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An Emerging Role for Epigenetic Dysregulation in Arsenic Toxicity and Carcinogenesis

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Abbreviations: AHCY, S-adenosylhomocysteine hydrolase; APL, acute promyelocytic leukemias; As, inorganic arsenic; AS3MT, arsenic (+3 oxidation state) methyltransferase; ChIP-on-chip, chromatin immunoprecipitation-on-chip; ChIP-seq, chromatin immunoprecipitation-sequencing; DEFB1, defensin, beta 1; DNMTs, DNA methyltransferases; H3K4me3, H3K4 tri-methylation; H3K9me2, H3K9 di-methylation; H3K27me3, H3K27 tri-methylation; HATs, histone acetyltransferases; HDACs, histone deacetylases; MAT1A, methionine adenosyltransferase I, alpha; miRNA, microRNAs; MMA, DMA and TMA, mono-, di- and trimethylated arsenicals; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; PBL, peripheral blood leukocytes; SAH, S-adnosyl-homocysteine; and SAM, S-adenosyl methionine.

Abstract

Objective: Exposure to arsenic, an established human carcinogen, through consumption of highly contaminated drinking water, is a worldwide public health concern. Multiple mechanisms by which arsenical compounds induce tumorigenesis have been proposed including oxidative stress, genotoxic damage and chromosomal abnormalities. Recent studies have suggested that epigenetic mechanisms may also mediate toxicity and carcinogenicity resulting from arsenic exposure. Our objective was to examine the evidence supporting the roles of the three major epigenetic mechanisms, DNA methylation, histone modification and microRNA expression, in arsenic toxicity, and in particular carcinogenicity. A further goal was to identify future research directions necessary to clarify epigenetic and other mechanisms in humans.

Data sources and synthesis: We conducted a PubMed search of arsenic exposure and epigenetic modification through April, 2010, and summarized the *in vitro* and *in vivo* research findings, both from our group and others, on arsenic-associated epigenetic alteration and its potential role in toxicity and carcinogenicity.

Conclusions: Arsenic exposure has been shown to alter methylation level of both global DNA and promoter of genes, histone acetylation, methylation, and phosphorylation, and microRNA expression in studies mainly analyzing a limited number of epigenetic endpoints. Systematic epigenomic studies in human populations exposed to arsenic or in arsenic-associated cancer have not yet been performed. Such studies would help to elucidate the relationship between arsenic exposure, epigenetic dysregulation and carcinogenesis and are becoming feasible due to recent technological advancements.

Background on Arsenic and Its Genotoxic Effects

The International Agency for Research on Cancer classified arsenic, a toxic metalloid, as a group 1 carcinogen more than 20 years ago (IARC 1987). It is widely accepted that exposure to arsenic is associated with lung, bladder, kidney, liver, and non-melanoma skin cancers (IARC 2004; Pershagen 1981; Smith et al. 1992; Smith and Steinmaus 2009). High levels of arsenic have also been associated with the development of multiple other diseases and deleterious health effects in humans, such as skin lesions (dyspigmentation, keratosis); peripheral vascular diseases; reproductive toxicity; and neurological effects (Abernathy et al. 1999).

Exposure to arsenic typically results from either oral arsenic consumption through contaminated drinking water, soil and food, or arsenic inhalation in an industrial work setting. Arsenic contaminated drinking water has been associated with increased mortality of bladder and lung cancer in Chile (Marshall et al. 2007), and increased mortality of both non-cancerous causes and cancers in Bangladesh (Sohel et al. 2009). In the human arsenic metabolic pathway, inorganic pentavalent As (AsV) is converted to trivalent As (As^{III}), with subsequent methylation to mono- and dimethylated arsenicals (MMA, DMA) (Drobna et al. 2009). The general scheme is as follows:

$$As^{V}O_4^{3-} + 2e \rightarrow As^{III}O_3^{3-} + Me^{+} \rightarrow MMA^{V}O_3^{2-} + 2e \rightarrow MMA^{III}O_2^{2-} + Me^{+} \rightarrow$$

$$DMA^{V}O_2^{-} + 2e \rightarrow DMA^{III}O^{-}$$

It is largely agreed that methylated arsenicals, especially MMA^{III}, are more toxic than inorganic As^{III} both *in vivo* (in animals) (Petrick et al. 2001) and *in vitro* (human cells lines) (Styblo et al. 2002). Multiple mechanisms by which arsenical compounds induce

tumorigenesis have been proposed including oxidative stress (Kitchin and Wallace 2008), genotoxic damage and chromosomal abnormalities (Moore et al. 1997a; Zhang et al. 2007a), co-carcinogenesis with other environmental toxicants (Rossman et al. 2004), as well as epigenetic mechanisms, in particular, altered DNA methylation (Zhao et al. 1997).

It is generally believed that arsenic does not induce point mutations, based on negative findings in both bacterial and mammalian mutagenicity assays (Jacobson-Kram and Montalbano 1985; Jongen et al. 1985). Arsenic does induce deletion mutations but arsenical compounds vary in their potency (Moore et al. 1997b). With respect to arsenic's ability to induce chromosomal alterations in humans, studies in the early 90's showed that the cell micronucleus assay could be used as a biological marker of genotoxic effect of arsenic exposure (Smith et al. 1993). Later studies validated this assay and demonstrated higher frequencies of micronuclei in individuals who were chronically exposed to arsenicals (Moore et al. 1997a). Analysis of chromosomal alterations in the bladder tumor DNA of 123 patients, who had been exposed to arsenic in drinking water, showed that tumors from patients with higher estimated levels of arsenic exposure had higher levels of chromosomal instability than tumors from patients with lower estimated levels of exposure, suggesting that bladder tumors from arsenic-exposed patients may behave more aggressively than tumors from unexposed patients (Moore et al. 2002). Based on these findings overall, a plausible and generally accepted mechanism for arsenic carcinogenicity is the induction of structural and numerical chromosomal abnormalities through indirect effects on DNA. However, as has been demonstrated for multiple tumors, including urothelial and hematological malignancies (Fournier et al. 2007; Muto et al. 2000), it is likely that inter-related genetic and epigenetic

mechanisms together contribute to the toxicity and carcinogenicity of arsenic (Hei and Filipic 2004; Zhao et al. 1997).

