ENVIRONMENTAL EPIGENETICS (A BACCARELLI, SECTION EDITOR)

Arsenic Exposure and Epigenetic Alterations: Recent Findings Based on the Illumina 450K DNA Methylation Array

Maria Argos[1]

Abstract Arsenic is a major public health concern worldwide. While it is an established carcinogen and associated with a number of other adverse health outcomes, the molecular mechanisms underlying arsenic toxicity are not completely clarified. There is mounting evidence from human studies suggesting that arsenic exposure is associated with epigenetic alterations, including DNA methylation. In this review, we summarize several recent human studies that have evaluated arsenic exposure using the Illumina HumanMethylation 450K BeadChip, which interrogates more than 485,000 methylation sites across the genome. Many of these studies have observed novel regions of the genome associated with arsenic exposure. However, few studies have evaluated the biological and functional relevance of these DNA methylation changes, which are still needed. We emphasize the need for future studies to replicate the identified DNA methylation signals as well as assess whether these markers are associated with risk of arsenic-related diseases.

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✉ Maria Argos
argos@uic.edu

[1] Division of Epidemiology and Biostatistics, School of Public Health, University of Illinois at Chicago, 1603 West Taylor Street, MC923, Chicago, IL 60612, USA

Introduction

An estimated >200 million people worldwide, including ~17 million in the USA, consume drinking water contaminated with arsenic at levels associated with a variety of adverse health effects and shortened lifespan [1, 2]. Furthermore, recent studies have suggested that food-derived inorganic arsenic exposure is also an emerging public health concern [3–7]. Epidemiologic research has established arsenic as a potent human carcinogen and has associated arsenic exposure with cancers of the skin, lung, bladder, kidney, liver, and possibly prostate [8]. Arsenic exposure has also been associated with detectably increased risk of cardiovascular disease (including coronary and ischemic heart disease, acute myocardial infarction, and hypertension) [9–11], respiratory disease [12, 13], diabetes mellitus [14–16], and impaired neurodevelopment [17]. Recent studies have observed increased infant mortality and low birth weight to be associated with prenatal arsenic exposure at moderate-to-high doses [18, 19]. With respect to its frequency, toxicity, and potential for human exposure, arsenic holds the highest ranking since 1997 on the Agency for Toxic Substances and Disease Registry (ATSDR) Substance Priority List [20].

The mechanisms underlying arsenic toxicity and resulting human health effects are not completely clarified; however, epigenetic events have been hypothesized as a possible mediating pathway. There has been mounting evidence of support from animal and human studies to show that arsenic contributes to epigenetic modifications of the genome [21]. DNA methylation is an epigenetic event with a hypothesized role in gene expression, development, and disease [22]. In humans, methylation is typically of the DNA base cytosine, which is modified reversibly by adding a methyl group ($-CH_3$) to its 5-carbon position [23]. This modification occurs on cytosines that precede a guanosine in the DNA sequence, referred to as

the CpG dinucleotide. Short regions of 0.5–4 kb in length, known as CpG islands, are rich in CpG content. These islands are typically found in or near promoter regions of genes where transcription is initiated. In normal somatic cells, the vast majority of CpG dinucleotides in the genome are methylated, whereas CpG islands often remain unmethylated, allowing gene expression to occur. Whereas in disease pathways, this pattern of CpG methylation is thought to be disrupted, with increased methylation within promoter regions of genes causing abnormal gene silencing, in addition to global hypomethylation of genomic DNA, which promotes chromosomal instability, translocation, and gene disruption [24]. Unlike CpG island regions, there is greater biologic variability in methylation of CpG dinucleotides in CpG shores (within 2 kb of a CpG island), CpG shelves (2–4 kb from a CpG island), as well as isolated CpG loci in the genome [25]. DNA methylation levels are influenced by various factors including genetic, environmental, and dietary factors [26–28].

