Clinical management of pancreatic cancer aided by histone signatures

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Characterization of histone-related chemical modifications in formalin-fixed paraffin-embedded and fresh-frozen human pancreatic cancer xenografts using LC-MS/MS

Monika Bauden¹, Theresa Kristl², Roland Andersson¹, György Marko-Varga³ and Daniel Ansari¹

Post-translational modifications (PTMs) of histones including acetylation, methylation, and ubiquitination are known to be involved in the epigenetic regulation of gene expression and thus can have an important role in tumorigenesis. A number of PTMs have been linked to pancreatic cancer and are frequently studied as potential targets for cancer therapy or diagnosis. The availability of biobank-stored, formalin-fixed, paraffin-embedded (FFPE) materials and advanced proteomic analytical tools make it possible to detect histone-related PTMs using predicted mass shifts caused by specific modification. It is, however, important to take into account the fact that formaldehyde (FA) present in the FFPE material is chemically reactive and may undergo condensation reactions, for example, with terminal amino groups and active CH functionalities of the studied proteins. As supported by the results of this study, the possibility to misinterpret such protein condensation product as endogenous PTMs should be taken into consideration in all proteomic analytical work involving FFPE materials. In this study, we used liquid chromatography-tandem mass spectrometry to assess preassumed modification of the lysine residues of histone proteins in FFPE or fresh-frozen (FF) tumor xenografts, derived from the human pancreatic cancer cell line, Capan-1. Here we report modifications with a defined mass shift of +14.016, +28.031, +42.011, or +114.043 Da, corresponding to apparent methylation, dimethylation, acetylation, or ubiquitination that were differentially distributed between the groups. The identified modifications were significantly more frequent in FFPE samples as compared with FF samples. Our results indicate that FFPE tissue processing may result in persistent chemical modifications of histones, which correspond in mass shift of important PTMs. Herein, we highlight the importance to investigate and report FA-formed modifications in FFPE-treated tissues, as well as the necessity of careful manual examination of observed modifications to eliminate false-positive PTMs.

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In eukaryotic nuclei, chromosomal DNA is tightly packed in a compact chromatin, a macromolecular structure composed of repetitive fundamental units, nucleosomes. Nucleosomes are in turn formed of 166 bp DNA wrapped around a histone octamer core consisting of histone H3–H4 tetramer and two copies of histone H2A–H2B dimers. Adjacent linker histones such as histone H1 can further package the nucleosome into the higher-order chromatin. Basic histones are often subjected to post-translational modifications (PTMs) located on both the tail and the globular domain of the protein.¹⁻⁴ PTMs of histones, including acetylation, methylation, and/or ubiquitination typically occurring on lysine, arginine, or serine residues, are known to be involved in epigenetic regulation of gene expression and thus have an important role in tumorigenesis.⁵ A number of PTMs of histones, linked to diverse malignancies including pancreatic cancer, are frequently studied as potential targets for cancer therapy. Novel cancer-specific histone PTMs could also provide information for the discovery of biomarkers that would be suitable for diagnostic, prognostic, or predictive purposes.⁶⁻⁸

Owing to the insufficient availability of required amounts of fresh tissue biopsies, studies are often based on archived formaldehyde-fixed, paraffin-embedded (FFPE) tissue blocks, serving as an important clinical resource.

In this respect, the fixing with paraformaldehyde (PFA) followed by embedding in paraffin is a classical standard
method in pathology for preservation and morphology analysis of clinical tissue material. Several techniques have been developed to extract effectively proteins from FFPE tissue, allowing subsequent proteomic analyses of the digested proteins.9–12 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) approaches are commonly applied to detect PTM sites on histones, by tracing predicted mass shifts caused by a particular modification.13

FFPE-preserved tissues offer many advantages as compared with fresh-frozen (FF) biopsies, in particular regarding the high stability at room temperature and the extensive availability from global biobanks. Formaldehyde (FA) fixation of the tissue also provides an outstanding structural preservation of the tissue morphology, which is not possible when the tissue is stored at ultralow temperatures.14 It is however important to consider that FA present in FFPE materials may undergo condensation reaction with, for example, free N-terminal amino groups, other amino- and thiol-residues, as well as with active CH functionalities of the proteins, forming attached methyl and methyol groups, via intermediate Schiff bases or imines and crosslinking methylene bridges between amino groups.15,16 In addition, as reported previously, N-terminal amino groups and particularly lysine side chains account for a great majority of FA fixation-induced modifications. Methylation corresponding to a mass shift of +14.016 Da, methylene adducts (+12 Da), methylol adducts (+30.0106 Da), and formylation (+27.9949 Da) were identified as the most significant.17–19

