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Targeted Proteomic Analyses of Histone H4 Acetylation Changes Associated with Homologous-Recombination-Deficient High-Grade Serous Ovarian Carcinomas

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Abstract

Approximately 20% of high-grade serous ovarian cancers are homologous-recombination (HR)-deficient due to genetic and epigenetic mutations of HR pathway genes including the tumor suppressor genes *BRCA1* and 2. HR deficiency (HRD) compromises cells' ability to efficiently repair DNA damage, but it also increases sensitivity to chemotherapeutic treatment strategies; however, not all ovarian cancer patients with HRD tumors exhibit positive responses to chemotherapy. Our previous iTRAQ-based comprehensive proteomic characterization of high-grade serous ovarian carcinomas found that lower levels of histone H4 acetylation at Lys12 and Lys16 (H4-K12acK16ac) were associated with HRD tumors compared with non-HRD tumors. In the current study, we developed and validated an H4-K12acK16ac parallel-reaction-monitoring (PRM)-targeted mass-spectrometry-based assay to analyze acetylation changes of histone H4 and to determine the association of these changes with total H4, histone acetyltransferase, and histone deacetylase (HDAC) levels. Whereas the levels of H4 and histone acetyltransferases were stable irrespective of HRD status, the levels of histone H4 acetylation and one HDAC, HDAC6, were elevated in the HRD tumors. Relative H4 acetylation levels were also analyzed by an antibody-based approach in additional ovarian tumors. It is possible that specific H4 acetylation at Lys12 and Lys16 associated with HRD could inform chemotherapeutic treatment modalities to improve ovarian cancer patients' treatment response.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval of the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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Supplemental Tables S1–S3 captions. (PDF)

Table S1: H4-K12acK16ac peak area ratios from the PRM analysis of HRD and non-HRD ovarian tumors. (XLSX)

Table S2: Z-scores of H4-K12acK16ac measured by iTRAQ and PRM in HRD and non-HRD ovarian tumors. (XLSX)

Table S3: Global protein iTRAQ ratios of H4, histone acetyltransferases and histone deacetylases (HDACs) measured in HRD and non-HRD ovarian tumors. (XLSX)

Keywords

parallel reaction monitoring (PRM); histone; lysine acetylation; ovarian cancer; homologous recombination; targeted assays; quantification; mass spectrometry

INTRODUCTION

Epithelial ovarian cancer is the most lethal gynecological malignancy in the United States, and the 5-year survival rate of patients with advanced stage ovarian cancer is only 26.9%.¹ A significant challenge in chemotherapeutic treatment regimens for ovarian cancer patients is that the response to platinum-based chemotherapy is often very poor because of the development of chemoresistance, and thus novel treatment strategies are needed.

A recent advance in translational oncology research is that the mutational status of a solid tumor can predict the therapeutic efficacy for a specific drug in a molecularly defined subset of patients.^{2,3} Genetic variants in genes involved in the distribution, metabolism, accumulation, or repair of lesions can influence the response of drugs that are used in the treatment of ovarian cancer.⁴ An impactful example of the study of the genomic prediction of therapeutic efficacy is that of inhibitors of poly(ADP-ribose) polymerase (PARPi) that have emerged as a novel class of anticancer drugs to treat homologous recombination deficiency (HRD)-related ovarian cancer associated with loss of *BRCA1/2* function due to mutations or the down-regulation of the expression of *BRCA1/2* and associated genes.⁵ PARP inhibitors interrupt the DNA repair process by impairing two mechanisms of PARP: (1) blocking binding sites of single-strand breaks (SSBs) with its own zinc finger domains, consequently directly blocking DNA access by PARP, and (2) preventing the transfer of ADP-ribose to form PAR chains to block the formation of the base excision repair complex.⁶⁻⁸