Furthermore, evidence shows cell- and tissue-specific patterns of DNA methylation, with the largest variability in DNA methylation across tissues for CpG islands located within the body of a gene as well as CpG dinucleotides located outside of CpG islands [29]. This presents an important consideration for epigenetic epidemiologic studies. First, the cellular composition of the biological specimen needs to be considered. For example, in blood-based DNA methylation studies, extracted DNA is derived from a mixture of different leukocytes (i.e., monocytes, lymphocytes, granulocytes) found in the blood. Individual cell types in blood differ not just because of specific surface markers that have been traditionally used to differentiate immune cells, but also systematically differ in DNA methylation patterns at a subset of CpG sites. Therefore, differentially methylated loci associated with an environmental factor of interest may be observed not due to a direct impact on DNA methylation but due to the effect of the environmental factor on a shift in the distribution of white blood cells, as illustrated in Fig. 1. This shift in cellular composition would therefore confound the association of interest between the environmental factor and alterations in DNA methylation. In a recently proposed novel statistical method, the DNA

methylation patterns at a subset of CpG sites across the genome have been used for immunophenotyping to infer the proportions of white blood cell types in a given measured sample [30•] so that this potential source of confounding can be evaluated. Arsenic-related cell type shifts in blood have been observed in previous studies detailed below, although these cell type shifts have been estimated to account for a small proportion of the overall variability in DNA methylation. Second, the correlation of DNA methylation patterns in the measured surrogate biological tissue with the target tissue of interest needs to be considered. In human studies, access to target tissues of interest is often not feasible; therefore, studies are more commonly conducted using blood as a surrogate tissue. For the subset of CpG sites that are not correlated between leukocytes and the cells of the target tissue, differentially methylated CpG sites associated with the environmental factor in the target tissue will not be detected using DNA methylation measures from blood, missing potentially important genes and molecular pathways associated with the exposure. Conversely, differentially methylated CpG sites associated with the environmental factor in blood may be observed that are not relevant for the target tissue of interest. This is a more challenging consideration to overcome and may be informed by future publically available databases such as the Genotype-Tissue Expression Project (GTEx) as well as future studies with access to diverse tissues to enable the evaluation of tissue-specific patterns of DNA methylation.

Several human studies have examined global DNA methylation in blood in relation to arsenic exposure using surrogate markers of global DNA methylation, such as long interspersed nucleotide element-1 (LINE-1), Alu element methylation, methyl incorporation assays, or luminometric methylation assays (LUMA). The findings from these studies have been inconsistent, although they included a number of differences in exposure measures and doses across studies [31–40]. A number of studies have also evaluated arsenic in relation to candidate gene-specific DNA methylation, most frequently assessing p16 and p53 promoter methylation [36, 39, 41–45]. Studies evaluating global and candidate gene DNA methylation in relation to arsenic have been the subject of previous detailed reviews [21, 46, 47].
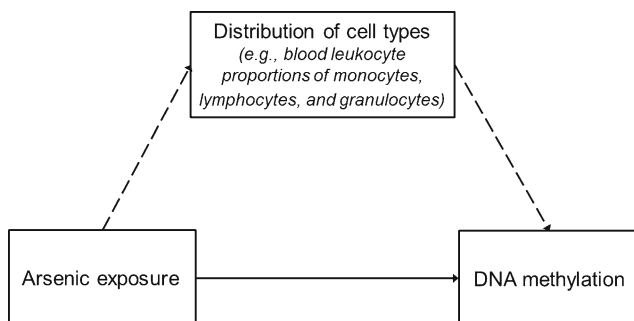
Epigenome-Wide DNA Methylation Studies Using Illumina 450K Platform

Relatively few epigenome-wide association studies have been conducted to investigate DNA methylation alterations in relation to arsenic exposure in humans. Published studies to date have been primarily focused on two main areas of research including the evaluation of 1) cord blood DNA methylation in relation to in utero arsenic exposure [48•, 49•, 50•, 51•] and 2) white blood cell DNA methylation in adults in relation to a

