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Quantitative mass spectrometry reveals the epigenome as a target of arsenic

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Abstract

Recent studies reveal that posttranslational modifications on chromatin proteins, especially histones, organize genomic DNA and mediate various cellular responses to environmental influences. Quantitative mass spectrometric analysis is a powerful approach to reveal these dynamic events on chromatin in a systematic manner. Here, the effects of arsenic exposure on histone epigenetic state were investigated in human UROtsa cells, and a reduction in acetylation level on several histone H3 and H4 lysine residues was detected. Furthermore, MYST1 was shown to be the major histone acetyltransferase for H4 Lys16 and protect UROtsa cells from arsenic toxicity.

Keywords

Histone modifications; Mass spectrometry; Quantitation; Arsenic toxicity; Histone acetyltransferase

1. Introduction

1.1. Environmental influences and epigenetic regulation

Epigenetic mechanisms, heritably regulating the organization and expression of genetic information, are of essential importance for the development, functioning and survival of organisms. Recent discoveries have suggested epigenetic regulation as a key mediator in the profound interactions between the environment and organisms, while disruption in epigenetic regulatory mechanisms has been implicated in the initiation and progression of

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Conflict of interest statement

The authors declare no conflict of interest.

human diseases, such as cancer, mental disorders and metabolic syndromes [1–4]. Among epigenetic regulatory mechanisms, histone posttranslational modifications have been implicated as particularly important in defining the epigenetic status of a cell [5].

Histones proteins, including H1, H2A, H2B, H3 and H4, are the major protein components of chromatin. They constitute the major packaging device for genomic DNA, and also orchestrate spatial organization of chromatin. The N-terminal tails of histones, are subject to a diverse array of modifications, which modulate nucleosome:nucleosome interactions [6] and regulate affinities of chromatin-associated proteins [7–10]. In addition, histone modifications are functionally linked and integrated (often referred as ‘histone cross-talk’) to determine the ultimate transcriptional outcome, integrating upstream signaling networks activated by various environmental stimuli. Recently, a growing body of evidence has suggested that the epigenetic marking system functions as the molecular substrate for environmental and behavioral stimuli to exert their influence on organisms [11]. For instance, prolonged cold temperature leads to heterochromatin formation on the promoters of flowering repressors in the vernalization responses of plants [12–14]; UV irradiation induces a series of histone modifications to decondense chromatin and to facilitate DNA repair [15,16]. The profound interactions between organisms and the environment are further underlined by age-correlated drifts of epigenetic marks between identical twins [17].

1.2. Mass spectrometric analysis of histone modifications

Although site- and modification-specific antibodies are powerful tools to elucidate the cellular functions of the epigenetic marks on histone proteins, mass spectrometry has gradually become the method-of-choice for systematic, high-throughput analysis of histone modifications, revealing novel modifications [18] and novel modification sites [19–21]. Challenge exists, however due to the large dynamic range of modifications at different sites (i.e. residues) with different modification nature (e.g. methylation vs. acetylation) and modification state (e.g. monomethylation vs. trimethylation). For example, minor cross-reactivity towards another modification state of an otherwise highly specific antibody can illuminate false positive signals, or mask significant changes and generate false negatives in quantitation analyses.

Structural elucidation of the modifications by mass spectrometry is chemically specific and unambiguous. This level of specificity is therefore advantageous over traditional antibody approach. Recent application of effective dissociation technologies (electron capture dissociation and electron transfer dissociation) on high resolution mass spectrometers has made possible the structural characterization of multiple posttranslational modifications directly on intact proteins or large histone N-terminal tails [22–24]. Insights from these studies can unveil functional interactions between histone modifications and assist in deciphering complex histone codes [25,26]. In addition, mass spectrometry can be used in conjunction with isotope labeling or label-free techniques to provide quantitative information on histone epigenetic states. This approach is useful to reveal dynamic changes in histone modifications as responses towards environmental stimuli, which leads to the discovery of new protective pathways mediated by chromatin-modifying and remodeling enzymes [27,28].