Because of the therapeutic advantages they confer in HRD tumors, many PARP inhibitors are in various stages of clinical trials. However, HRD can be manifested in several ways other than genomic *BRCA1/2* mutational analysis.^{9–12} A vital key to the success of ovarian cancer treatment strategies based on the genomic prediction of therapeutic efficacy, such as PARPi treatment, is a more thorough understanding of ovarian cancer etiology. Although there has been extensive genomic and transcriptomic characterization of ovarian cancer aimed at defining the genomic landscape and assisting the development of targeted therapies,^{13,14} less is known about how the ovarian cancer genome drives the cancer proteome with effects on clinical outcomes. Thus there is a great need for these types of studies given that functions encoded in the genome are executed at the protein level with the potential for further modulation by post-translational modifications (PTMs).¹⁵

Lysine acetylation is a reversible PTM that is controlled by the opposing activities of lysine acetyltransferases and deacetylases.¹⁶ The dysregulation of histone acetylation has been implicated in various human diseases including cancer, and thus histone deacetylases (HDACs) are attractive anticancer therapeutic targets.¹⁷ Previously, we conducted an isobaric tags for relative and absolute quantitation (iTRAQ)-based comprehensive proteomic characterization of high-grade serous ovarian carcinomas toward the determination of the underlying molecular mechanisms that are associated with HRD in ovarian cancer to identify putative biomarkers that could be used to stratify ovarian cancer patients for treatment.¹⁸ One of our main observations was that lower levels of histone H4 acetylation at Lys12 and Lys16 (H4-K12acK16ac) correlated with HRD status. Indeed, the loss of H4 monoacetylation in cancer cells has been demonstrated to occur predominantly at Lys16.¹⁹ Our iTRAQ data indicating lower H4-K12acK16ac levels in HRD tumors was supported by the results from a sequential window acquisition of all theoretical mass spectra (SWATH-MS) analysis of the same tumors.¹⁸

Parallel reaction monitoring (PRM) is a targeted proteomics strategy where all product ions of the target peptides are simultaneously monitored at high resolution and high mass accuracy, thus enabling high-quality quantitative measurements.^{20,21} The use of PRM to quantify changes in histone modifications was first introduced by Tang et al. in 2014.²² In the current study, we developed, validated, and deployed a targeted mass-spectrometry-based H4-K12acK16ac PRM assay to verify our initial proteomic findings, demonstrating that lower H4-K12acK16ac levels are associated with HRD status in ovarian tumors. The results from the H4-K12acK16ac PRM assay were further verified by applying the PRM assay and Western blot to additional ovarian tumors. We also compared the expression of acetylated H4 to the total H4 protein level as well as that of histone acetyltransferases and histone deacetylases (HDACs), and we observed an HRD-associated level of HDAC6 overexpression, which supports a potential role for the effectiveness of HDAC inhibitor treatment in HRD tumors that are characterized by low levels of H4 lysine acetylation.

EXPERIMENTAL SECTION

Materials

Tris(2-carboxyethyl)phosphine (TCEP), urea, LC-MS grade water, and LC-MS grade acetonitrile were acquired from Thermo Fisher Scientific. Sodium citrate, Tween-20,

iodoacetamide, ammonium bicarbonate, and formic acid were obtained from Sigma-Aldrich. Sequencing-grade trypsin was obtained from Promega. All chemicals were of analytical grade unless otherwise specified.

Clinical Specimens

The TCGA frozen ovarian tumor tissue specimens were obtained through the CPTAC Biospecimen Core Resource, as previously described.¹⁸ The *BRCA* mutant ($n = 4$) and WT ($n = 3$) ovarian tumor tissues were obtained from Dr. Ie-Ming Shih from the Department of Gynecology and Obstetrics, Johns Hopkins Medical Institutions and Dr. Douglas Levine from the Department of Gynecologic Oncology, Laura and Isaac Perlmutter Cancer Center, New York University Langone Medical Center, according to IRB-approved protocols.

Western Blotting

Ten μg protein from the *BRCA* WT ($n = 3$) and mutant ($n = 4$) ovarian tumor tissue homogenized in urea buffer (8 M urea, 0.8 M NH_4HCO_3 , pH 8.0) was separated on 4–12% Bis-Tris gels (Thermo Fisher Scientific), followed by electrophoretic transfer to nitrocellulose membranes. Nonspecific binding sites on the blot were blocked with 5% milk/TBS-T, followed by overnight incubation at 4 °C in primary antibody (rabbit anti-Histone H4 acetyl K16, 1:1500, Abcam; rabbit anti-histone H4 acetyl K12, 1:10 000, Abcam; rabbit anti-Histone H4, 1:1000, Abcam; rabbit anti-actin-HRP, 1:10:000, Sigma). Goat anti-rabbit secondary antibody was used at a 1:10 000 dilution. Immunoreactive bands were visualized using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific). Densitometric analysis was conducted using ImageJ (NIH).