Fig. 1 Illustration of the potential impact of cellular heterogeneity in epigenetic studies of arsenic exposure in relation to DNA methylation

variety of arsenic constructs including arsenical skin lesion status [52, 53•], urinary arsenic species [54], toenail arsenic concentration [55•], and blood arsenic and urinary total arsenic concentrations [56•]. Only one study has evaluated epigenome-wide DNA methylation patterns in tissue other than blood, which was a study of arsenic-related urothelial carcinomas [57]. Among these studies, all but three were conducted using the same epigenome-wide DNA methylation platform, the Illumina HumanMethylation 450K BeadChip (Illumina, San Diego, CA, USA), which interrogates 485, 577 methylation sites per sample [48•, 49•, 50•, 51•, 53•, 55•, 56•]. Of the remaining studies, two used the Affymetrix GeneChip Human Promoter 1.0R array (Affymetrix, Santa Clara, CA, USA) [52, 54] and one used the previous generation Illumina HumanMethylation 27K BeadChip [57]. As summarized in Table 1, the studies utilizing the Illumina 450K platform are the further focus of this review.

In Utero Arsenic Exposure and Umbilical Cord Blood DNA Methylation

Four epigenome-wide association studies to date have investigated umbilical cord blood DNA methylation in relation to prenatal arsenic exposure [48•, 49•, 50•, 51•]. In a study of low-dose prenatal arsenic exposure, Koestler et al. evaluated maternal urinary arsenic concentration at 24–28 weeks gestation in relation to umbilical cord blood DNA methylation among 134 mother-child dyads from the New Hampshire Birth Cohort Study [49•]. Maternal urinary total arsenic concentration was the primary measure of in utero arsenic exposure (median (interquartile range)=4.1 µg/L (1.8–6.6)) in the study. At a nominal significance threshold ($P<0.05$), 18 % of interrogated CpG loci were associated with maternal urinary arsenic concentration, although none remained statistically significant after accounting for multiple comparisons. The authors employed a novel statistical method to infer major leukocyte components of whole blood based on a subset of DNA methylation loci [30•] and observed a significant positive association between maternal urinary total arsenic concentration and proportion of $CD8^+$ T lymphocytes in cord blood.

A study of moderate- to high-dose prenatal arsenic exposure was conducted by Kile et al. to evaluate maternal well water arsenic concentration in relation to umbilical cord blood DNA methylation among 44 mother-child dyads from a prospective birth cohort study in Bangladesh [48•]. Maternal well water arsenic concentration at ≤16 weeks gestation was the primary measure of in utero arsenic exposure (median (range)=12 µg/L (<1.0–581)). A positive significant association between maternal well water arsenic concentration and an intergenic DNA methylation locus on chromosome 19, cg00498691 ($P=5.89\times10^{-8}$), was observed, after accounting for multiple comparisons. Additionally, maternal well water arsenic concentration was observed to have a significant

positive association with the proportion of $CD8^+$ T lymphocytes and a negative association with $CD4^+$ T lymphocytes in cord blood, based on the Houseman et al. statistical method [30•].

Another study of moderate- to high-dose arsenic exposure in Bangladesh was conducted by Broberg et al. to evaluate maternal urinary total arsenic concentration in relation to umbilical cord blood DNA methylation among 127 mother-child dyads from a prenatal supplementation trial [51•]. Maternal urinary total arsenic concentrations during early (5–14 gestational weeks) and late (26–36 gestational weeks) gestation were separately evaluated as the primary measures of in utero arsenic exposure (early-gestation median (5–95 percentiles)= 66 µg/L (20–457) and late-gestation median (5–95 percentiles)=89 µg/L (18–562)). Arsenic exposure measured in early gestation showed stronger association with DNA methylation than that in late gestation, and significant associations were only observed in boys. Three CpG sites in boys (PLIN5 cg15255455, LRRC26 cg13659051, and RPS6KA2 cg17646418) were significantly associated with early-gestation maternal urinary total arsenic concentration based on false discovery rate (FDR) <0.05. Among the top 500 nominally associated CpG loci in relation to early-gestation maternal urinary total arsenic concentration, the authors observed evidence of enrichment for hypomethylated loci among boys.