1.3. Arsenic toxicity and aberrant epigenetic landscape

Arsenic is a potent environmental carcinogen, strongly associated with lung, skin, bladder and other internal cancers in humans. However, the underlying mechanisms for the association between chronic arsenic exposure and the increased risk of cancers still largely remain elusive. Multiple cellular pathways activated by arsenic exposure lead to the direct modulation of chromatin or chromatin proteins, suggesting epigenome as a target of arsenic for tumor transformation. For instance, arsenic methylation process can deplete the cellular level of S-adenosyl-L-methionine (SAM), the methyl donor, and result in global DNA hypomethylation [29]. Arsenic can generate reactive oxygen species, cause oxidative stress and activate transcription factors, including NF- κ B and AP-1 [30]. The strong thiol chelating activity of arsenic also contributes to the inhibition of many enzymes, especially DNA-binding chromatin enzymes such as poly(ADP-ribose) polymerase [31]. In addition, dysregulation in histone modifications has been detected in arsenic toxicity, including up-regulation in histone H3 phosphorylation and acetylation at the loci of c-jun and c-fos proto-oncogenes [32], and histone H3 Lys9 hyperacetylation in a hepatocarcinoma cell line [33]. In this study, we carried out comparative quantitation analysis of histone modifications and detected a global increase in histone acetylation level upon arsenic exposure in human UROtsa cells.

2. Materials and methods

2.1. Stable isotope labeling

UROtsa cells were metabolically labeled using SILAC (Stable Isotope Labeling of Amino acid in Cell culture) technique. UROtsa cell cultures were grown for 5 passages in SILAC RPMI 1640 media containing either light or heavy lysine ($U\text{-}^{13}\text{C}_6$, 98%, $U\text{-}^{15}\text{N}_2$, 98%; Cambridge Isotope Laboratories Inc., Andover, MA), supplemented with 10% dialyzed FBS, glutamine and antibiotics.

2.2. Histone sample preparation

The UROtsa cell cultures labeled with either heavy or light lysine were treated with arsenic agent (sodium arsenite, As^{III} ; or monomethylarsine oxide, MMA^{III}) or left untreated, respectively. Equal numbers of cells from treated and untreated samples with different lysine isotope labels were mixed. Histone proteins were acid-extracted, separated by 4–20% gradient SDS-PAGE and visualized by Coomassie blue staining as described previously [27]. In-gel digestion of the histone bands were carried out using the procedure described at <http://msf.ucsf.edu/ingel.html>.

2.3. Mass spectrometric analysis and database search

The tryptic digests of core histone proteins were analyzed by nano-flow rate LC-MS and LC-MS/MS analysis, as described previously [20]. Peptides were separated by a $75\text{ }\mu\text{m} \times 15\text{ cm}$ reverse-phase C12 capillary column at 330 nL/min. A binary LC gradient of 2–30% B in 35 min (B = 0.1% formic acid in acetonitrile; A = 0.1% formic acid) was used; and the LC eluent was connected directly to a QSTAR XL mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The centroided peak lists of MS/MS spectra were

searched against the National Center for Biotechnology Information (NCBI) protein database using the Batch-Tag program in UCSF Protein Prospector. Protein N-terminus and lysine acetylation; lysine mono-, di- and trimethylation; arginine mono- and dimethylation; phosphorylation were considered as variable modifications.

2.4. Peptide quantitation

Quantitative calculation of identified peptides was carried out by the Search Compare program in Protein Prospector, which uses averaged peak areas of light and heavy-isotope peptides in MS scans for SILAC ratio calculation. The peptide elution windows were manually inspected and adjusted if necessary to ensure good ion statistics for quantification. SILAC ratio of each peptide was normalized against the average SILAC ratio of all confidently identified peptides in each histone protein.