Protein Extraction and Tryptic Digestion

Approximately 50 mg of each of the TCGA ovarian tumor tissues was sonicated in 1.5 mL of 8 M urea, 0.8 M NH_4HCO_3 , pH 8.0. Proteins were reduced with 10 mM TCEP for 1 h at 37 °C and subsequently alkylated with 12 mM iodoacetamide for 1 h at RT in the dark. Samples were diluted 1:4 with deionized water and digested with trypsin at a 1:50 enzyme-to-protein ratio. After 12 h of digestion at 37 °C, another aliquot of the same amount of trypsin was added to the samples and further incubated at 37 °C overnight. The digested samples were then acidified, cleaned up (SCX and C18), and dried in a vacuum centrifuge. The FFPE ovarian tissue sections were processed and subjected to trypsin digestion according to the procedure described in Tian et al.²³

Parallel Reaction Monitoring Analysis

Tier 2 assay development was conducted using crude light and heavy stable isotope-labeled peptide standards (SIS; ~60% chemical purity, >99% isotopic purity, Thermo Fisher Scientific PEPotec SRM peptide library). Histone H4-K12acK16ac peptide sequence: $\text{gGLGKacGGAKacR}_{17}$. The SIS peptide incorporated a fully atom-labeled ^{13}C and ^{15}N isotope at the C-terminal arginine (R). Peptides were provided in 0.1% TFA/ 50% ACN and stored at –80 °C until use.

PRM analysis was conducted using a Dionex UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific).

The peptides were injected (6 μL) onto a C18 trap column (300 μm I.D. $\times$ 5 mm packed with Acclaim PepMap 100, 5 μm , 100 Å C18; Thermo Fisher Scientific) at a loading pump flow rate of 5 $\mu\text{L}/\text{min}$, followed by separation on a 75 μm I.D. $\times$ 50 cm EASY-Spray analytical column packed with 2 μm Acclaim PepMap RSLC C18 (Thermo Fisher Scientific). Mobile phase A was 2% ACN/0.1% formic acid in water, and mobile phase B was 90% ACN/0.1% formic acid. The column was heated to 42 °C. Separations were performed at 250 nL/min across a 20 min linear gradient from 4–40%B.

An EASY-Spray source (Thermo Fisher Scientific) with zero dead volume nanoViper fittings was used with the Q-Exactive. The spray voltage was 1.8 kV and the capillary temperature was 250 °C. The mass spectrometer was operated in a targeted-MS² acquisition mode with a maximum IT of 100 ms, 1 microscan, 70 000 resolution, 5e⁵ AGC target, 2.0 m/z isolation window, and 29% normalized collision energy. Intrarun mass calibration was conducted using lock masses of 445.12003 m/z and 371.10123 m/z . A scheduled PRM method was created to monitor the 2+ charge state of each peptide with a 5 min retention time window. For the PRM analysis of the ovarian tumor specimens, each injection consisted of 1 μg of peptides from each specimen spiked with 200 fmol of the heavy H4-K12acK16ac peptide.

PRM Assay Characterization: Response Curve

Response (calibration) curves were generated using light peptides spiked into and serially diluted with a biological matrix consisting of tryptic peptides derived from digested human ovarian tumor tissue. The heavy peptides were spiked in at a constant concentration of 33.3 fmol/ μL (200 fmol on column) to enable normalization. Peak area ratios (light/heavy) were used as the dependent variables to generate the response curves. The seven-point response curves covered four orders of magnitude in abundance range, and they were run in triplicate in order of increasing concentration with three blank runs prior to the first replicate run of the curve and two blank runs following each curve. The assays were characterized based on several metrics including LOD, LLOQ, and linearity.