Epigenetic Modifications Induced by Arsenic

Epigenetic alteration, which is not a genotoxic effect, leads to heritable phenomena that regulate gene expression without involving changes in the DNA sequence (Feinberg and Tycko 2004), and thus could be considered a form of potentially reversible DNA modification. Recent mechanistic studies of arsenic carcinogenesis have directly or indirectly shown the potential involvement of altered epigenetic regulation in gene expression changes induced by arsenic exposure. We recently showed that urinary defensin, beta 1(DEFB1) protein levels were significantly decreased among men highly exposed to arsenic in studies conducted in Nevada, U.S. and in Chile (Hegedus et al. 2008). DNA methylation is thought to play a role in regulating *DEFB1* expression (Sun et al. 2006). Follow-up studies are underway in our laboratory to determine if reduced levels of *DEFB1* in exposed populations are due to arsenic-induced targeted gene silencing. Several studies have observed extensive changes in global gene expression in individuals following arsenic exposure (Andrew et al. 2008; Bailey et al. 2009; Bourdonnay et al. 2009; Xie et al. 2007). Further, maternal exposure to arsenic has been shown to alter expression of transcripts in the mouse fetus (Liu et al. 2008) and human newborn (Fry et al. 2007). Since epigenetic processes are major regulators of gene expression, these findings suggest that dysregulation of epigenetic processes could contribute mechanistically to arsenic-induced changes in gene expression and cancer, affecting both people exposed to arsenic directly and those of future generations in a heritable manner, without directly altering the genome. Dysregulation of epigenetic processes could also

contribute to vascular disease (Yan et al. 2010) and neurological disorders (Urdinguio et al. 2009).

Many groups have directly examined the association of arsenic exposure on epigenetic phenomena, and as the technologies used to study the various epigenetic modifications are developing rapidly, we believe that a review of current findings from the literature is warranted. While epigenetic alterations may contribute to effects of arsenic on both cancer and non-cancer outcomes, this perspective summarizes the recent *in vitro* and *in vivo* research findings on the potential role of arsenic-mediated epigenetic alterations in arsenic-induced toxicity and carcinogenicity. Three major epigenetic mechanisms proposed to play roles in arsenic-induced carcinogenesis are discussed: altered DNA methylation, histone modification, and microRNA (miRNA) expression. Future directions that can further inform our understanding of the epigenetic and overall mechanisms underlying the effects of arsenic are also proposed.

Arsenic Exposure and DNA Methylation

Introduction

DNA methylation is tightly regulated in mammalian development and is essential for maintaining the normal functioning of the adult organism (Schaefer et al. 2007). Altered DNA methylation has been associated with multiple human diseases (Robertson 2005). Global genomic DNA hypomethylation is a hallmark of many types of cancers (Esteller et al. 2001), resulting in illegitimate recombination events and causing transcriptional deregulation of affected genes (Robertson 2005). In mammalian systems, DNA methylation occurs

predominantly in cytosine-rich gene regions, known as CpG islands, and serves to regulate gene expression and maintain genome stability (Yoder et al. 1997). DNA methyltransferases (DNMTs) are responsible for transferring a methyl group from the S-adenosyl methionine (SAM) cofactor to the cytosine nucleotide, producing 5-methylcytosine and s-adenosyl homocysteine (Figure 1) (Razin and Riggs 1980). Three different families of *DNMTs* have been identified so far, namely, *DNMT1*, *2* and *3* (Robertson and Wolffe 2000).

Mechanisms of arsenic-induced changes in DNA methylation

An association between arsenic-induced carcinogenesis and DNA methylation was proposed because arsenic methylation and DNA methylation both utilize the same methyl donor, SAM (Figure 1). SAM is a coenzyme involved in more than 40 metabolic reactions that require methyl group transfers (Chiang et al. 1996; Loenen 2006; Reichard et al. 2007). As SAM is the unique methyl group donor in each conversion step of biomethylation of arsenic, long term exposure to arsenic may lead to SAM insufficiency and global DNA hypomethylation (Coppin et al. 2008; Goering et al. 1999; Zhao et al. 1997). Further, as SAM synthesis requires methionine, an essential amino acid in humans, dietary methyl insufficiency could exacerbate effects of arsenic on DNA methylation (Figure 1) (McCabe and Caudill 2005). Indeed, human exposure to arsenic often occurs in relatively resource-poor populations in developing countries that also may have low dietary intakes of methionine (Anetor et al. 2007). In addition to its effect on SAM availability, arsenic can directly interact with DNMTs and inhibit their activities. Several studies have shown that arsenic exposure leads to a dose-dependent reduction of *DNMTs* mRNA levels and activity of DNMTs both *in vitro* and *in*

vivo, including *DNMT 1*, *DNMT 3A* and *DNMT 3B* (Ahlborn et al. 2008; Cui et al. 2006b; Fu et al. 2007; Reichard et al. 2007).

Arsenic and global DNA hypomethylation

Global DNA hypomethylation is expected to result from arsenic exposure through both SAM insufficiency and reduction of *DNMTs* gene expression (Reichard et al. 2007). Arsenic exposure has been reported to induce DNA hypomethylation *in vitro* and in animal studies, as summarized in Table 1. For example, rats (Uthus and Davis 2005) and mice (Chen et al. 2004; Okoji et al. 2002; Xie et al. 2004) exposed to As^{III} for several weeks displayed global hepatic DNA hypomethylation. Similarly, exposure of fish to As^{III} for 1, 4, and 7 days resulted in sustained DNA hypomethylation compared to non-exposed fish (Bagnyukova et al. 2007). Studies in cell lines *in vitro* yielded similar results, with a dose-dependent reduction in global genomic DNA methylation resulting from As^{III} exposure (Table 1) (Benbrahim-Tallaa et al. 2005; Chen et al. 2004; Coppin et al. 2008; Reichard et al. 2007; Sciandrello et al. 2004; Zhao et al. 1997). In contrast to the animal and *in vitro* findings, there are limited human population studies available. A cross-sectional study of 64 people reported by Dr. Majumdar et al. indicated that people exposure to arsenic contaminated water (250 to 500 μg/L) was associated with a global DNA hypermethylation (Majumdar et al. 2009). However, the participants in the highest estimated exposure group (>500 μg/L) had methylation levels that were comparable to those in the two lowest groups. The one possible reason for this inconsistency may because the actual intake of arsenic into the body is different in the participants whose exposures were estimated based on the concentrations in their drinking water. Another well designed study published in 2007 assessed the relationship between