3. Results

3.1. Arsenic treatment of UROtsa cells

To interrogate epigenetic landscape alterations induced by arsenic exposure, we used SILAC metabolic labeling of human UROtsa cells and carried out relative quantitation of histone modifications when exposed to arsenic in a dosage- and time-dependent manner. The UROtsa cell line was originally isolated from human uroepithelium and provides a good model for bladder epithelium and arsenic-induced bladder cancer. We treated the cells at around 80% confluence with As^{III} or MMA^{III} at concentrations up to 24 h IC₅₀ values [28]. Concentrations used for low dose treatments (1 μ M is equivalent to 75 μ g/L) were higher than the current maximum contaminant level of 10 μ g/L; but is comparable to the detected levels of As^{III} or MMA^{III} in human urine samples when exposed to arsenic [34]. Long term As^{III} exposure at 1 μ M has been shown to induce malignant transformation of UROtsa cells without causing excessive cell death [35].

3.2. A decrease in histone H4 K16 acetylation level upon arsenic exposure

Quantitative mass spectrometric analysis revealed a significant reduction in histone H3 and H4 acetylation level. For example, a dose- and time-dependent decrease in the SILAC ratio was detected for a tetra-acetylated N-terminal peptide from histone H4, GKacGGKacGLGKacGGAKacR (Table 1A). The sequence and the modified residues of this peptide can be conclusively determined by tandem mass spectrum (Fig. 1A). The presence of four lysine residues in this peptide shifted the m/z value of the doubly-charged signal from heavy isotope samples (heavy Lys: ¹³C₆, ¹⁵N₂) by 16 Da, from m/z 719.89²⁺ to 735.91²⁺ (Fig. 1B). Treatment with MMA^{III} for 24 h resulted in only a slight decrease in the SILAC ratio at 0.3 and 1 μ M, but a significant decrease at 3 μ M. Low dose treatment (0.3 and 1 μ M) for a prolonged period of time (7 days) also led to substantial decreases in tetra-acetylation on H4. Interestingly, an H4 N-terminal tri-acetylated peptide, GKacGGKacGLGKacGGAK, only displayed a slight decrease upon arsenic treatment (data not shown). Since the only difference between these two peptides lies in Lys16 acetylation, the drastic reduction in tetra-acetylated peptide abundance is mainly due to the large decrease in H4 Lys16 acetylation level. Quantitative Western blotting data with antiacetyl H4 Lys16 antibody was consistent with mass spectrometric results. Since MYST1 is a major

histone acetyltransferase for histone H4 K16 in human cells, the function of MYST1 in arsenic exposure was then investigated. As expected, reduced H4 Lys16 acetylation was observed after *MYST1* knockdown. Furthermore, knockdown of *MYST1* sensitized human UROtsa cells to both As^{III} and MMA^{III} and decrease UROtsa cell viability, suggesting a protective role of MYST1 in alleviating arsenic toxicity [28].

3.3. A decrease in histone H3 acetylation level upon arsenic exposure

Besides histone H4 Lys16, we also detected a decrease in acetylation level for some lysines at the N-terminal region of H3 upon arsenic exposure (Table 1B and C). Although to a relatively less extent, the trend of changes in two H3 di-acetylated peptides (KacSTGGKacAPR and KacQLATKacAAR) was similar to that of H4 Lys16 in a dose- and time-dependent manner. To confirm that the changes in the SILAC ratios were caused by arsenic treatment rather than isotope labeling, experiments were repeated but with reversed labeling. Reduced SILAC ratios for these acetylated peptides were observed again (Fig. 2, H3 peptide KacSTGGKacAPR). In human cells, acetylations on H3 K9, K14, K18 and K23 can be catalyzed by histone acetyltransferases, Gcn5 and p300/CBP (CREB-binding protein). Interestingly, changes are more pronounced in the globular region (i.e. K18 and K23) than the tail region (i.e. K9 and K14), suggesting a modulation towards chromatin structure than transcription. Consistent with this notion, changes in H4 K16 acetylation (correlated to chromatin structure) are also more drastic rather than other three acetylations at H4 N-terminal tail (correlated with transcription).