PRM Assay Characterization: Reproducibility

Assay performance reproducibility (intraday, interday, and total assay CV) was measured across 5 days in triplicate at three levels, low, medium, and high, to approximate 2 $\times$ LLOQ, 50 $\times$ LLOQ, and 100 $\times$ LLOQ, respectively. The total assay CV was calculated as the square root of the sum of squares of the intra and interday CVs. The run order was randomized to more accurately reflect the variability in assay performance.

Data and Statistical Analysis

The raw PRM data were processed using Skyline.²⁴ All assay details, assay parameters, response curves, repeatability data, detailed standard operating protocols, and additional assay-specific resources can be located on the CPTAC Assay Portal <https://assays.cancer.gov/> using the search term “CPTAC-1055”. Links to the assay development data on Panorama are available through the CPTAC-1055 assay page on the Assay Portal. Exported Skyline data from the PRM analysis of the ovarian tumor tissue specimens were

analyzed by a Mann-Whitney U-test to determine the statistical significance of the peak area ratios. *p*-values <0.05 were considered significant.

RESULTS AND DISCUSSION

Histone H4-K12acK16ac PRM Assay Development

To verify our previous observation that lower levels of H4-K12acK16ac are directly correlated with HRD status in high-grade serous ovarian tumors, we developed and validated a targeted mass spectrometry-based assay to measure the relative abundance of H4-K12acK16ac. Crude light and heavy H4-K12acK16ac peptides (H4 amino acids 9–17) were synthesized, and a PRM assay was developed as a Tier 2 assay according to the framework for targeted MS-based assay “fit-for-purpose” validation established by the National Cancer Institute Clinical Proteomic Tumor Analysis Consortium (NCI CPTAC).^{25–28} By definition, Tier 2 assays have a moderate-to-high degree of analytical validation, include labeled internal standards for every analyte, and have high specificity, moderate to high precision (20–35% CV), and high repeatability.

To ensure the specificity of the PRM assay, all of the transitions from the light and heavy H4-K12acK16ac peptides that were selected to be monitored (y_7^{1+} , y_6^{1+} , and y_6^{2+}) included both of the modified lysine residues (K12 and K16, Figure 1A). After establishing the scheduled method using these three transitions, the assay was validated by running a response curve in triplicate that covered four orders of magnitude (1 fmol/ μ L to 1 pmol/ μ L, Figure 1B). On the basis of the response curve data, the estimated limit of detection (LOD) and lower limit of quantification (LLOQ) of the assay were determined to be ~2.6 fmol/ μ L (Figure 1C). LOD, the lowest analyte concentration at which the signal is distinguishable from the noise, was calculated based on the average signal from three blank injections + 3* standard deviation of the peak area ratios observed for the lowest concentration. LLOQ, the lowest concentration of the analyte at which quantitative measurements can be made, was determined to be the lowest concentration at which the CV was <20%. It should be noted that because unpurified synthetic peptides were used to develop and validate the assay, the precise amount of each peptide standard was unknown. (Approximate amounts based on the values reported by the peptide vendor were used for all subsequent calculations.) Accordingly, the reported values for LOD and LLOQ are not absolute values.

To determine the performance of the H4-K12acK16ac assay across several days, a mini-validation of repeatability experiment was conducted wherein the assay was run at three concentration levels (low, medium, high) in triplicate on 5 days. The assay performance was assessed based on the intraday, interday, and total CVs (Figure 1C). In agreement with the acceptable performance criteria for Tier 2 assays, the Total CVs of the assay at the low, medium, and high concentration levels were all <20%. All assay resources are located on the CPTAC Assay Portal <https://assays.cancer.gov/> (assay no. CPTAC-1055).