A study of moderate- to high-dose prenatal arsenic exposure was also conducted by Rojas et al. to evaluate maternal urinary total arsenic concentration in relation to umbilical cord blood DNA methylation among 38 mother-child dyads from the Biomarkers of Exposure to Arsenic (BEAR) birth cohort study in Mexico [50•]. Maternal urinary total arsenic concentration at the time of delivery was the primary measure of in utero arsenic exposure (median (range)=32.57 µg/L (6.2–319.7)) [58]. There were 4771 differentially methylated CpG sites (34 % hypomethylated and 66 % hypermethylated) associated with maternal urinary total arsenic concentration based on FDR<0.05. Among the arsenic-related differentially methylated CpG sites, there was evidence of enrichment for 3′UTR and gene body regions. Corresponding gene expression data were also evaluated for the 38 umbilical cord blood samples, and only weak correlations were observed for a subset of arsenic-associated CpG loci with mRNA transcript levels. The subset of CpG probes associated with gene expression changes were subsequently evaluated in relation to birth outcomes, with associations observed for gestational age, placental weight, and head circumference; the subset of genes associated with both differential DNA methylation and gene expression were also observed to be enriched for transcription factor binding sites compared to genes with altered expression but no correlation with DNA methylation.

Table 1 Recent studies examining the relationship between arsenic exposure and DNA methylation using the Illumina 450K array

Study	Sample size	Country	Arsenic assessment	DNA methylation assessment	Differentially methylated CpG loci
Koestler et al. [49•]	134 mother-child dyads	USA	Maternal urinary total arsenic concentration at 24–28 weeks gestation	Umbilical cord blood	NS
Kile et al. [48•]	44 mother-child dyads	Bangladesh	Maternal well water arsenic concentration at ≤16 weeks gestation	Umbilical cord blood	cg0498691 ($P=5.89\times10^{-8}$, FDR=0.021)
Rojas et al. [50•]	38 mother-child dyads	Mexico	Maternal urinary total arsenic concentration at time of delivery	Umbilical cord blood	cg16754643 ($P=8.72\times10^{-9}$; FDR=0.002)[a] cg16039651 ($P=1.58\times10^{-8}$; FDR=0.002) cg03553613 ($P=2.44\times10^{-8}$; FDR=0.002) cg02193513 ($P=2.96\times10^{-8}$; FDR=0.002) cg25605771 ($P=3.30\times10^{-8}$; FDR=0.002) cg05549970 ($P=4.02\times10^{-8}$; FDR=0.002) cg01040719 ($P=4.77\times10^{-8}$; FDR=0.002) cg13582500 ($P=7.02\times10^{-8}$; FDR=0.003) cg19788417 ($P=8.76\times10^{-8}$; FDR=0.003) cg21969548 ($P=9.81\times10^{-8}$; FDR=0.003)
Broberg et al. [51•]	127 mother-child dyads	Bangladesh	Maternal urinary total arsenic concentration at 5–14 and 26–36 weeks gestation	Umbilical cord blood	Early-gestation exposure for boys cg15255455 ($P=1.3\times10^{-7}$; FDR=0.031) cg13659051 ($P=1.3\times10^{-7}$; FDR=0.031) cg17646418 ($P=2.3\times10^{-7}$; FDR=0.036)
Liu et al. [55•]	45 adults	USA	Toenail arsenic concentration	Whole blood	NS
Seow et al. [53•]	20 adults	Bangladesh	Incident arsenical skin lesion status	Whole blood	NS
Argos et al. [56•]	400 adults	Bangladesh	Blood arsenic and urinary total arsenic concentrations	Whole blood	cg04605617 ($P=3.40\times10^{-11}$)[b] cg01225779 ($P=2.37\times10^{-9}$) cg06121226 ($P=1.16\times10^{-8}$) cg13651690 ($P=9.16\times10^{-8}$)

NS no significant loci after accounting for multiple comparisons

[a] Only the top 10 of 4771 differentially methylated loci based on FDR<0.05 are presented

[b] Associations surpassing Bonferroni significance threshold in relation to urinary total arsenic concentration summarized