This decrease in H3 acetylation level is in contrast to a previous report, where H3 hyperacetylation was detected following arsenic exposure [33]. However, this discrepancy can be attributed to the usage of different cell lines (UROtsa vs. HepG2) for different cancer models (bladder cancer vs. liver cancer). In an independent study, significant alterations in histone H3 acetylation level were detected at the promoter region of 53 genes (43 hypoacetylation, 10 hyperacetylation) during arsenical-induced malignant transformation of UROtsa cells [36]. Taken together, it is conceivable that arsenical-induced distortion of the epigenome landscape leads to the pathological silencing of key growth controlling genes and facilitates tumorigenesis.

4. Discussion

Recent studies reveal that posttranslational modifications on chromatin proteins, especially histones, modulate alterations in the accessibility of DNA in response to environmental exposures, such as flavoring agents [37], sun light [27], arsenic [28,36] and endocrine-disrupting chemicals [38]. Quantitative mass spectrometry provides a powerful tool to reveal these dynamic events taking place on chromatin. Metabolic labeling techniques (e.g. SILAC) incorporate isotopes for quantitation at early stage of the workflow, and thus have the potential to minimize variations introduced by sample handling. However, direct metabolic labeling of tissue samples is usually not amenable. Potentially, relative quantification of different tissue samples can still be achieved through normalization against a common SILAC labeled sample. Another challenge is how to elucidate key regulatory events, which usually take place at low frequency. Conceivably, tight standard deviation in

quantitation will be critical to detect small changes in global histone modifications. More promising approaches might rely on the purification and comparison of specific sub-chromatin domains with specific functions.

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Fig. 1. Mass spectrometric analysis of histone H4 N-terminal tetra-acetylated peptide, GKacGGKacGLGKacGGAKacR. (A) Tandem MS spectrum of this peptide to reveal peptide sequence and modification. (B) SILAC ratios can be calculated from signals with light (m/z 719.89, control) or heavy (m/z 735.91, treatment) lysine. MMAIII treatment was applied to the heavy-isotope labeled sample. The SILAC ratio (treatment/control) of each experiment was normalized accordingly to the mean value of all identified peptides.

Fig. 2.

Mass spectrometric analysis of a histone H3 N-terminal di-acetylated peptide, KacSTGGKacAPR, with light (m/z 493.26) or heavy (m/z 501.27) lysine. MMAIII treatment (3 μ M, 24 h) was applied either to the heavy sample (forward labeling) or to the light sample (reverse labeling). The SILAC ratio (treatment/control) of each experiment was normalized accordingly to the mean value of all identified peptides.

Table 1

Peptides with altered SILAC ratios^a after arsenic treatment.

Time ^b	As ^{III}		MMA ^{III}			
	1 nM	3 μ M	10 μ M	0.3 μ M	1 μ M	3 μ M
(A) H4 peptide with acetylation on K5, K8, K12 and K16						
1 day	0.86	0.78	0.53	0.88	0.81	0.11
7 days	0.48	0.38	N/A ^c	0.55	0.56	N/A ^c
(B) H3 peptide with acetylation on K9 and K14						
1 day	1.04	1.05	0.70	1.01	0.97	0.18
7 days	0.78	0.48	N/A ^c	0.85	0.67	N/A ^c
(C) H3 peptide with acetylation on K18 and K23						
1 day	1.01	0.81	0.54	0.90	0.87	0.19
7 days	0.77	0.52	N/A ^c	0.50	N/A ^d	N/A ^c

^a SILAC ratio (treatment/control) has been normalized to the mean value of identified peptides. Heavy-isotope labeled samples are treated with arsenic.

^b Treatment period with arsenic agents.

^c Not available due to extensive cell death in arsenic treated samples.

^d Not available due to MS signal interference for quantitation.