It is clear that tissue specimens are subjected to a wide range of chemical processing before the final proteomic analysis, starting with formalin fixation and paraffin impregnation for tissue preservation, followed by heat-induced antigen retrieval to reverse the effect of the previous treatment as well as to improve the protein yield.20,21

The pattern of chemical modifications in tissue proteins after formalin treatment has been studied previously.18,19 It has, however, not yet been fully elucidated whether chemical reactions occurring during FFPE preservation of tissue could result in irreversible alterations of histone proteins to induce shifts in a multifold of masses that could be incorrectly interpreted as endogenously formed PTMs.

In this study, we evaluated and compared chemical modifications on the basic residues of the histone proteins originating from pancreatic tumor xenografts tissue, subjected to FFPE or FF processing. The focus was directed towards modifications resulting in a defined mass shift of +14.016, +28.031, +42.047, +42.011, and +114.043 Da, assigned as methylation, dimethylation, trimethylation, acetylation, or ubiquitination, respectively, considered as PTMs potentially causing epigenetic changes frequently occurring in diverse malignancies.

**MATERIALS AND METHODS**

**Materials**
The following chemicals and solvents were purchased from Sigma-Aldrich (St Louis, MO, USA); Tris-HCl, guanidine-HCl, ammonium bicarbonate (AMBIC), dithiothreitol (DTT), iodoacetamide (IAA), formic acid, and acetonitrile (ACN). PFA, xylene and paraffin were obtained from Histolab Products AB (Gotemburg, Sweden) and ethanol (EtOH) from Solveco (Rosenberg, Sweden). BCA assay, Pierce quantitative colorimetric peptide assay, and Pierce LC-MS grade water were obtained from Thermo Scientific (Rockford, IL, USA). Milli Q water was produced using in-house installed purification system Q-POD Millipore (EMD Millipore, Billerica, MA, USA).

Thermo Fisher Scientific (Bremen, Germany) was the supplier of analytical instruments used in this study, including EASY-nLC 1000 and quadrupole Orbitrap (Q Exactive) mass spectrometer equipped with a Thermo nanospray Flex ion source. The table centrifuge 5415R, speed vacuum Concentrator plus, and thermomixer Comfort were provided by Eppendorf AG (Hamburg, Germany).

**Pancreatic Cancer Cell-Derived Tumor Xenograft Model**
To establish the homogeneity of the experimental material, human xenografts were generated in genetically identical NMRI-nu mice (Janvier Labs, Saint-Berthevin Cedex, France) by inoculation of human pancreatic cancer cell line Capan-1 (ATCC, Manassas, VA, USA) originating from liver metastasis derived from pancreas adenocarcinoma in the head of the human pancreas. Briefly, the cells from the same passage were detached using TrypLe 10x (Gibco, Life Technologies, Grand Island, NY, USA) harvested, and pelleted at 300 g for 3 min. After the pellet dissociation, by gently pipetting, the cell concentration and viability was determined using 0.06% Trypan blue. A total of 25 × 10⁶ viable cells were then resuspended in 1.25 ml serum-free Iscove’s modified Dulbecco’s medium (Gibco) and stored on ice until inoculation. Fifty microliter cell suspension containing 1 × 10⁶ tumor cells was then subcutaneously injected through a 27 3/4-gauge needle into the right flank of the individual animals, within 1 h after harvesting. The tumors were resected 2 weeks after inoculation. The animals were housed in standardized pathogen-free conditions in individually ventilated cages and provided illimitable access to food, drinking water, standard rodent chew, and nesting material. All animal handling was performed in a dedicated room and received proper animal care in accordance with the guidelines of the Swedish Government and the Lund University (Lund, Sweden). This study (No. M 273-12) was approved by the local ethical committee at Lund University.

**Tissue Samples**
Tissue samples were obtained from solid human pancreatic subcutaneous tumor xenografts developed from inoculated human pancreatic cancer cell line, Capan-1. Directly after the necropsic excision, each tumor was divided in the middle to create two parts of the same tumor. One part of the individual tumor was then snap frozen in liquid N₂ and stored at −80 °C until further use. The corresponding tumor half was fixed in 4% PFA for 48 h at 4 °C, dehydrated in...
graded series of ethanol and xylene, embedded in paraffin, and stored at room temperature until analysis. Samples were stored for 12 weeks before protein extraction. This study comprised two sample cohorts that included nine FFPE and FF samples, respectively.