arsenic and DNA methylation in a cross-sectional study with 294 participants (Pilsner et al. 2007). They reported that a positive association was observed between urinary arsenic and DNA hypermethylation in the study population. Plasma folate level apparently has a significant effect on the level of DNA methylation since a dose-response relation was evident only among participants with adequate folate levels (≥9 nmol/L) when estimates were stratified according to plasma folate level after control for other factors. In a separated but closely related cross sectional study, the authors found that individuals with hypomethylation of PBL DNA were 1.8 (95% CI, 1.2–2.8) times more likely of having skin lesions two years later after adjusting for age, urinary As and other factors (Pilsner et al. 2009). The authors speculated that "*adequate folate may be permissive for an adaptive increase in genomic methylation of PBL DNA associated with As exposure, and that individuals who are similarly exposed but in whom the increase in genomic DNA methylation does not occur (or cannot be sustained) are at elevated risk for skin lesions*". Further studies are required to determine if exposure to As^{III} has differential effects on the status of DNA methylation across tissues, cells, and species.

Arsenic and gene promoter methylation

While the effects of arsenic exposure on global genomic DNA methylation remain unclear, DNA hypo- or hyper-methylation of promoters of some genes has been reported in human skin cancer (Chanda et al. 2006) and bladder cancer (Chen et al. 2007; Marsit et al. 2006c) associated with arsenic exposure. It also observed in human cell lines (Chai et al. 2007; Fu and Shen 2005; Jensen et al. 2008; Mass and Wang 1997), animal cell lines (Chen et al. 2001, Takahashi et al. 2002), animals (Cui et al. 2006; Okoji et al. 2002; Waalkes et al. 2004) and humans (Chanda et al. 2006; Chen et al. 2007; Marsit et al. 2006b; Zhang et al. 2007b)

exposed to arsenic (Table 2). While this gene specific effect observed in these studies could be due to the study bias in which researchers only examined a small group of genes, the repeatedly reported similar methylation pattern in the same genes after arsenic exposure might be also suggest that arsenic could selectively target specific genes. However, little is known about how DNA methylation is targeted to specific regions (Jones and Baylin 2002). Hypo- and hyper-methylation of genes could mediate carcinogenesis through up-regulation of oncogene expression or down-regulation of tumor suppressor genes, respectively. Both observations have been reported. Hypomethylation of the promoter region of oncogenic *Hras1* and an elevated *Hras1*mRNA level was demonstrated in mice treated with sodium arsenite (Okoji et al. 2002). Similar results on the mRNA expression and promoter hypomethylation of *Hras1* and *c-myc* were also observed *in vitro* (Chen et al. 2001; Takahashi et al. 2002). The evidence has linked over-expression of *Esr1* gene with estrogen-induced hepatocellular carcinoma in mice (Couse et al. 1997). Arsenic exposure leads to over-expression of the *Esr1*gene resulting from hypomethylation of its promoter region, indicating an association between over-expression of *Esr1* and arsenic hepatocarcinogenesis (Chen et al. 2004; Waalkes et al. 2004).

Dose-dependent hypermethylation at the promoter region of several tumor suppressor genes (*p15*, *p16*, *p53 and DAPK* etc) was induced by arsenic exposure *in vitro* and *in vivo* (Boonchai et al. 2000; Chanda et al. 2006; Fu and Shen 2005; Mass and Wang 1997; Zhang et al. 2007b). In a population-based study of human bladder cancer in 351 patients, *RASSF1A* and *PRSS3* promoter hypermethylation was positively associated with toenail arsenic concentrations, and promoter hypermethylation in both genes also was associated with

invasive (versus non-invasive low grade) cancer (Marsit et al. 2006b), an outcome recapitulated in arsenic-induced lung cancer in A/J mice, in which the arsenic exposure reduced the expression of *RASSF1A* resulting from hypermethylation of its promoter region and was associated with arsenic-induced lung carcinogenesis (Cui et al. 2006a). *DAPK* is a positive mediator of gamma-interferon induced programmed cell death and tumor suppressor candidate. A study analyzed 38 patients with urothelial carcinoma, and the author reported that hypermethylation of *DAPK* gene was observed in 13 of 17 tumors in patients living in arsenic-contaminated areas when compared with 8 of 21 tumors from patients living in arsenic non-contaminated areas (Chen et al. 2007). This hypermethylation of *DAPK* gene was observed *in vitro* study too when the immortalized human uroepithelial cells were exposed to arsenic (Chai et al. 2007). The increase of DNA hypermethylation of promoter in *p16* gene was observed in arseniasis patients when compared to people with no history of arsenic exposure (Zhang et al 2007b). In another study which examined the methylation status of promoters in *p53* and *p16* in DNA extracted from peripheral lymphocytes, an increase of methylation in both *p53* and *p16* genes was observed and associated with an estimated arsenic exposure in a dose dependent manner. However, this same study also showed that the subjects from the highest arsenic exposure group exhibited a hypomethylation of both *p53* and *p16* genes (Chanda et al. 2006). Chronic exposure to arsenic *in vitro* has been shown to induce malignant transformation in several human cell types (Benbrahim-Tallaa et al. 2005; Zhao et al. 1997), in which the alteration of DNA methylation level has been shown to be involved (Jensen et al. 2008; Jensen et al. 2009a; Zhao et al. 1997).

