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Global mapping of post-translational modifications on histone H3 variants in mouse testes

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ABSTRACT

Mass spectrometry (MS)-based characterization is important in proteomic research for verification of structural features and functional understanding of gene expression. Post-translational modifications (PTMs) such as methylation and acetylation have been reported to be associated with chromatin remodeling during spermatogenesis. Although antibody- and MS-based approaches have been applied for characterization of PTMs on H3 variants during spermatogenesis, variant-specific PTMs are still underexplored. We identified several lysine modifications in H3 variants, including testis-specific histone H3 (H3t), through their successful separation with MS-based strategy, based on differences in masses, retention times, and presence of immonium ions. Besides methylation and acetylation, we detected formylation as a novel PTM on H3 variants in mouse testes. These patterns were also observed in H3t. Our data provide high-throughput structural information about PTMs on H3 variants in mouse testes and show possible applications of this strategy in future proteomic studies on histone PTMs.

1. Introduction

The nucleosome—the basic repeating element of the chromatin—consists of eight core histones and one linker histone wrapped by a DNA fragment. Histone variants encoded by different genes are diversely expressed during cell cycles [1–3]. Histone H3, one of the core histones, has a number of variants (H3.1, H3.2, H3.3, CENP-A, H3t, H3.X, H3. Y, and H3.5) that are characterized using proteomic approaches [4,5]. These histone H3 variants are subjected to post-translational modifications (PTMs) for regulation of several biological activities such as transcription and chromatin remodeling [4]. Proteomic approaches using antibody or mass spectrometry (MS) have facilitated detection of several PTMs such as methylation, acetylation, phosphorylation, O-GlcNAcylation, and ubiquitination on H3 variants [4]. The characterization of histone PTMs has traditionally relied on the antibody-based analysis, owing to its sensitivity; however, this method is associated with several shortcomings such as cross-reactivity and uncertain specificity. In particular, antibody-based approaches are unable to distinguish variants with similar but small alterations in their amino acid sequences [1–3,6].

Mass spectrometry has become a general and powerful tool ensuring rapid, sensitive, accurate, and comprehensive characterization of various PTMs of amino acid residues on histone variants [1–4,6,7]. In particular, MS-based bottom-up strategy involving a digestion step was reported to be unsuitable for comprehensive analysis, owing to the loss of information about PTMs of short peptides (3–4 amino acids) generated during digestion. However, recent advances with tandem mass spectrometry (MS/MS) provide sensitive analysis as well as high-throughput and informative data for identification of specific or novel modified sites on histones [2,6,7].

The expression of histone H3 variants, including H3t, has been detected during spermatogenesis [5,8]. Studies have reported the importance of acetylation and methylation of the N-terminal lysine residues of histone H3 in the regulation of chromatin structure during spermatogenesis [5,8]. Acetylated and methylated lysine as well as phosphorylated serine have been identified in H3 variants during spermatogenesis, using antibody-based techniques [9–13]. In addition, MS-based approaches characterized lysine modifications on H3 variants during spermatogenesis [14,15]; however, these methods have limited potential for identification of variant-specific and novel PTMs. The histone H3t is mainly enriched in small amounts from somatic cells during spermatogenesis [5,8]. Our previous study showed successful separation and characterization of H3t and its K9 mono-, di-, and tri-methylations from mouse testes, using top-down strategies [16].

Abbreviations: MS, mass spectrometry; PTMs, post-translational modifications; HCD, high-energy collision dissociation; TCA, trichloroacetic acid; DTT, dithiothreitol; H2SO4, sulfuric acid; HPLC, high performance liquid chromatography; RP, reverse phase; HFBA, heptafluorobutyric acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; ESI-TRAP, electrospray TRAP; FDR, false discovery rate; MALDI, matrix-assisted laser desorption/ionization; ISD, in source decay

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However, no information on the comprehensive mapping of other PTMs on H3t has been reported. Studies on the identification of PTMs on histone H3 variants have become important for understanding the chromatin structure and its role in biological activities. Specific and comprehensive analysis is required to study the structure and PTMs of histone H3 variants during spermatogenesis.

In this study, we comprehensively characterized PTMs on H3 variants in mouse testes, using MS-based bottom-up approach. Our data showed global mapping of modified lysine in H3 variants. These patterns were observed not only at histone tails (K9, K14, K18, K23, K36, and K112) but also at K56 and K79. Most lysine sites were mainly methylated and acetylated. In addition, we identified formylation as a novel PTM on H3 variants in mouse testes by higher-energy collisional dissociation (HCD) fragmentation. We also observed these PTMs on H3t. Our approach enables high-throughput analysis of PTMs as well as differentiation of similar modified masses at the same lysine, such as tri-methylation versus acetylation on K9 and K27 and di-methylation versus formylation on K23. These data will be useful for the investigation of overall assessment of PTM on histones with accuracy and specificity.