**Protein Extraction**

**FFPE tissue**

Eight 10 μm tissue sections with an area up to 80 mm² were cut from FFPE tissue blocks according to the standard methodology, collected in 2 ml maximum recovery microtubes (Axygen, Union City, CA, USA), deparaffinized, and extracted. Briefly, the sections were incubated two times for 10 min at 97 °C in 1 ml EnVision FLEX retrieval solution (pH 8) (Daco Denmark A/S, Glostrup, Denmark) diluted 1:50 according to the manufacturer’s instructions. After the careful paraffin and retrieval solution removal, the deparaffinized tissues were sonicated with a probe in an extraction buffer (150 μl of 500 mM Tris–HCl (pH 8) and 150 μl of 6 M guanidine–HCl in 50 mM AMBIC) for 20 min on ice, followed by centrifugation for 1 min at 14 000 r.c.f. at 4 °C, to remove debris. The soluble proteins in the supernatant were reduced with 15 mM DTT for 60 min at 60 °C, alkylated for 30 min at room temperature using 50 mM IAA, and precipitated overnight with ice-cold absolute ethanol, with the ratio of one part sample and nine parts 99.5% EtOH. The next day, the precipitated samples were centrifuged for 15 min at 14 000 r.c.f. at room temperature. The pelleted proteins were air-dried, dissolved in 250 μl 50 mM AMBIC, and quantified by its protein content using the BCA assay. The absorbance was measured at 540 nm using the multiScan Plate Reader (Thermo Scientific). One hundred micrograms of protein was digested overnight at 37 °C using Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) with a protein ratio of 1:100. Speed vacuum-dried peptides that were missing in the corresponding FFPE and FF samples were excluded from the analysis. The findings were further processed as described above.

**FF tissue**

The respective tissue samples were individually grounded into a fine powder using mortar filled with liquid N₂ and pestle. The tissue powder was thereafter transferred into 2 ml maximum recovery microtubes filled with ice-cold extraction buffer supplemented with protease and phosphatase inhibitors. The samples were kept on ice until the reduction step. Additional steps including extraction of nuclear proteins followed by histone purification could be omitted, as the number of identified histone variants was not increased. The samples were further processed as described above.

**LC-MS/MS Analysis**

The peptide analysis was performed using a high-performance liquid chromatography system EASY-nLC 1000 connected to a quadrupole Orbitrap (Q Exactive) mass spectrometer equipped with a Thermo Nanospray Flex ion source.

For each sample, the tryptic peptides were dissolved in 0.1% formic acid to obtain a concentration of 0.25 μg/μl. One microgram of sample was injected and measured with a flow rate of 300 nl/min and separated with a 150 min gradient of 4–40% ACN in 0.1% formic acid using a two-column setup, including the Acclaim PepMap RSLC 75 μm × 25 cm as analytical column and Acclaim PepMap 100, 75 μm × 2 cm as precolumn. Each sample was measured in duplicate.

The Q Exactive system was operated in the positive data-dependent acquisition mode. For peptide identification a full MS survey scan was performed in the Orbitrap. Fifteen data-dependent higher energy collision dissociation MS/MS scans were performed on the most intense precursors. The spray voltage was set to 1.75 kV with the capillary temperature of 300 °C. Moreover, the S-lens RF level was fixed at 50. The MS1 survey scans of the eluting peptides were executed with a resolution of 70 000, recording a window between m/z 400.0 and 1600.0. The automatic gain control (AGC) target was set to 1e6 with an injecting time of 100 ms. The normalized collision energy was set at 25.0% for all scans. The resolution of the data-dependent MS2 scans was fixed at 17 500 and the values for the AGC target and inject time were 1 × 10⁶ and 120 ms.

**Identification of Proteins and Detection of Chemical Modifications**

The Proteome Discoverer software, version 1.4, obtained from Thermo Fisher was used to identify the proteins including information about the number of unique peptides, sequence coverages, and modifications. The selection of spectra contained the following settings: min precursor mass 350 Da; max precursor mass 5000 Da; s/n threshold 1.5. Parameters for Sequest HT searches were as follows: precursor mass tolerance 10 p.p.m.; fragment mass tolerance 0.002 Da; trypsin; 1 missed cleavage site; Uniprot human database; dynamic modifications: acetyl (+42.011 Da; K); methyl (+14.016 Da; K, R); dimethyl (+28.031 Da; K, R); trimethyl (+42.047 Da; K, R), glygly (+114.043 Da; K), and oxidation (+15.995 Da; M, P) fixed modification: carbamoylmethylation (+57.021 Da; K). The percolator was used for the processing node and the false discovery rate value was set to 0.01. The list of obtained histone proteins was then exported to Excel for further analysis.