Verification of Low H4-K12acK16ac Levels in HRD versus non-HRD Ovarian Tumors

After the development and validation of the Tier 2 H4-K12acK16ac PRM assay, we used the assay to assess the abundance of H4-K12acK16ac in the subset of the TCGA high-grade

serous ovarian tumors that we analyzed by iTRAQ- and SWATH-based proteomics.¹⁸ Of the initial total of 122 TCGA high-grade serous ovarian tumors to which we had access, only 97 were analyzed by PRM due to the availability of tumors containing a sufficient amount of peptides remaining after other proteomic analyses including iTRAQ and SWATH. Among the 97 ovarian tumor tissue specimens analyzed by PRM, 80 tumors (45 HRD, 35 non-HRD) were detected with H4-K12acK16ac signals above the LLOQ. The median relative abundance of H4-K12acK16ac (determined by the PRM peak area ratio of the endogenous light/synthetic spiked-in heavy peptide) in the non-HRD tumors was 1.34-fold higher than the HRD tumors, and the overall difference in the level of H4-K12acK16ac between the HRD and non-HRD tumors was significant ($p = 0.029$, Mann-Whitney U-test; Figure 2A, Supplemental Table 1). These results verify our previous observation that lower H4-K12acK16ac levels are associated with HRD status in ovarian tumors.

With the exception of one tumor, the relative abundance of H4-K12acK16ac in all of the HRD tumors was within two standard deviations of the mean. The relative abundance of H4-K12acK16ac in one of the HRD tumors exceeded four standard deviations of the mean, and thus it could be considered as an outlier. The relatively high level of H4-K12acK16ac in this tumor could be indicative of the intratumor heterogeneity of the specimen, wherein the H4-K12acK16ac levels could vary according to cell type. Omitting this tumor from the panel of HRD tumors increases the significance of the H4-K12acK16ac level between the HRD and non-HRD tumors to $p = 0.017$, and the magnitude of the median relative abundance of H4-K12acK16ac in the non-HRD tumors compared with the HRD tumors increases to 1.41-fold. A systematic analysis of the purity of the TCGA ovarian tumors indicated that this specimen had an intermediate Consensus Measurement of Purity Estimator (CPE) score of 0.798 (on a scale of 0 to 1.0, with a score of 1.0 indicating the highest level of purity).²⁹ However, it should be noted that tumor purity was not considered as a covariate in the present study, and thus conclusive statements cannot be made regarding any potential causal associations between tumor purity and H4-K12acK16ac levels.

Reflecting the increased sensitivity of PRM as a targeted MS method compared with an untargeted method such as iTRAQ, H4-K12acK16ac was only detected in 70% of the tumors that were analyzed via iTRAQ (85/122) compared with 82% of the tumors that were analyzed via PRM (80/97). There were 64 tumors (34 HRD, 30 non-HRD) in which H4-K12acK16ac was detected by both iTRAQ and PRM (Figure 2B). Of the 21 tumors in which H4-K12acK16ac was exclusively detected by iTRAQ, 6 were also analyzed by PRM and had undetectable H4-K12acK16ac levels. The unique detection of H4-K12acK16ac in 16 tumors by PRM and 21 tumors by iTRAQ is likely indicative of the fundamental differences in targeted proteomics strategies compared with shotgun proteomics approaches with one obvious difference that our targeted proteomics approach entailed 1D online LC-MS/MS and the shotgun proteomics approach used 2D LC-MS/MS with offline basic reverse-phase fractionation, followed by online LC-MS/MS.^{30–34}

To compare the iTRAQ versus PRM quantification of H4-K12acK16ac, the iTRAQ ratios (signal intensity of the H4-K12acK16ac in an individual sample normalized to a common control sample) and the PRM peak area ratios (endogenous light-peptide XIC peak area normalized to the spiked-in heavy-peptide XIC peak area) were converted to z-scores, and

the Pearson correlation coefficient (r) was 0.35 ($p < 0.005$), indicating a positive correlation between the iTRAQ- and PRM-based measurements of H4-K12acK16ac despite the fundamental differences in the respective methods of quantification (Supplemental Table 2). Additional statistical analysis was performed to further analyze the similarity between the PRM and iTRAQ data. A p value of 0.60 was obtained when conducting a Wilcoxon signed-rank test for paired samples to determine whether the true median difference between the PRM and iTRAQ measurements of the samples was 0. Thus this p value indicates that the null hypothesis of a median difference of 0 between the paired PRM and iTRAQ measurements of the same samples cannot be rejected.