Future Research Directions

There are notable differences across these studies making them challenging to synthesize; however, these differences also raise important issues for further exploration in future research. The studies varied with respect to the window of prenatal arsenic exposure assessment, with the study by Broberg et al. [51•] evaluating both early- and late-gestation exposures and suggesting that early prenatal exposure may have stronger effects on cord blood methylation. The studies also varied in the arsenic exposure levels of the study populations, which ranged from low- to moderate- and high-dose exposures. Additional studies will help to determine whether differentially methylated loci associated with low-dose exposure can be replicated in populations with high-dose exposure and vice versa. The study by Broberg et al. [51•] was the only analysis to present results stratified by sex, suggesting there may be important sex-specific differences in DNA methylation alterations, which should be systematically explored in future studies. Koestler et al. [49•] and Kile et al. [48•] observed an immunotoxic effect of prenatal arsenic exposure. Although the effect of arsenic on altered leukocyte cell types was estimated to explain a small percentage of the variability in methylation observed in those studies and is not believed to explain the association results observed, future studies should consider this issue as a source of potential confounding in DNA methylation analyses. Finally, a major innovation in the study by Rojas et al. [50•] was to evaluate the association of differentially methylated loci with gene expression, enrichment for transcription factor binding sites, and birth outcomes, providing additional mechanistic support through functional evidence and disease risk for the identified loci.

Arsenic and White Blood Cell DNA Methylation in Adults

Three epigenome-wide association studies have evaluated arsenic exposure in relation to white blood cell DNA methylation in adults using the Illumina 450K platform. In a prospective study of low-dose arsenic exposure, Liu et al. evaluated toenail arsenic concentration in relation to white blood cell DNA methylation 13 years later among 45 participants from the Coronary Artery Risk Development in Young Adults (CARDIA) study [55•]. Toenail arsenic concentration, a biomarker of longer-term arsenic exposure, was the main arsenic exposure measure of interest, with individuals sampled from the lowest (<0.0649 μg/g) and highest (≥0.1442 μg/g) quartiles of exposure for this study. No statistically significant associations were observed based on a strict Bonferroni P value threshold. The effect of arsenic on white blood cell proportions using the Houseman et al. statistical method [30•] was evaluated and did not reveal any significant associations with cell type proportions, although there was a modest positive association of arsenic exposure with CD8$^+$ T lymphocyte proportions, as reported previously by others [48•, 49•].

Seow et al. conducted a prospective study among 10 incident skin lesion cases and 10 controls among adults in Bangladesh [53•]. Skin lesion cases were defined as the presence of at least one type of arsenical skin lesion. DNA methylation was measured at both baseline (2001–2003) and follow-up (2009–2011) in each study participant to identify DNA methylation changes associated with incident skin lesions based on percent methylation difference between the baseline and follow-up assessments. No differentially methylated loci were identified, after accounting for multiple comparisons, likely due to the small sample size of the study.

In the largest study to evaluate arsenic toxicity in relation to epigenome-wide DNA methylation to date, Argos et al. evaluated blood arsenic and urinary total arsenic concentrations in relation to white blood cell DNA methylation among 400 Bangladeshi adults with manifest arsenical skin lesions in cross-sectional analyses [56•]. Blood arsenic (mean=9.3 μg/L, standard deviation (SD)=11.3 μg/L) and urinary total arsenic (mean=302 μg/g, SD=364.5 μg/g) concentrations were the primary measures of arsenic exposure in the study. Statistically significant associations based on a Bonferroni significance threshold ($P < 1 \times 10^{-7}$) were observed for *PLA2G2C* cg04605617, *SQSTM1* (*p62*) cg01225779, *SLC4A4* cg06121226, and *IGH* cg13651690. A subset of these methylation loci were replicated in an independent study sample. Additionally, urinary total arsenic concentration was significantly inversely associated with proportions of CD4$^+$ T lymphocytes and natural killer T cells when comparing the highest versus lowest quartiles of exposure, based on the Houseman et al. statistical method [30•]. There was evidence of enrichment for arsenic-related differentially methylated loci in ocean (isolated CpG loci in the genome) and CpG island shore regions (within 2 kb from a CpG island). The authors also evaluated the correlation of the top differentially methylated loci in relation to peripheral blood mononuclear cell gene expression and observed evidence of possible methylation-related gene regulation for a subset of these differentially methylated loci.