**Statistical Analysis**

Histone proteins identified in less than six samples in each respective group as well as histone modifications expressed on peptides that were missing in the corresponding FFPE and FF sample were excluded from the analysis. The findings were further assessed in the presence or absence of respective histone modification and analyzed as categorical data. P-values at a significance level of 0.05 were computed using Fisher’s exact test with GraphPad Prism v.6.0 (GraphPad
RESULTS

Histone Protein Identification
Proteins were extracted from nine resected Capan-1 cell line developed pancreatic tumor xenografts, preserved as FF or FFPE samples. Proteins, detected in both groups on a high confidence level, enabled a direct comparison. Peptides associated with the main core histone protein families could be linked to several subtypes of H2A and H2B, H3 and H4, as well as a number of variants of linker histone proteins including H1.0, H1.1, H1.2, H1.5, and H1x. In both of the experimental groups, however, proteins sharing a common unspecific peptide were identified because of high sequence homology within the main histone families. For each histone protein, the yields of the unique peptides were concordant in both groups. In the FFPE group, the total number of peptides associated with the respective proteins of interest was slightly increased as compared with the FF group. No significant differences were observed between the groups regarding the sequence coverage of the histone core proteins. On the contrary, the FFPE samples exhibited twice as high sequence coverages of linker histone proteins, specifically the H1.1, H1.2, and H1.5, in comparison with the FF samples.

Overall, the identity and the number of histone proteins were expressed in principal consistent in both the FF and the FFPE samples. Specific histone variants identified are summarized in Table 1 along with the number of detected peptides and sequence coverages.

Identification of Histone Modifications
Five preassumed modifications, with a mass shift of +14.016, +28.031, +42.011, +42.047, and +114.043 Da, equivalent to apparent methylated (Me), dimethylated (Me2), acetylated (Ac), trimethylated (Me3), and ubiquitinated (Ub) peptides, respectively, were searched for in the MS spectrum of both of the FF and the FFPE samples. An example of apparent methylation, a modification associated with a mass shift of +14.016 Da is illustrated in Figure 1.

In total, 20 modification sites and 33 individual modifications, located on lysine (K) residues, were identified in FFPE samples, as compared with five modification sites and six distinct modifications in the corresponding FF specimens. The comparison was made by including peptides detected in both groups. The modifications were distributed in both of the globular domain and the terminal tails of the core histones and in the globular domain of the linker histone family H1, as illustrated in Figure 2. No modifications were found on arginine residues.

In FFPE samples, between one and five modified sites were detected in the respective H1.1, H1.2, H1.5, H2A, H2B, H3, and H4 proteins. A maximum of three different modifications were annotated per site. Lysine methylation (+14.016 Da, 48%) was the most prominent modification, followed by ubiquitination (+114.043 Da, 39%) and acetylation (+42.011 Da, 9%). Trimethylation was identified only sporadically in a limited number of FFPE-treated samples. Five individual modifications (25%) were consistently detected in all FFPE samples. The remaining modifications were unevenly spread within the group (Figure 3).

Ten modifications, with a mass shift of +14.016 Da (62.5%), one modification, with a mass shift of +42.011 Da (33%), and five modifications, with a mass shift of +114.043 Da (38.5%), were significantly increased in the FFPE group.

In FF specimens, five modifications (83%) were consistently detected in all samples. A modification with a mass shift of +114.043 Da was detected in one sample only. One modification with a mass increase of +42.011 Da (33%), located on H3K24, was significantly more frequent in FF samples. A modification with a mass shift of +28.031 Da situated on H3K80 was identified in the FF group only.

In total, five individual modifications were detected in both FFPE and FF groups. All identified modifications and their distribution, within the respective group, are summarized in Table 2.

DISCUSSION
Histone modifications, especially methylation, acetylation, and ubiquitination, are recognized to impact biological processes and cause epigenetic changes associated with diverse malignancies. At present, it is widely accepted that histone-related PTMs are involved in the epigenetic regulation in pancreatic cancer, and epigenetic cancer profiling is still an active area of interest.