Summary

Arsenic does not fall into the classic model of carcinogenesis, as it is not efficient at inducing point mutations or initiating and promoting the development of tumors in experimental animals, one likely mechanism by which arsenicals operate is through the disruption of normal epigenetic control at specific loci, which may result in aberrant gene expression and cancer (Andrew et al. 2008; Xie et al. 2007). Though there is increasing evidence that arsenic exposure alters methylation levels in both global DNA and promoter of some genes, the current available studies are essentially descriptive, and difficult to interpret due to the complexity of the study populations and limited information provided in the papers. Studies are needed that systematically investigate DNA methylation on a genome-wide level in arsenic exposed cell lines and in target tissues, such as exfoliated bladder cells, from well characterized arsenic exposed human populations, or in tumor tissue from arsenic-associated cancers. Such studies would help to clarify potential effects of arsenic exposure on DNA methylation and carcinogenesis.

Arsenic Exposure and Histone Modification

Introduction

Chromatin is structured within the cell nucleus in units called nucleosomes, in which DNA is packaged within the cell. The nucleosome core particle consists of stretches of DNA (~146bp) wrapped in left-handed superhelical turns around a histone octamer consisting of 2 copies each of the core histones H2A, H2B, H3, and H4 (Luger et al. 1997). While H1 does not make up the nucleosome "bead", H1 plays a role in keeping in place the DNA that has wrapped around the nucleosome (Figure 2). From a structural and functional perspective, histones have different characteristics depending on the number of amino acids and the number and

type of covalent modifications in these residues. These covalent modifications are found in the tails of the histone chains and include acetylation, methylation, phosphorylation, citrullination, ubiquitination, sumoylation, ADP ribosylation, deimination and proline isomerization (Kouzarides 2007) (Figure 2) and influence many fundamental biological processes. To date, published studies on histone modifications and arsenic toxicity have focused on acetylation, methylation and phosphorylation.

Histone acetylation

Histone acetylation is a dynamic and reversible event (Glozak and Seto 2007), in which the acetylation status of lysine residues in the histone tail are regulated by two antagonistic enzyme classes, histone acetyltransferases (HATs) (Sterner and Berger 2000) and histone deacetylases (HDACs) (Cress and Seto 2000). Utilizing acetyl-coenzyme A (acetyl-CoA) as an acetyl group donor, HATs enzymatically transfer a single acetyl group to the ε-amino group of specific lysine side chains within the histone's basic N-terminal tail region, while HDACs remove the acetyl group from the lysine residues.

Evidence for an association between altered histone acetylation and arsenic-induced toxicity continues to be strengthened. In the early 1980's, arsenic exposure was shown to significantly reduce histone acetylation in Drosophila (Arrigo 1983). More recently, changes in histone H3 acetylation has observed in association with As^{III} and MMA^{III} induced malignant transformation of human urothelial cells in vitro, and these modifications apparently are arsenic specific because the co-occurring changes in both As^{III} and MMA^{III} induced malignant transformation are significantly higher than by random chance (Jensen et al. 2008). Further,

DNA hypermethylation was identified in a number of the hypoacetylated promoters identified in the study, suggesting that arsenic coordinately targets genes through dysregulation of different epigenetic mechanisms contributing to malignant transformation (Jensen et al. 2008). Recently, we showed that the global level of H4K16 acetylation in human bladder epithelial cells was reduced in a dose- and time-dependent manner by both As^{III} and MMA^{III} treatment. Moreover, knockdown of *MYST1*, the gene responsible for H4K16 acetylation, resulted in an increased cytotoxicity to arsenical exposure in human bladder epithelial cells, suggesting that H4K16 acetylation may be important for resistance to arsenic-induced toxicity (Jo et al. 2009).

Interestingly, As^{III} exposure has also been shown to induce elevated histone acetylation, which was reportedly responsible for the up-regulation of genes involved in apoptosis or the response to cell stress after exposure to arsenic (Li et al. 2002; Li et al. 2003). This result probably is mediated by HDACs. It has been demonstrated that As^{III} inhibits *HDACs* that correlates with increased global histone acetylation (Ramirez et al. 2008). The level of inhibition is comparable to that of the well-known *HDACs* inhibitor trichostatin A (Drummond et al. 2005). Together, these studies clearly provide evidence that histone acetylation is dysregulated by arsenic exposure, but further work needs to be done to understand the underlying mechanisms and to clarify the net effect of altered histone acetylation on arsenic-induced toxicity and carcinogenesis.

Histone methylation

Like acetylation, histone methylation is also a reversible process. However, unlike acetylation, which occurs only on lysine residues at the histone tail, histone methylation occurs on both lysine and arginine residues (Martin and Zhang 2005; Wysocka et al. 2006). In mammals, histone methylation is usually found on histone H3 and H4, though it also occurs on H2A or H2B. Arginine methylation is catalyzed by the enzyme, arginine N-methyltransferase (Wysocka et al. 2006), while lysine methylation is catalyzed by two different classes of proteins, the SET-DOMAIN-containing protein family and the non-SET-domain proteins, DOT1/DOT1L (Martin and Zhang 2005). Histone methylation can occur in the mono-methyl, symmetrical di-methyl, asymmetrical di-methyl state, and in the tri-methyl group states, in contrast to the single acetyl group added during acetylation (Klose and Zhang 2007). Histone methylation was considered a static modification until recent years, when enzymes were found capable of antagonizing histone arginine methylation or directly removing a methyl group from a lysine residue of histone. These enzymes include peptidylarginine deiminase enzymes and amine oxidase and JmjC-domain-containing histone demethylase enzymes (Klose and Zhang 2007).

Accumulating evidence implicates the aberrant loss or gain of histone methylation in tumorigenesis (Schneider et al. 2002). In the early 1980's, Arrigo first reported that exposure to arsenic in Drosophila cells led to a complete abolishment of methylation of histones H3 and H4 (Arrigo 1983), and the effect on H3 was later confirmed by other investigators (Desrosiers and Tanguay 1986; Desrosiers and Tanguay 1988). The response to arsenic exposure in the mammalian cell is more complex, and As^{III} treatment can lead to differential effects on the methylation of H3 lysine residues, including increased H3K9 di-methylation (H3K9me2) and

H3K4 tri-methylation (H3K4me3) and decreased H3K27 tri-methylation (H3K27me3) (Zhou et al. 2008). A recent report showed that 1 μM arsenite significantly increased H3K4me3 after 24 hour and 7 days exposure in human lung carcinoma A549 cells. Importantly, the tri-methyl H3K4 remained elevated, apparently inherited through cell division, 7 days after the removal of arsenite (Zhou et al. 2009). Elevated H3K9me2, mediated by increased levels of histone methyltransferase G9a protein (Zhou et al. 2008), correlates with transcriptional repression (Peterson and Laniel 2004), and has been shown to be involved in the silencing of tumor suppressers in the cancer cell lines (Esteve et al. 2007; McGarvey et al. 2006). However, data on the patterns of histone methylation induced by arsenic exposure are limited and further studies are required to decipher the relationship between altered histone methylation and gene expression, as well as its effect on arsenic-induced carcinogenesis.

