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Global-scale profiling of differential expressed lysine acetylated proteins in colorectal cancer tumors and paired liver metastases

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ABSTRACT

Lysine acetylated modification was indicated to impact colorectal cancer (CRC)'s distant metastasis. However, the global acetylated proteins in CRC and the differential expressed acetylated proteins and acetylated sites between CRC primary and distant metastatic tumor remains unclear. Our aim was to construct a complete atlas of acetylome in CRC and paired liver metastases. Combining high affinity enrichment of acetylated peptides with high sensitive mass spectrometry, we identified 603 acetylation sites from 316 proteins, among which 462 acetylation sites corresponding to 243 proteins were quantified. We further classified them into groups according to cell component, molecular function and biological process and analyzed the metabolic pathways, domain structures and protein interaction networks. Finally, we evaluated the differentially expressed lysine acetylation sites and revealed that 31 acetylated sites of 22 proteins were downregulated in CRC liver metastases compared to that in primary CRC while 40 acetylated sites of 32 proteins were upregulated, of which HIST2H3AK19Ac and H2BLK121Ac were the acetylated histones most changed, while TPM2 K152Ac and ADH1B K331Ac were the acetylated non-histones most altered. These results provide an expanded understanding of acetylome in CRC and its distant metastasis, and might prove applicable in the molecular targeted therapy of metastatic CRC.

Biological significance: This study described provides, for the first time, that full-scale profiling of lysine acetylated proteins were identified and quantified in colorectal cancer (CRC) and paired liver metastases. The novelty of the study is that we constructed a complete atlas of acetylome in CRC and paired liver metastases. Moreover, we analyzed these differentially expressed acetylated proteins in cell component, molecular function and biological process. In addition, metabolic pathways, domain structures and protein interaction networks of acetylated proteins were also investigated. Our approaches shows that of the differentially expressed proteins, HIST2H3AK19Ac and H2BLK121Ac were the acetylated histones most changed, while TPM2 K152Ac and ADH1B K331Ac were the acetylated non-histones most altered. Our findings provide an expanded understanding of acetylome in CRC and its distant metastasis, and might prove applicable in the molecular targeted therapy of metastatic CRC.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer in the world, with nearly 1.4 million new cases diagnosed in 2012 [1]. In 2014, an estimated 71,830 men and 65,000 women will be diagnosed with colorectal cancer and 26,270 men and 24,040 women will die of the disease [2]. The invasion and distant metastasis were the major determinants of worse prognosis of colorectal cancer [3]. Recently, accumulative novel antitumor drugs were developed to inhibit the advanced cancer progression, especially the molecular targeted drugs [4–8]. However, the overall response rate of most current molecular targeted drugs to the treatment for colorectal cancer was less than 50% [9,10]. Further, side effects are one of the issues of concern [11]. Thus, new targeted molecules of colorectal cancer still need to be investigated. Evidence showed that protein acetylation might be a candidate of therapeutic targets for cancer [12,13]. Epigenetic alteration which leads to modified gene expression was involved in colorectal cancer, initially and during progression [14–16]. Protein acetylation including histone and non-histone proteins, such as transcriptional factors, has been shown to play an important role in the epigenetic regulation of various cancers, and exhibited tissue specificity [12,17,18]. Mutation and/or aberrant expression of various histone deacetylase (HDAC) have also been observed in human disease, in particular cancer, making them a

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candidate of therapeutic targets for many human cancers [13]. Histone deacetylase inhibitors (HDIs) have emerged as promising cancer therapeutic agents. To date, two HDIs have been approved for cancer therapy (cutaneous T-cell lymphoma); vorinostat (SAHA, Zolinza) and romodepsin (FK228, Istodax) by the Food and Drug Administration (FDA) [19]. Therefore, targeting protein acetylation represents a potentially promising strategy for human cancers. However, there is no information about the target of acetylation in the colorectal cancer treatment. The main reason is that the critical acetylated protein and site remains unclear in the colorectal cancer.

Proteomic methods are largely based on the use of mass spectrometry (MS), a highly specific, effective, and universal technique that does not require complicated multi-step sample preparation [20]. Proteomics analyses the composition, amounts, isoforms, and posttranslational modifications of cellular proteins [21]. Karczmarski and colleagues [22] used MS-based analysis to quantify global alterations of histone post-translational modifications (PTMs) in normal and colon cancer samples and showed that histone H3 lysine 27 acetylation (H3K27Ac) was associated with colon cancer. However, the differentially expressed acetylated proteins and acetylated sites between CRC primary and distant metastatic tumor remain unclear. In this study, we used integrated approach involving tandem mass tag (TMT) labeling and mass spectrometry-based quantitative proteomics to quantify dynamic changes of protein acetylation including histone or non-histone proteins between matched colon cancer primary and liver metastatic tumor samples. It might throw a new light on the metastatic CRC molecular targeted treatment.