Although proteomic-based analyses are undeniably a powerful tool for epigenetic research, the discovery of novel PTMs using FFPE samples may, however, be a challenging assignment. Apart from all parameters that needs to be taken into consideration when designing a sequence alignment algorithm, FFPE treatment may, for example, generate various lysine modifications that are randomly distributed thorough the MS/MS spectra.

In this study, we investigated the manifestation of specified histone modifications situated on, for example, side chains of basic amino acids in FFPE and FF processed pancreatic cancer xenograft tissues. We focused on modifications with a defined mass shift of +14.016, +28.031, +42.047, +42.011, and +114.043 Da, interpreted as apparent methylation, dimethylation, trimethylation, acetylation, and ubiquitination, respectively. Accordingly, we performed a traditional bottom-up proteomic analysis, in which we searched the LC-MS/MS data for methylated, acetylated, and ubiquitinated basic residues of histone proteins, based on the mass shift caused by the corresponding modification. In this study, a complete analysis of histone modifications was, however, not possible to achieve. The sequence of histone proteins, and especially
Table 1 Histone protein identification in FF (A) and FFPE (B) samples

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<th>Accession</th>
<th>Description</th>
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<th>Coverage % (median (range))</th>
<th>No. of unique peptides (median (range))</th>
<th>No. of peptides (median (range))</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q71DI3</td>
<td>Histone H3.2</td>
<td>54.50</td>
<td>54.42 (41.91–61.76)</td>
<td>1 (1–2)</td>
<td>7 (6–8)</td>
</tr>
<tr>
<td>P84243</td>
<td>Histone H3.3</td>
<td>52.39</td>
<td>51.84</td>
<td>1 (1–2)</td>
<td>7 (6–8)</td>
</tr>
</tbody>
</table>

MS analysis of chemical modifications of histone proteins
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H1 variants, is highly rich in both lysine and arginine, making the bottom-up analysis quite challenging. The digestion with trypsin resulted, in some cases, in low sequence coverages and short peptides yields (≤3 amino acids) that were not possible to evaluate.28 We believe, however, that the sequence coverage and the number of identified modifications, detected within the obtained unique peptides, were sufficient to confirm our hypothesis.

Apart from the fixation procedure, where FF tissues were snap frozen in non-reactive liquid N2 and FFPE specimens were subjected to 4% FA solution, all samples were thereafter treated equally, using an optimized protocol for high protein and peptide recovery. The only exception during sample preparation was the heat treatment in the presence of the retrieval solution, which was applied exclusively to FFPE samples to remove paraffin and reverse the crosslinks generated by FA fixation.29 Despite the heat treatment applied to FFPE samples, we expected to find a broad modification array in FFPE samples as FA is known to react chemically with various functional groups of proteins, glycoproteins, or

### Table 1 Continued

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Coverage % (mean)</th>
<th>Coverage % (median range)</th>
<th>No. of unique peptides (median range)</th>
<th>No. of peptides (median range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q6NX12</td>
<td>Histone H3.3C</td>
<td>31.11</td>
<td>31.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P62805</td>
<td>Histone H4</td>
<td>53.08</td>
<td>53.4 (52.43–53.40)</td>
<td>8 (7–10)</td>
<td>8 (7–10)</td>
</tr>
</tbody>
</table>

Abbreviations: FF, fresh-frozen; FFPE, formalin-fixed, paraffin-embedded.

Sequence coverage of histone proteins found in nine FF and nine FFPE samples is demonstrated both as mean and median with range noted within parentheses. Total number of unique peptides as well as total number of all identified peptides are reported as median with range noted within parentheses. Histone protein variants found in both groups are indicated by bold text. Histone protein variants found only in respective group are indicated by italic text.

**Figure 1** Tandem mass spectrometry (MS/MS) spectrum alignment illustrating a specific mass shift of +14.016 interpreted as methylation, caused by a chemical or post-translational modification.
nucleoproteins in a crosslinking matter, creating diverse peptide adducts.\textsuperscript{14}

The data presented here clearly show that the frequency of identified lysine modifications of the histone proteins was significantly higher in FFPE samples as compared with the corresponding FF samples. Hence, we suggest that such chemical alterations of, for example, lysine residues may result from FFPE processing.

