increase in CpG island methylation level, and blue represents a relative decrease in methylation level.

Notably, we have also identified an arsenic-methylated tumor suppressorome (Figure 2C), a pivotal clue in unravelling a possible epigenetic mode of arsenic-induced disease. The tumor suppressorome is a complex of 17 known or putative tumor suppressors silenced in human cancers. It comprises the following hypermethylated genes: *C11orf70* (chromosome 11 open reading frame 70), *CENPE* (centromere protein E, 312 kDa), *EEF1E1* (eukaryotic translation elongation factor 1 epsilon 1, also known as p18), *ENDOG* (endonuclease G), *FOXF1* (forkhead box F1), *HOXB5* (homeobox B5), *HOXB9* (homeobox B9), *hsa-mir-126* (human microRNA 126), *MMP15* (matrix metallopeptidase 15 (membrane inserted)), *MSX1* (msh homeobox 1, also known as HOX7), *POLD4* (polymerase (DNA-directed), delta-4, also known as p12), *PRDM2* (PR domain containing 2, with *ZNF* domain, also known as RIZ), *RNF20* (ring finger protein 20), *SMARCD2* (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2), *SUFU* (suppressor of fused homologue (Drosophila)), *TBR1* (T-box, brain, 1), and *TSC22D3* (TSC22 domain family, member 3).

Among the members of the tumor suppressorome, of particular interest are those with known associations to arsenic-induced diseases such as cancer of the bladder, kidney, lung, liver, and prostate, as well as cardiovascular disease and diabetes mellitus (Figure 2C, Supporting Information, Table 5). Interestingly, the expression levels of specific members of the tumor suppressorome have previously been shown to be altered via iAs exposure. For example, iAs exposure *in vitro* results in the downregulation of both *MSX1*[10] and *CENPE*.[11] In this study, we find that the CpG islands within the promoter regions of the identified genes are hypermethylated in individuals with skin lesions. As mentioned, DNA hypermethylation of the promoter regions of three genes has been reported in arsenic-induced disease.[8,9] Notably, the results from our study vastly increase this list of gene targets. Examination of these gene targets would be the next step in understanding how epigenetic changes regulate gene expression and, subsequently, cause dysregulation leading to disease.

In this study we have analyzed epigenetic changes in the peripheral blood lymphocyte DNA from iAs exposed and diseased individuals and do not directly measure alterations in target organs. Recent studies support the utilization of lymphocyte

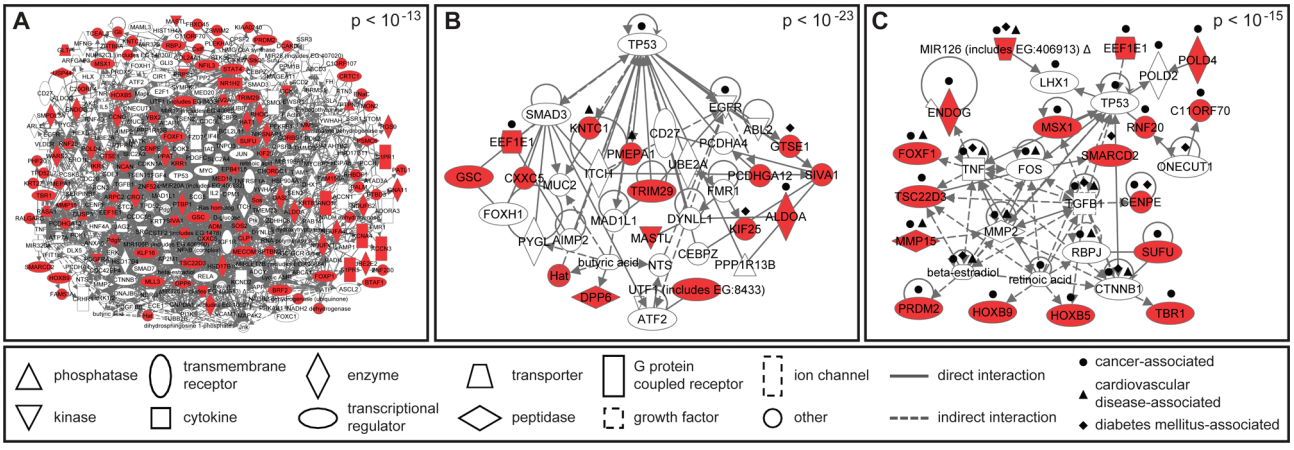

Figure 2. Epigenetically modified iAs-induced networks. (A) Large interacting network of hypermethylated genes. (B) Tumor protein p53 (tp53)-associated network. (C) The iAs-induced tumor suppressorome. *P*-values are shown in the top right corners of each network. Networks are displayed with symbols representing products of hypermethylated genes (red symbols) or the proteins associated with these genes (clear symbols).

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DNA to detect genomic and epigenetic biomarkers of organ-specific disease.[12,13] In future research, it will be possible to compare the epigenetic alterations of the tumor suppressorome from tissue samples of arsenic-exposed individuals.

In conclusion, these results demonstrate that a large number of genes are epigenetically modified in the lymphocyte DNA of individuals exposed to iAs with related arsenicosis. It is likely that the pathways we have identified here are influenced at the transcriptional level resulting in the repression of their activity in exposed individuals. Our findings demonstrate the significant effects of iAs on the epigenome. The identified methylation sites and differential DNA methylation patterns may serve as biomarkers of adverse health effects associated with iAs exposure. Through the identification of differential patterns of methylation, we hope to detail arsenic effects in humans in order to understand arsenic-induced disease and to identify potential methods for disease prevention.

■ ASSOCIATED CONTENT

Supporting Information. Experimental methods and detailed gene lists. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

Microarray data have been submitted to NCBI's Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/) and are available under accession number GSE26073.

Author Contributions

#These individuals contributed equally to this work.

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■ ABBREVIATIONS

iAs, inorganic arsenic; C11orf70, chromosome 11 open reading frame 70; CDKN2A/p16, cyclin-dependent kinase inhibitor 2A; CENPE, centromere protein E, 312 kDa; EEF1E1, eukaryotic translation elongation factor 1 epsilon 1, also known as p18; ENDOG, endonuclease G; FOXF1, forkhead box F1; FDR, false discovery rate; HOXB5, homeobox B5; HOXB9, homeobox B9; hsa-mir-126, human microRNA 126; MIRA-Chip, methylated CpG Island Recovery-Chip assay; MMP15, matrix metallopeptidase 15 (membrane inserted); MSX1, msh homeobox 1, also known as HOX7; p53, tumor protein p53; POLD4, polymerase (DNA-directed), delta-4, also known as p12; PRDM2, PR domain containing 2, with ZNF domain, also known as RIZ; RASSF1A, ras association (RalGDS/AF-6) domain family member 1; RNF20, ring finger protein 20; SMARCD2, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 2; SUFU, suppressor of fused homologue (*Drosophila*); TBR1, T-box, brain, 1; TSC22D3, TSC22 domain family, member 3.

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