Histone phosphorylation

All four core histone proteins, H2A, H2B, H3 and H4 and the linker histone H1 can be post-translationally modified by phosphorylation. Cyclin dependent kinases (CDKs) are believed to be responsible for H1 phosphorylation (Swank et al. 1997). Multiple kinases are able to phosphorylate H2A and H2B, such as ataxia telangiectasia mutated (ATM) for H2AX, etc (Burma et al. 2001). Phosphorylation of H3 has been specifically implicated in cell cycle progression and regulation of gene expression (Houben et al. 2007). Similarly, phosphorylation of histone H4 (Serine 1) increases during the cell-cycle, and is believed to be regulated by casein kinase 2 (CSNK2) (Barber et al. 2004).

Histone phosphorylation may also contribute to arsenic-induced carcinogenesis. While all four core histones, H2A, H2B, H3, and H4, are targets of protein kinases (Peterson and Laniel 2004), the most well studied histone phosphorylation event is that of H2AX, a form of H2A which makes up to 25% of the total H2A pool in mammals. A recent study demonstrated that arsenic trioxide induces apoptosis by upregulation of phosphorylated H2AX and may be one of the mechanisms by which arsenic trioxide acts as an antineoplastic agent (Zykova et al. 2006) (Figure 2). Little is known about histone phosphorylation and arsenic carcinogenesis. Studies have suggested that H3 phosphorylation induced by arsenic exposure might be responsible for the up-regulation of oncogenes *c-fos and c-jun* (Li et al. 2003), and induction of a protoapoptotic factor, caspase 10 (Li et al. 2002). Another important metal with epigenetic effects, nickel, has been shown to induce phosphorylation of histone 3, specifically H3S10, via the activation of the JNK/SAPK pathway (Ke et al. 2008). As it is known that arsenite exposure activates JNK and p38/Mpk2 kinase by inhibition of the corresponding protein phosphatases (Cavigelli et al. 1996), phosphorylation of histone H3 via the JNK/SAPK pathway might be a common mechanism of metal-induced histone modification.

Summary

Different types of histone modifications have been shown to coordinately impact gene regulation and expression. For example, *WNT5A* gene expression is upregulated in As^{III} and MMA^{III} induced malignant transformation in uroepithelial cells in association with the enrichment of permissive histone modifications and reduction of repressive modifications in the *WNT5A* promoter region (Jensen et al. 2009b). Two histone modifications, di-methylation of lysine 4 and acetylation of lysine 9 and 14 of histone H3 are associated with transcriptional

competency while the other two modifications, tri-methylation of lysine 27 and di-methylation of lysine 9 of histone H3 are correlated with transcriptional repression (Peterson & Laniel 2004). Although we are still in the early stages of elucidating the association between histone modifications induced by arsenic and their effects on arsenic carcinogenicity, newly available techniques such as mass-spectrometry-based histone modification analysis and genome-wide sequencing offer the potential to systematically characterize the altered histone modifications induced by arsenicals and the subsequent changes in gene expression.

Arsenic Exposure and MiRNA Expression

Introduction

In the past several years, a small class of non-protein coding RNAs, called miRNA, which participate in diverse biological regulatory events and which are transcribed mainly from non-protein coding regions of the genome, has been discovered by several laboratories, (Bartel 2004; He and Hannon 2004). Over 700 human miRNAs have been identified to date as documented by the miRBase Database (Release 14) (miRBase 2009) and it is predicted that many more exist. Each miRNA is thought to target several hundred genes, and as many as 30% of mammalian genes are regulated by miRNA (Lewis et al. 2005). MiRNAs deactivate gene expression by binding to the 3' untranslated region of mRNA with incomplete base pairing (Wightman et al. 1993). The exact mechanisms by which expression is repressed are still under investigation but may include the inhibition of protein synthesis, the degradation of target mRNAs, and the translocation of target mRNAs into cytoplasmic processing bodies (P-bodies) (Jackson and Standart 2007). Due to the suppressive effect of miRNA on gene expression, a reduction or elimination of miRNAs that target oncogenes could result in the

inappropriate expression of those oncoproteins, for example, it has been shown that *RAS* oncogene is regulated by the let-7 miRNA family (Johnson et al. 2005). Conversely, the amplification or overexpression of miRNAs that have a role in regulating the expression of tumor suppressor genes could reduce the expression of such genes. A prime example of this is the observation of the miR-34 family on the p53 tumor suppressor pathway (He et al. 2007).

Altered miRNA expression and arsenic exposure

Despite the significant progress made towards understanding the biogenesis and mechanisms of action of miRNA, much less is known about the effect of environmental exposures, in particular carcinogens such as arsenic, on miRNA expression. Several studies have shown that exposure to exogenous chemicals can alter miRNA expression (Kasashima et al. 2004; Pogribny et al. 2007; Shah et al. 2007). *In vitro* exposure of cells to ROS-generating metal sulfates, iron- and aluminum-sulfate, led to the up-regulation of a specific set of miRNAs, including miR-9, miR-125b and miR-128 (Lukiw and Pogue 2007). ROS generation resulting from arsenic exposure is thought to play a large role in arsenic induced carcinogenesis and toxicity (Flora et al. 2007; Hei and Filipic 2004) and could potentially alter these miRNAs in a similar manner. In a recent study, Marsit and colleagues examined the roles that arsenic and folate deficiency play in miRNA expression (Marsit et al. 2006a). Human lymphoblast TK6 cells that had been treated with sodium arsenite and cells that had been grown in folate-deficient media over a six-day period, showed similarly altered expression of five miRNAs when compared to untreated controls, suggesting a common mechanism of dysregulation. One such potential mechanism is aberrant DNA methylation occurring as a result of SAM depletion (Caudill et al. 2001; Loenen 2006), which arises under conditions of arsenic