To further confirm the results from our proteomic studies, we conducted Western blot analysis of Histone H4 acetylation in additional *BRCA* WT ($n = 3$) and *BRCA* mutant ($n = 4$) ovarian tumors. The loss of H4 monoacetylation in human tumor cells has been demonstrated to occur predominantly at K16 prior to K12, and the loss of H4 acetylation at K16 is a common hallmark of cancer.¹⁹ Our results indicated lower H4-K16ac expression levels in the *BRCA* mutant compared with the WT tumors when normalized to total H4 levels ($p = 0.034$), whereas the levels of H4-K12ac normalized to total H4 levels were unchanged (Figure 3A). Neither of these monoacetylated H4 peptides were detected in the TCGA ovarian tumors using an iTRAQ-based global proteomic approach, suggesting that the diacetylated H4-K12acK16ac peptide could have a higher level of relative abundance compared with the monoacetylated H4 peptides.

We also applied our developed H4-K12acK16ac PRM assay to the additional *BRCA* WT ($n = 3$) and mutant ($n = 4$) ovarian tumor specimens, and the average relative abundance of H4-K12acK16ac was approximately two times higher in the *BRCA* WT versus the mutant tumors (Figure 3B). However, the difference between the relative abundance levels of H4-K12acK16ac in the *BRCA* WT and mutant tumors was not significant. The lack of a significant difference in the H4-K12acK16ac levels could be reflective of the relatively small sample size ($n = 7$) compared with that of the TCGA tumor specimens ($n = 97$).

Decreased H4 Acetylation in HRD Tumors Is Associated with Elevated HDAC6 Levels

To determine whether the decreased H4 acetylation in HRD tumors is regulated at the total protein level or at the post-translational level, it is important to quantify not only the modified form of the peptide(s) of interest but also the corresponding unmodified form of the peptide(s) and the total protein.³⁵ However, in the case of the H4-K12acK16ac peptide, the corresponding unmodified peptide cannot be readily detected by a standard shotgun proteomics sample preparation workflow using trypsin as the cleavage enzyme. The lack of an acetyl group on unmodified K12 and K16 enables trypsin to cleave at those sites, consequently generating three peptides with low masses: ${}^9\text{GLGK}_{12}$: 187.73 m/z (2^+); ${}^{13}\text{GGAK}_{16}$: 166.69 m/z (2^+); and R_{17} : 175.12 m/z (1^+). These masses are below the lower full MS1 scan range cutoff of 400 m/z that was used to acquire the iTRAQ data for the TCGA high-grade serous ovarian tumors.¹⁸ Additionally, the mass-spectrometric method that was used to acquire the iTRAQ data included precursor ion charge-state screening wherein singly charged ions were rejected to enable the preferred selection of ions with z 2, which are predominantly tryptic peptides.

The quantification of the total H4 protein level in our global proteomic iTRAQ data set (33 unique peptides) indicated the lack of a significant difference in the relative abundance of this protein based on the HRD status of the ovarian tumors (Supplemental Table 3). Statistical analysis of the total H4 protein-normalized H4-K12acK16ac levels indicated significantly lower levels in the HRD versus non-HRD tumors ($p = 0.015$, Mann-Whitney U-test). This observation supports our hypothesis that it is the post-translational acetylation modification of H4 and not the level of the unmodified protein that is associated with HRD status.

We also analyzed the global levels of the histone acetyltransferases (EP300, KAT2A, KAT5, KAT6A, KAT7, and KAT8) and deacetylases (HDAC1-10) with known roles in H4 acetylation^{36,37} regardless of whether they were detected across all of the 122 TCGA ovarian tumor specimens (Supplemental Table 3). The relative abundance levels of the acetyltransferases did not differ significantly between the HRD and non-HRD tumors. Interestingly, the level of hMOF/KAT8, an important histone acetyltransferase that is responsible for the basal level of H4-K16 acetylation,³⁸ was unchanged. This could suggest a more influential role of HDACs in the difference between H4-K16 acetylation levels in high-grade serous ovarian tumors.