Future Research Directions

There are several important research considerations for future evaluations of arsenic toxicity in relation to DNA methylation alterations in adults. The study by Argos et al. [56•] was a cross-sectional analysis, whereas Liu et al. [55•] and Seow et al. [53•] took longitudinal approaches to evaluate arsenic toxicity with respect to DNA methylation alterations. Future studies should consider repeated measure analyses to evaluate the persistence of arsenic-related DNA methylation alterations. These studies all examined DNA methylation alterations in blood. The extent to which DNA methylation patterns in blood correlate with the target tissue and prove to be

useful markers of disease risk or early markers of disease detection needs to be further explored [59]. Only one previous study by Yang et al. [57] evaluated epigenome-wide DNA methylation alterations in target tissue. Therefore, future studies should be designed to evaluate not only DNA methylation alterations in target organ tissues but also whether blood-based differentially methylated loci are associated with risk of arsenic-related diseases.

Research Considerations

With the emergence of epigenetic epidemiology, several research considerations are outlined for future studies evaluating arsenic epigenetics, which highlight many of the successes as well as limitations of the existing epigenome-wide DNA methylation studies evaluating arsenic toxicity.

One of the major concerns for the advancement of arsenic epigenetics and epigenetic epidemiology in general has been the interpretation of DNA methylation signals from human tissues due to heterogeneous cellular composition. This issue has been widely discussed in the literature [60]. The implications for arsenic epigenetics are such that if arsenic exposure is associated with shifts in cell types, in either blood or other tissues, then observed associations between arsenic and DNA methylation may be confounded if cellular heterogeneity is not taken into account [61]. Although arsenic-related cell type shifts in blood have been estimated to account for a small proportion of the overall variability in DNA methylation [49•], statistical methods have been proposed to account for cellular heterogeneity [30•, 62•, 63] and should be employed in future arsenic epigenetic studies to improve interpretation of study findings.

The vast majority of epigenome-wide association studies have evaluated the effect of arsenic on cord or venous blood DNA methylation. The results of blood-based studies should be interpreted cautiously since it is not established whether arsenic-related DNA methylation patterns are also present in target organ tissues. To address this, future studies should evaluate arsenic in relation to the methylome of target tissues when biological specimens are accessible. Additionally, studies examining DNA methylation alterations in white blood cells can be enhanced by evaluating differentially methylated loci in relation to arsenic-related disease outcomes in order to generate evidence on whether a differentially methylated locus identified in blood represents a biomarker of exposure or biomarker of early biological effect.

Existing epigenome-wide association studies of arsenic have been conducted in study samples with wide-ranging arsenic exposure. It is not well-established whether DNA methylation patterns observed in populations with low-dose arsenic exposure are the same as methylation patterns observed in moderate- and high-dose exposures. Additionally, future studies may shed light on the biological impacts of timing of arsenic exposure, whether differentially methylated regions vary if exposure is during in utero life, childhood, adolescence, or adulthood. Aging has an important effect on DNA methylation [64]; therefore, susceptibility of the methylome to arsenic exposure may dramatically vary by timing of arsenic exposure. Furthermore, it is not known to what extent DNA methylation alterations persist after arsenic exposure has been remediated. It will be particularly interesting for future studies to examine changes in methylation patterns among individuals with changes in arsenic exposure through prospective studies with repeated measures.

Many of the previous epigenome-wide DNA methylation studies in relation to arsenic presented findings from small study samples, which were not adequately powered to detect modest methylation effects. Studies with relatively larger sample sizes are needed to discover novel differentially methylated loci associated with arsenic as well as to replicate observed methylation signals.

Finally, epidemiologic studies are increasingly measuring multiple types of molecular or "omic" data that can be combined in order to conduct integrative molecular studies of DNA methylation and gene expression. When possible, future studies should evaluate the functional relevance of differentially methylated loci on corresponding gene expression, as was undertaken by Rojas et al. [50•] and Argos et al. [56•] to show that a subset of differentially methylated loci were associated with gene expression, which increases confidence that the identified loci might be related to disease development.

Conclusions

Epigenome-wide DNA methylation studies are a promising discovery-based approach for the identification of novel genes and pathways that may be associated with arsenic toxicity. An overview of the existing literature as well as several considerations for future studies has been presented that highlight the existing knowledge gaps in arsenic epigenetics as well as address the limitations of the existing studies. Future studies should integrate functional assessments of DNA methylation alterations as well as characterize disease risk associated with differentially methylated regions.

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