exposure and folate deficiency. However, the authors did not observe a significant decrease in global methylation between the treated and control groups, suggesting more subtle or targeted effects. The induced changes in miRNA expression were not stable and returned to baseline levels upon removal of the stress conditions, suggesting that chronic exposure may be necessary to permanently alter expression of miRNAs (Marsit et al. 2006a). Arsenic trioxide (As_2O_3), a treatment option for acute promyelocytic leukemia (APL) (Zhou et al. 2005), induces the re-localization and degradation of the nuclear body protein PML, as well as the degradation of PML-RARalpha in APL cells (Shao et al. 1998). APL patients treated with all-trans retinoic acid release a group of miRNAs transcriptionally repressed by the APL-associated PML-RAR oncogene (Saumet et al. 2009), suggesting that arsenicals may produce similar effects on miRNA expression in APL patients.

Summary

Overall, these studies show that environmental carcinogen exposures can lead to altered miRNA expression profiles, which may be associated with the process of carcinogenesis. Further studies are necessary to clarify whether chronic exposure to arsenic is capable of altering miRNA expression and what biological effects are related to the altered miRNA expression.

Epigenomic Approach Proposed for Future Studies

Emerging evidence suggests that arsenic acts through several epigenetic mechanisms. The characterization of genome-wide patterns of DNA methylation, post-translational histone modification, and miRNA expression following arsenic exposure *in vitro* and *in vivo*, represents a new frontier toward our understanding of the mechanisms of arsenic toxicity and

carcinogenesis. Emerging epigenomic technologies such as ChIP (chromatin immunoprecipitation)-on-chip and ChIP-sequencing (ChIP-seq), global methylation and miRNA microarrays, as well as whole genomic DNA sequencing platform will facilitate these efforts (Schones & Zhao, 2008). ChIP-on-chip or Chip-Seq is used primarily to determine how proteins interact with DNA, and has the potential to clarify how epigenetic changes, particularly histone modifications, induced by arsenic exposure, regulate gene expression (Park 2009). Mass spectrometry (MS) offers an unbiased approach to mapping the combinations of histone modifications and requires highly sensitive and precise mass measurements: the difference in mass between a trimethylation and acetylation is only 36 millidaltons, for example. Using LC-MS in a recent study, we identified acetylation of H4K16 as a histone modification that is significantly reduced post arsenic treatment, especially with long-term exposure (Jo et al. 2009).

With the rapid development of array and sequencing-based DNA-methylation profiling technologies, global DNA methylation profiling has clearly come of age. As epigenetic modifications do not alter gene sequence but rather, gene expression, transcriptomics may eventually allow the characterization of the expression profiles of epigenetically labile genes. Identification of the genes dysregulated through epigenetic mechanisms by arsenic exposure, will further elucidate the associated biological processes and disease states. Proteomics using both conventional “bottom-up” and newer cutting-edge “top-down” mass spectrometry approaches to detect labile posttranslational modifications that are often lost in conventional MS/MS experiments will allow further clarification of the resulting phenotype. The difference between these two approaches is that the materials introduced into the mass spectrometer are

either peptides generated by enzymatic cleavage of one or many proteins in “bottom-up” approach, or intact protein ions or large protein fragments in “top-down” approach. Integration of epigenetic, transcriptomic and proteomic datasets generated by these techniques will facilitate a more thorough understanding of the interplay of these processes under normal conditions and during arsenic exposure. Indeed, the importance of a comprehensive understanding of the epigenome has been recognized by the scientific community and is reflected in the NIH Roadmap Initiative established in 2007 with the goal of developing comprehensive reference epigenome maps and new technologies for comprehensive epigenomic analyses (NIH 2007).

Conclusion and Future Directions

While experiments in suitable model systems could complement the human studies, as discussed earlier, there may be differences between epigenetic effects in animals and humans, and between various tissues and cell types. Thus, studies in human populations exposed to high levels of arsenic will be necessary to understand how individual differences in arsenic methylation and genetic background, as well as environmental factors such as diet and age, influence the epigenetic response to chronic arsenic exposure. Studies will also be required across various tissue and cell types to identify and validate the levels and patterns of epigenetic markers in these cells. Accessible tissues such as blood may not represent a good surrogate of target tissues such as bladder, kidney, and lung. High-resolution methylation data have shown that tissues have distinct epigenetic profiles (Christensen et al.2009; Illingworth et al. 2008) and aging and environmental exposures may alter methylation in a tissue-specific manner (Christensen et al. 2009). Thus, epigenetic profiles from disease-relevant tissues such

as exfoliated bladder cells from exposed and unexposed disease-free individuals could allow early effects to be identified. Such cells could also be analyzed from individuals with arsenic- and non-arsenic associated cancers to identify arsenic-associated tumorigenic profiles. It was recently shown that it may be possible to detect bladder cancer using gene expression signatures in exfoliated bladder urothelia (Rosser et al. 2009). Similarly, the effects of inhaled arsenic on epigenetic profiles in bronchial airway epithelial cells could be examined in exposed and unexposed disease-free individuals and those with lung cancer, as was recently done using miRNA profiling for cigarette smoke exposure (Schembri et al. 2009).

In conclusion, a comprehensive epigenomic approach may elucidate the mechanisms of arsenic-induced carcinogenesis. Such an approach would also facilitate the discovery of biomarkers of arsenic exposure and early effect, associated disease and disease progression, as well as factors that confer susceptibility.