2. Materials and methods

2.1. Extraction of histone H3 variants

Mouse (BALB/c) testes combined with epididymides, purchased from Funakoshi Co. (Tokyo, Japan), were homogenized following separation. Whole histones were extracted from testes and epididymides by the trichloroacetic acid (TCA) precipitation method with minor modifications [17]. Briefly, all steps were carried out at 4 °C. Testes (148.7 mg) and epididymides (183.9 mg) tissues were suspended in hypertonic lysis buffer (10 mM Tris-Cl pH 8.0, 1 mM KCl, 1.5 mM Mg $(CH_3COO)_2$, 1 mM dithiothreitol [DTT], and protease inhibitors) for 30 min. Lysed cells were centrifuged at 10,000 $\times g$ for 10 min and the pelleted intact nuclei resuspended in 0.4 N sulfuric acid (H_2SO_4) for 30 min. The nuclear debris was pelleted by centrifugation at 16,000 $\times g$ for 10 min to obtain a supernatant enriched with histones. The supernatant was precipitated by incubation with TCA at a final concentration of 33% for 30 min. The precipitate was subjected to centrifugation at 16,000 $\times g$ for 10 min. The histone pellet thus obtained was washed with acetone, dried at room temperature, and resuspended in 100 µL MilliQ water.

2.2. Separation of each H3 variant by high performance liquid chromatography (HPLC)

Histone H3 variants were separated with Agilent HPLC 1100 series (Agilent Technologies, Santa Clara, CA, USA), using a C4 reverse-phase (RP) column (2.0 mm × 15.0 mm, 3-µm particle size; GL sciences, Tokyo, Japan). A 20-µL sample was injected and detected at UV wavelength of 215 nm. Heptafluorobutyric acid (HFBA, Thermo-Fisher Scientific, Waltham, MA, USA) was used as an ion-pairing reagent. The mobile phase A (5% acetonitrile with 0.1% HFBA) and B (90% acetonitrile with 0.1% HFBA) were delivered at a flow rate of 0.1 mL/min, using the following gradient parameters: 0 min (to 5 min, 15% solvent B); 5 min (to 15 min, 15–48% solvent B); 15 min (to 25 min, 48% solvent B); 25 min (to 100 min, 48–62% solvent B); 100 min (to 120 min, 62–100% solvent B); 120 min (to 130 min, 100% solvent B); 130 min (to 135 min, 100–15% solvent B); 135 min (to 145 min, 15% solvent B). The fraction collector (Gilson, Middleton, WI, USA) was programmed to collect fractions from 30 to 116 min at an interval of 1 min.

2.3. In-gel digestion

Each band containing histone proteins was excised from the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and destained using 30% acetonitrile. Disulfide bonds of proteins were reduced with 50 mM DTT at 37 °C for 2 h, followed by their alkylation with 100 mM acrylamide at room temperature for 30 min. Histones were subjected to digestion by incubation of gel pieces with 20 µL of buffer (20 mM Tris-HCl, pH 8.0) and trypsin (0.01 µg/µL) at 37 °C for 12–15 h.

2.4. Characterization of PTMs by MS

After digestion, the sample was analyzed by a Q-Exactive mass spectrometer (Thermo-Fisher Scientific, Bremen, Germany) with HCD using a linear gradient of 0–100% solvent B over 30 min (solvent A: 0.1% trifluoroacetic acid [TFA]; solvent B: 80% acetonitrile with 0.1% TFA). The MS was directed using Xcalibur software version 3.0.63 (Thermo-Fisher Scientific). A nanoelectrospray (Thermo-Fisher Scientific) was applied with a spray voltage of 1.9 kV and capillary temperature of 275 °C. We acquired HCD spectra (at a range of 300–2000 m/z) with a resolution of 70,000 at m/z 400 Da. MS/MS spectra were obtained in a data-dependent manner. Spectra of histone peptides were searched against the protein databases (NCBInr and SWISS-PROT), using the MASCOT program (version 2.5, Matrix Science, UK).

2.5. Data processing

We processed MS/MS files as *.mgf files, using Proteome Discoverer (version 2.1.0.81, Thermo-Fisher Scientific). Converted files were searched through MASCOT against the databases (NCBI and SWISS-PROT), using following parameters: monoisotopic mass values; trypsin as the enzyme for digestion, with two missed cleavages; 1.2 Da of peptide mass tolerance; and 0.02 Da of fragment mass tolerance. Peptide charge was set at +1, +2, and +3 and the instrument set to electrospray iontrap (ESI-TRAP) with decoy mode. Variable modifications were as follows: mono- (+14.016 Da), di- (+28.031 Da), tri-methylation (+42.047 Da), formylation (+27.995 Da), acetylation (+42.011 Da), propionylation (+56.026 Da), and butyrylation (+70.042 Da) of lysine and oxidation (+15.995 Da) of methionine. Identified peptides were approved at a false discovery rate (FDR) less than 1%, with a minimum score of 15. HCD spectra were manually examined for exact characterization of poor matched results from MASCOT search data.

3. Results and discussion

3.1. Separation of histone H3 variants

We successfully separated histone H3 variants from mouse testes and epididymides, using HPLC with HFBA (Fig. 1). In our past study, we had employed MS-based analysis for verification of each separated histone H3 variant [16,18]. Epididymis is a tube connected to the testis where sperms flow and get temporarily stored [5]. We used epididymides to compare patterns of H3 histone variants, including H3t, during spermatogenesis. H3t was clearly separated in mouse testes and undetectable in the epididymides (Fig. 1). In addition, higher H3.2 signal was observed in the epididymides compared to that in the testes (Fig. 1).

3.2. Identification of PTMs on H3 variants in mouse testes

We characterized PTMs on H3.1, H3.2, H3.3, and H3t, using MS-based bottom-up approach. Several studies have employed antibody-based immunoassays for the identification of PTMs such as methylation and acetylation during spermatogenesis, owing to their sensitivity. However, antibody-based assays exhibit cross-reactivity and uncertain specificity, which limit their application for high-throughput PTM