Whereas there was a lack of an HRD-associated difference in acetyltransferase expression, we observed elevated levels of HDAC6 expression in the HRD versus non-HRD tumors (Figure 4). Among the 10 HDACs that were quantified by iTRAQ, only HDAC6 had significantly increased expression in the HRD tumors ($p = 0.020$, Mann-Whitney U-test). HDAC6 overexpression is known to cause resistance to chemo-therapeutic drugs, and it is also associated with poor prognosis in lung adenocarcinoma.³⁹ Analysis of the association between total H4 protein-normalized H4-K12acK16ac levels and HDAC6 abundance indicated lower levels in the HRD versus non-HRD tumors ($p = 0.018$, Mann-Whitney U-test), thus providing additional evidence of an association between decreased H4-K12acK16ac levels and increased HDAC6 levels in the HRD tumors. In a separate study using a pathway-based differential dependency network approach to assess HRD-associated differences in acetylation-related proteins,⁴⁰ we previously showed that the levels of HDAC1 and associated proteins were increased at the pathway level together with decreased acetylation of H4 in HRD tumors at the PTM level.¹⁸

CONCLUSIONS

Our previous iTRAQ-based global proteomic study of high-grade serous ovarian carcinomas indicated that the dual acetylation of H4 at Lys12 and Lys16 correlates with HRD status. This observation was verified in the current study using PRM as an orthogonal analytical mass-spectrometry-based method. Similar trends in H4 acetylation levels were confirmed by Western blot using additional ovarian tumor tissue expressing mutant or WT *BRCA*. Given that HRD is associated with susceptibility to PARP inhibitors and that HDAC inhibitors are attractive anti-ovarian-cancer therapeutic targets,^{17,41} it is possible that specific H4 acetylation events associated with HRD could inform PARPi or HDAC inhibitor chemotherapeutic treatment modalities to improve patients' treatment response.

We have presented multiple lines of evidence that lower H4-K12acK16ac levels are significantly associated with HRD. A requisite subsequent step for the possible future assessment of the role of H4-K12acK16ac in stratifying ovarian cancer patients for PARPi treatment would be to determine whether this statistical association is valid for patients who respond to PARPi treatment. Additionally, it would be beneficial to conduct further studies toward the elucidation of the mechanism(s) that underlie the lower H4-K12acK16ac levels that are observed in ovarian cancer patients with HRD tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CPTAC	Clinical Proteomic Tumor Analysis Consortium
CV	coefficient of variation
HRD	homologous recombination deficiency
iTRAQ	isobaric tags for relative and absolute quantitation
LLOQ	lower limit of quantification
LOD	limit of detection
PARP	poly(ADP-ribose) polymerase
PRM	parallel reaction monitoring
PTM	post-translational modification
RT	retention time
SIS	stable isotope-labeled peptide standard
XIC	extracted ion chromatogram

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Figure 1.

Validation of H4-K12acK16ac PRM assay. (A) Representative XICs of the light and heavy peptides. Insets show XICs of the transitions for each peptide. (B) Response curve. The peak area ratio (light/heavy) for each transition is plotted versus the theoretical concentration (fmol/μL). The curve was run in triplicate. (C) Assay validation parameters determined from the response curve and the 5 day repeatability experiment. *Concentrations are approximate as the assay was developed and validated using crude peptides (~60% purity).

Figure 2.

Lower relative abundance of H4-K12acK16ac in HRD versus non-HRD tumors. (A) H4-K12acK16ac was measured by PRM in 45 HRD and 35 non-HRD tumors. (B) Quantifiable levels of H4-K12acK16ac were detected in 64 (34 HRD and 30 non-HRD) of the tumors that were analyzed by iTRAQ and PRM.

Figure 3.

Detection of H4-K12ac and H4-K16ac in an independent set of WT and mutant *BRCA* ovarian tumor tissues. (A) Western blot analysis of H4-K12ac, H4-K16ac, total H4, and actin expression. Densitometric analysis (lower panel). (B) PRM analysis of the H4-K12acK16ac expression in the same tissues. n.s., not significant.

Figure 4. HRD-associated elevated expression of HDAC6. Among the 10 HDACs that were quantified by iTRAQ, only HDAC6 had significantly increased expression in the HRD compared with the non-HRD tumors.