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Table 1. Arsenic Exposure and Global DNA Methylation

	Arsenicals	Dose	Time (Weeks)	Global DNA methylation	References
Human Cells					
Human prostate epithelial cell line RWPE-1	As^{III}	5 µM	16	Hypo	Coppin et al. 2008
Human prostate epithelial cell line RWPE-1	As^{III}	5 µM	29	Hypo	Benbrahim-Tallaa et al. 2008
Human HaCaT keratinocytes	As^{III}	0.2 µM	4	Hypo	Reichard et al. 2007
Animal Cells					
TRL 1215 rat liver epithelial cell line	As^{III}	125-500 nM	18	Hypo	Zhao et al. 1997
V79-Cl3 Chinese hamster cells	As^{III}	10 µM	8	Hypo	Sciandrello et al. 2004
Animal Studies					
Goldfish	As^{III}	200 µM	1	Hypo	Bagnyukova et al. 2007
Fisher 344 Rat	As^{III}	50 µg/G	12	Hypo	Uthus and Davis 2005
129/SvJ mice	As^{III}	45 ppm	49	Hypo	Chen et al. 2004
C3H mice	As^{III}	85 ppm	1.5	Hypo	Waalkes et al. 2004
C57BL/6J mice	As^{III}	2.6-14.6 µg/G	18.5	Hypo	Okoji et al. 2002
Homozygous Tg.AC mice	As^{III}	150 ppm	17	Hypo	Xie et al. 2004
	As^{V}	200 ppm			
	MMA^{V}	1500 ppm			
	DMA^{V}	1200 ppm			
Human Subjects*					
Human	As^{III}	2-250 µg/L	None[#]	Hyper	Pilsner et al. 2007 Majumdar et al. 2009
Human	As^{III}	2-250 µg/L	None[#]	Hypo (in Skin lesion patients)	Pilsner et al. 2009

* More information was provided in the main text. [#] Data not available

Table 2. Arsenic Exposure and Gene-Specific (promoter) Methylation Status

	Arsenicals	Dose	Time (Weeks)	Genes		References
				Hyper	Hypo	
Human Cells						
Human UROtsa cells	As^{III}	1 μM	9	*DBC1, FAM83A, ZSCAN12 & C1QTNF6*		Jensen et al. 2008
	MMA^{III}	50 nM				
Human uroepithelial SV-HUC-1 cells	As^{III}	2, 4, & 10 μM	24 or 52	*DAPK*		Chai et al. 2007
Human myeloma cell line U266	As^{III}	1 and 2 μM	0.4	*P16*		Fu and Shen 2005
Human lung adenocarcinoma A549 cells	As^{III}	0.08-2 μM	0.3	*P53*		Mass and Wang 1997
	As^{V}	30-300 μM	0.3			
Animal Cells						
Syrian hamster embryo cells	As^{III}	3-10 μM	0.3		*c-myc & c-Ha-ras*	Takahashi et al. 2002
	As^{V}	50-150 μM	0.3			
TRL 1215 rat liver epithelial cells	As^{III}	125-500 nM	8 or 18		*c-myc*	Chen et al. 2001
Animal Studies						
C57BL/6J mice	As^{III}	2.6-14.6μg/G	18.5		*c-Ha-ras*	Okoji et al. 2002
A/J mice	As^{V}	100 ppm	74	*p16 & RASSF1*		Cui et al. 2006
C3H mice	As^{III}	85 ppm	1.4		*ER-alpha*	Waalkes et al. 2004
Human Subjects						
Human	As^{III}	None*	None*	*DAPK*		Chen et al. 2007
Human	As^{III}	Variable[#]	None*	*p53&P16*		Chanda et al. 2006
Human	As^{III}	None*	None*	*p16*		Zhang et al. 2007b
Human	As^{III}	Variable^	None*	*RASSF1A & PRSS3*		Marsit et al. 2006b

* Data not available

[#] Study subjects were grouped based on historical arsenic concentration in drinking water, and the range of arsenic concentration in drinking water was from < 50μg/L to >300μg/L.

^ The toenail arsenic concentration of study subjects was estimated from <0.01μg/L to >50 μg/L.

Figure Legends

Figure 1, Simplified scheme of SAM synthesis and its involvement in arsenic and DNA methylation. Human arsenic metabolic pathway involves multiple steps and here shows the intermediate steps and metabolites.

Figure 2, Histone modifications affected by As^{III} and MMA^{III} exposure. The structure of a nucleosome is shown and major posttranscriptional histone modifications are listed on the left. Modifications of specific histone proteins that were reported in the literature as altered by arsenic exposure are listed on the right.