2. Materials and methods

2.1. Patient tissue samples

Three paired colon cancer tissue samples and liver metastases samples (three biological replicates) were obtained from patients undergoing coloproctectomy and immediately snap-frozen and stored at −80 °C until protein extraction. All samples were observed by two pathologists independently and identified as colorectal adenocarcinoma or metastatic cancer. All patients provided written informed consent before samples were collected. The study was approved by the local Research Ethics Committee of Peking University.

2.2. Crude protein extraction

The tissues were first grinded by liquid nitrogen and the powder was transferred to 50 mL centrifuge tube and precipitated with cold 10% TCA/acetone supplemented with 50 mM DTT, 0.1% Protease Inhibitor Cocktail Set IV for 2 h at −20 °C. After centrifugation at 20,000 g at 4 °C for 10 min, the supernatant was discarded. The remaining precipitate was washed with cold acetone supplemented with 50 mM DTT, 1 mM PMSF for three times. After air drying, the precipitate was resuspended in lysis buffer (8 M urea, 2 mM EDTA, 10 mM DTT). The sample was sonicated three times on ice using a high intensity ultrasonic processor (Scientz). The remaining debris was removed by centrifugation at 20,000 g at 4 °C for 10 min. The quality control of the extracted proteins was done by SDS-PAGE (Supplementary Fig. S1).

2.3. Trypsin digestion

For trypsin digestion, the supernatant was transferred to a new tube, reduced with 10 mM DTT for 1 h at 56 °C and alkylated with 55 mM IAA for 45 min at room temperature in darkness. The protein was precipitated with 3 volumes of prechilled acetone for 30 min at −20 °C. After centrifugation, the pellet was then dissolved in 0.5 M TEAB and sonicated for 5 min. The centrifugation step was repeated and the supernatant collected. Protein content was determined with 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions. The protein from each sample was then digested with trypsin overnight at 37 °C in a 1:50 trypsin-to-protein mass ratio.

2.4. Tandem mass tag (TMT) protein labeling

A total of 7.5 mg proteins were digested for enrichment (1.25 mg per plex TMT reagent and 6-plex TMT reagent was used). After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 6-plex TMT kit (Thermo). Briefly, one unit of TMT reagent (defined as the amount of reagent required to label 1.25 mg of protein) were thawed and reconstituted in ACN. The peptide mixtures were then pooled and incubated for 2 h at room temperature, desalted and dried by vacuum centrifugation. The six samples of 1 T, 1 M, 2 T, 2 M, 3 T and 3 M were labeled with TMT tag of 126, 127, 128, 129, 130 and 131, respectively.

2.5. Affinity enrichment

To enrich lysine acetylated peptides, tryptic peptides dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) were incubated with pre-washed antibody beads (purchased PTM Biolabs, Hangzhou, China, Cat. Number PTM-104, it is a mixture of rabbit-derived polyclonal and mouse-derived monoclonal anti-acetyllysine antibodies) at 4 °C overnight with gentle shaking. The beads were washed four times with NETN buffer and twice with ddH_2O. The bound peptides were eluted from the beads with 0.1% TFA. The eluted fractions were combined and vacuum-dried. The resulting peptides were cleaned with C18 ZipTips (Millipore) according to the manufacturer's instructions, followed by analyzing by LC-MS/MS.

2.6. LC-MS/MS analysis

Peptides were dissolved in 0.1% FA (solvent A), directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific, 75 μm × 2 cm, 3 μm, 100 Å). Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific, 50 μm × 15 cm, 2 μm, 100 Å). The elution was performed with a linear gradient of 5% ~ 35% solvent B (0.1% FA in 98% ACN) for 30 min and 35% ~ 80% solvent B for 10 min at a constant flow rate of 300 nL/min on an EASY-nLC 1000 UPLC system. The resulting peptides were analyzed by Q Exactive TM Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

The peptides were subjected to nanospray ionization (NSI) source followed by tandem mass spectrometry (MS/MS) in Q Exactive (Thermo) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. The collision mode for acquiring fragment ions is HCD. Peptides were selected for MS/MS using 27% normalized collision energy (NCE) with 12% stepped NCE; ion fragments were detected in the Orbitrap at a resolution of 17,500. The mass window for precursor ion selection is set as 2.0 *m/z*. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 3E4 in the MS survey scan with 15.0 s dynamic exclusion. The electrospray voltage applied was 1.8 kV. Automatic gain control (AGC) was used to prevent overfilling of the Orbitrap; 1E5 ions were accumulated for generation of MS/MS spectra. For MS scans, the *m/z* scan range was 350 to 1600 Da. The fixed first mass was set at 100 *m/z* for TMT quantification.

2.7. Database search

The resulting MS/MS data were processed using MaxQuant with integrated Andromeda search engine (Version 1.4.1.2). TMT were searched against Uniprot_human database (20,274 protein sequences,