Consistent with previously reported findings,\textsuperscript{19} the most pronounced identified modification in FFPE-processed tissue was lysine methylation, producing a mass shift of +14.016 Da. The reductive methylation reaction, using FA as a chemical reagent, is a well-established technique to chemically modify lysine residues.\textsuperscript{30} Furthermore, additional reactions can occur and thus contribute to the crosslinking action of FA.\textsuperscript{17} In addition, the Mannich reaction has been proposed to participate in the process of FA tissue fixation.\textsuperscript{31}

The Mannich reaction is known as a multicomponent reaction, in which FA reacts with an amine to yield a condensation product, such as a Schiff base or an attached methylol residue, which then attacks a substrate possessing nucleophilic properties. Another aspect of the reaction is the replacement of an active hydrogen atom in the substrate by an aminomethyl group. A number of compounds containing, for example, an NH residue may act as either substrate or amine component in Mannich reaction including amino acids or dimethylamines.\textsuperscript{32}

Peptide adducts with a mass increase of 114.043 Da, coinciding with the methylation array on the subtypes of H1, H2A, and H2B, accounted for the second next frequently identified modification.

We conclude that the distinct mass shift of +114.043 Da (apparent ubiquitination) could correspond to an arrangement of FA adducts consisting of four methylations (+56 Da), in any combination of mono- and dimethylations and two aminomethylated condensation products (+58 Da), formed by a Mannich reactions occurring within the same fragment but, at least partly, on different chemically reactive sites. A lysine modification with the mass shift of +58 Da was reported previously in connection to LC-MS/MS analysis of FFPE-processed tissue.\textsuperscript{19}

Peptide adducts with a mass increase of 42.011 Da (apparent acetylation), detected on lysines originating from the H1 linker family were considered as FA adducts composed

![Figure 2](https://www.laboratoryinvestigation.org/LaboratoryInvestigation/Volume97/March2017/images/fig2.png)

**Figure 2** Sequence alignments of histone proteins, visualizing the sites of detected modifications. The colors of the boxes correspond to the respective defined modifications with specific mass shifts. Modification sites detected only in fresh-frozen (FF) samples are marked with a dashed box. Modifications identified in both formalin-fixed, paraffin-embedded (FFPE) and FF samples are marked with ‘★’.
of a methylene bridge (+12 Da) and a hydroxymethyl/methylol group (+30.011 Da).

Above reported modifications, which are likely a condensation product resulting from FA reactions with tissue proteins, may be mistaken for endogenous PTMs when analyzing the FFPE tissue by MS.

All the remaining identified histone modifications, occurring exclusively in FFPE material, are hence most probably PTM artifacts resulting from chemical modifications induced by the FFPE tissue processing.

As may be concluded from the results of this study, only the modifications consistently found in both groups or being homogeneously distributed in FF samples, interpreted as H3K24Ac, H1.2K34Me, H3K80Me, H3K80Me2, and H1.1K55Me2, may be of biological origin.

Database searches for specific modifications indicate that acetylation of H3K24 occurs in the brain in response to a hypoxic environment. Tumor hypoxia is among other factors accepted as a clinical hallmark of pancreatic cancer and is associated with the progression of the disease. Hypothetically, the acetylation of H3K24 in tumor xenografts may be a resulting outcome of the oxygen-deprived tumor microenvironment. Although multitude PTMs have been identified within the globular domain of histones, it is still not fully proven how these modifications influence the nucleosomal structure and stability. Several PTM sites on H1 variants and the functional relevance of these alterations has been examined in a small number of sites. One example is the acetylation of lysine 34 on H1.4 linked to transcriptional activation and increased dynamic mobility, data generated from in vitro studies.

We speculate that the possible PTMs identified in xenografts developed from Capan-1 may possess similar functional properties. H3K24Ac and H1.2K34Me might be of special relevance for further investigation as they are located on the N-terminal of the histone tail. These results need,
However, to be verified with an orthogonal method and in a larger cohort of samples, which is beyond the scope of this study.

In summary, our results indicate that the FFPE tissue preservation may result in irreversible chemical modifications of the histone proteins originating from pancreatic tumor xenografts. Tissue proteins subjected to an extensive processing, including FA fixation, may undergo series of reactions, creating a mixture of condensation products. The possibility to mistake such condensation products for endogenous PTMs should be considered in all proteomic analysis of any tissue.

Modifications with a defined mass shift of +14.016, +28.031, +42.011, or +114.043 Da, corresponding to the apparent PTMs methylation, dimethylation, acetylation, and ubiquitination, exclusively present in FFPE-processed tissue, are thus most likely products resulting from FA associated reactions with the tissue proteins.

Most importantly, we have highlighted the importance to investigate and report chemical modifications of FFPE-treated tissues and the significance of careful manual examination of obtained modifications to eliminate false-positive PTMs.

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**DISCLOSURE/CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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