Figure 1

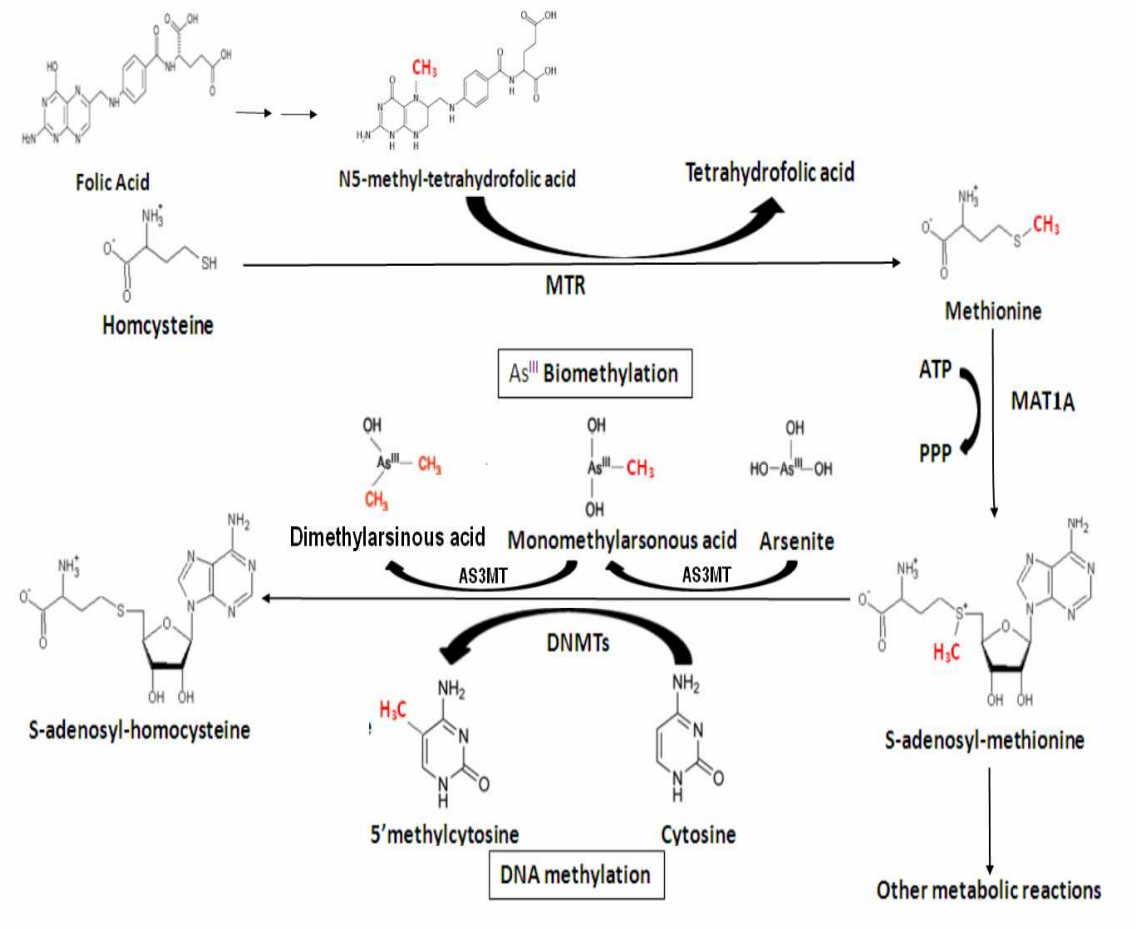
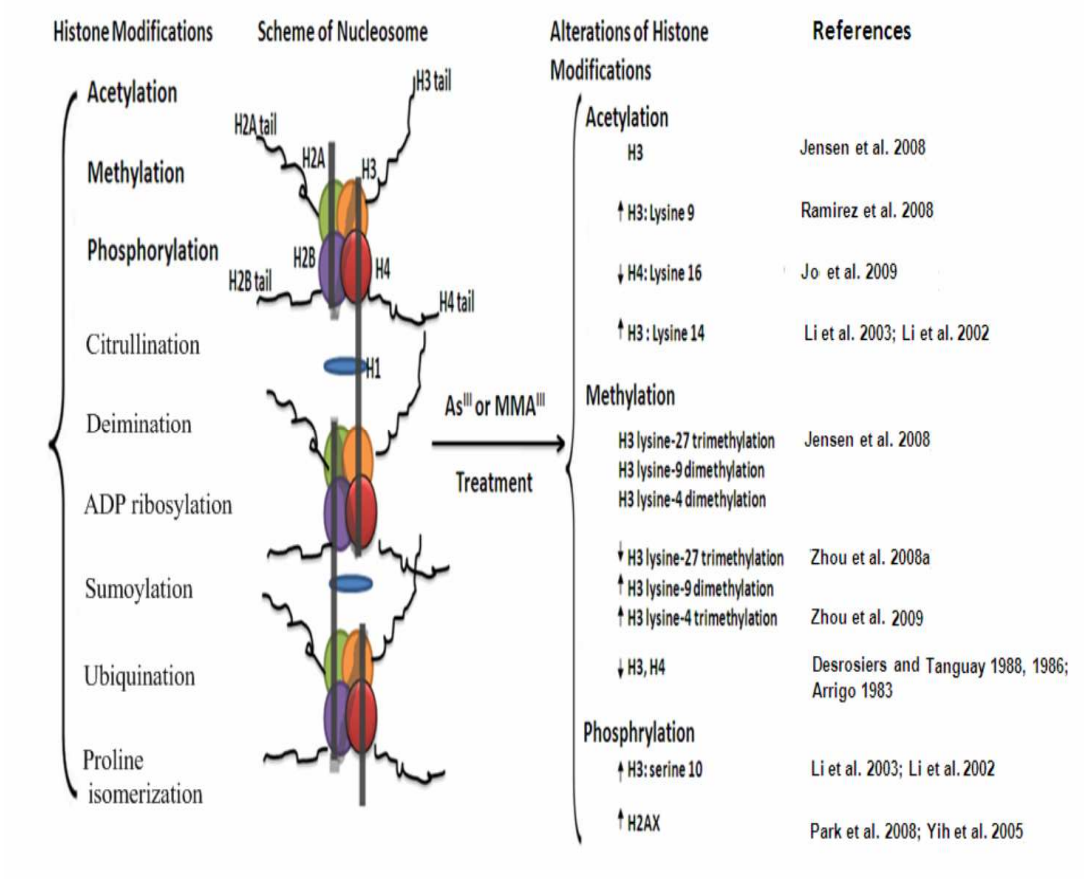

Folic Acid
N5-methyl-tetrahydrofolic acid
Tetrahydrofolic acid
Homcysteine
MTR
Methionine
As III Biomethylation
ATP
PPP
MAT1A
Dimethylarsinous acid
Monomethylarsonous acid
Arsenite
AS3MT
AS3MT
DNMTs
S-adenosyl-homocysteine
S-adenosyl-methionine
5'methylcytosine
Cytosine
DNA methylation
Other metabolic reactions

Figure 2


Histone Modifications
Scheme of Nucleosome
Alterations of Histone Modifications
References
Acetylation
Methylation
Phosphorylation
Citrullination
Deimination
ADP ribosylation
Sumoylation
Ubiquination
Proline isomerization
H3 tail
H2A tail
H2A
H3
H2B
H4
H2B tail
H4 tail
H1
As III or MMA III
Treatment
Acetylation
H3
Jensen et al. 2008
↑ H3: Lysine 9
Ramirez et al. 2008
↓ H4: Lysine 16
Jo et al. 2009
↑ H3 : Lysine 14
Li et al. 2003; Li et al. 2002
Methylation
H3 lysine-27 trimethylation
H3 lysine-9 dimethylation
H3 lysine-4 dimethylation
Jensen et al. 2008
↓ H3 lysine-27 trimethylation
Zhou et al. 2008a
↑ H3 lysine-9 dimethylation
↑ H3 lysine-4 trimethylation
Zhou et al. 2009
↓ H3, H4
Desrosiers and Tanguay 1988, 1986; Arrigo 1983
Phosphrylation
↑ H3: serine 10
Li et al. 2003; Li et al. 2002
↑ H2AX
Park et al. 2008; Yih et al. 2005