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The exposure to human breast cancer cells altered 14 post-translational modifications of human serum albumin

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ABSTRACT

Purpose

Serum albumin is in contact with practically all cells in the human body, including tumor cells in cancer patients. The purpose of this study was to explore whether cancer cells affect post-translational modifications (PTMs) of albumin.

Material and methods

Mass spectrometry was used to identify the PTMs. Purified human serum albumin was incubated with human breast cancer cells MDA-MB-231, MDA-MB-468, MCF7, or kept in water or in cell culture media. PTMs which were affected upon exposure of the albumin to cancer cells were identified. Three-dimensional analysis was performed to locate PTMs in albumin.

Results

We report here that an exposure to human breast cancer cells affected post-translational modifications (PTMs) of 14 peptides of human serum albumin (HSA). PTMs at 8 peptides were observed upon exposure of HSA to metastatic MDA-MB-231 and MDA-MB-468 breast cancer cells. PTMs at another 6 peptides were lost in MDA-MB-231 and MDA-MB-468 cells, while these 6 PTMs were observed in HSA exposed to conditionally tumorigenic MCF7 cells, or in HSA kept in water or a cell culture medium. Cancer cell altered phosphorylation, deamidation followed by methylation, acetylation, myristylation, palmitoylation, methylation, cysteine persulfide, and S-6-FMN cysteine modifications were detected in HSA. These PTMs locate predominantly in IB and IIA domains of HSA. Three-dimensional analysis showed that this region corresponds to the lipid-binding site and Sudlow's site 1.

Conclusion

Data reported here show that 14 PTMs of human serum albumin can be modified upon its exposure to human breast cancer cells.

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1. INTRODUCTION

Tumor cells modulate the matrix, the enzymatic and biochemical composition of the extracellular environment in a tumor [1, 2]. This modulation may include changes of post-translational modifications (PTMs) of proteins in the extracellular environment. PTMs may reflect the functional status of a tumor and serve as markers for the diagnostic and prognosis of cancer [3].

The number of reported PTMs in human serum albumin (HSA) is higher than 120 (reviewed in ref. [4]). The high number of PTMs indicates that there are many different forms of HSA due to different combinations of PTMs in a given HSA molecule. The diversity of PTM types is also broad and includes at least 30 types of PTMs, such as phosphorylation, acetylation, methylation, glycosylation, myristylation, palmitoylation, farnesylation, and PARylation [4-9]. Some of these PTMs in HSA have confirmed marker values for diagnosis and monitoring of diseases, e.g., the end glycation of HSA is the marker of diabetes [6-8]. Many other albumin PTMs do not have yet established links to physiological conditions. PTMs may be generated by enzymatic or chemical reactions. Phosphorylation, glycosylation, and ADP-ribosylation are examples of enzyme-dependent PTMs. Generation of advanced end glycation products is an example of non-enzymatic PTM. Oxidation of serum albumin at Cys34 is regarded as the marker of oxidative stress-related diseases [10, 11].

Serum albumin is the most abundant protein in human plasma [12, 13]. Albumin is highly abundant in the interstitial fluid of tumors, with albumin concentration reaching up to 60% of its concentration in plasma [12]. PTMs of albumin can be observed even in early-stage tumors, if these PTMs are stable in the interstitial fluid, lymph, and plasma. Protein acetylation and methylation are examples of such relatively stable PTMs [14]. Aggressive breast tumors often release circulating tumor cells in the bloodstream [15]. When the circulating tumor cells interact with albumin, it may be an additional opportunity to generate cancer relevant PTMs in albumin, in addition to PTMs that would be generated in the tumor. Therefore, presence of albumin in the interstitial fluid of tumors and in plasma, and the contact of albumin with tumor cells may lead to post-translational modifications of albumin. These tumor specific PTMs may serve as cancer markers.

Search for phosphorylation and acetylation in albumin from cancer patients identified a number of modified sites, even though the cancer

specificity of these PTMs has to be confirmed [4, 16-20]. The specificity issue is underlined by the possible impact of different non-cancerous conditions of the tested patients, e.g., diabetes, inflammatory diseases, renal or liver impairment. The use of an experimental model with only tumor cells may solve the specificity concern.

The published reports show that PTMs of albumin are frequent, and these PTMs may be a sensing mechanism for the status of a tumor. Therefore, it is important to explore whether human cancer cells affect PTMs profile of serum albumin. If PTMs of HSA are changed upon exposure to cancer cells, PTMs identification would be required for further clinical study of these PTMs as markers.

Here we report alterations of 14 PTMs of human serum albumin due to HSA exposure to human breast cancer cells.

2. MATERIALS AND METHODS

2.1. Materials

Human serum albumin was purchased from Abcam (Abcam ab205808, accession number P02768, 609 amino acids). Chemicals and reagents were purchased from Sigma Aldrich, Merk, and ThermoScientific, and were of analytical grade. This work was performed under an IBC permit from Qatar University (QU-IBC-2019/023). MCF7 (HTB-22), MDA-MB-231 (HTB-26), and MDA-MB-468 (HTB-132) human breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, USA).

The cells used in this study were in culture for less than one year. The cells were regularly tested for contaminations by mycoplasma, bacteria, and fungi. The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin, as recommended for these cells.

2.2. Preparation of albumin samples and electrophoresis

Aliquots of albumin were prepared in deionized (Millipore) water followed by glass-distilled water (pH 7.0-7.2), and stored frozen at -20^{0}C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described earlier [9]. 10% acrylamide gel was used. Dithiothreitol (DTT) and iodoacetamide (IAA) pre-treatment of samples is frequently used in SDS-PAGE to break Cys-Cys bonds in proteins. However, this pre-treatment may also modify cysteine PTMs, as such we did not do pre-treatment of albumin samples with dithiothreitol (DTT) and (IAA). This preserved PTMs that could be affected by DTT and/or IAA. Staining with Coomassie Brilliant Blue R-250 was used to detect proteins separated in gels.

2.3. Cell culture and electrophoresis of albumin

Cells were seeded in 10 cm dishes to form confluent. The cells were washed 2 times with serum-free DMEM, then incubated with fresh serum-free DMEM for 3 hrs. The medium was changed to the fresh serum-free DMEM with added 5% human serum albumin. The cells were incubated for 24 hrs. The medium was collected and centrifuged for 10 min at 21,000 g (15,000 rpm). 10 μL of the cleared medium was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described earlier [9].

2.4. Mass spectrometry

MALDI-TOF MS (Ultraflextreme, Bruker Daltonics, Bremen, Germany) mass spectrometry was used for the analysis of albumin samples. Albumin samples were prepared by in-gel digestion with porcine mass spectrometry-grade trypsin (sequence grade, Promega, USA), as described previously [9]. In brief, protein bands of interest in gels were excised and collected

in tubes. Gels were destained, washed with water, 50mM ammonium bicarbonate, and 4 times with 100% acetonitrile. After the removal of acetonitrile, the gels were air-dried. Solution of trypsin (5 to 10 µl; 0.1-0.2 µg) was added to the dried gel, and incubated for 18-24 hrs at 37^0C. Digest of the protein was extracted by adding 30-50 µl of 50% acetonitrile and 0.1% trifluoracetic acid (TFA) in water. Extraction was for 2-3 hrs under gentle agitation. The extracted digest was loaded on a MALDI target (polished steel) with matrix α-cyano-4-hydroxycinnamic acid (Sigma Aldrich, USA).

Matrix-assisted laser-desorption ionization (MALDI) mass spectrometry was performed with Ultraflextreme instrument (Bruker Daltonics, Bremen, Germany). Mass spectra were collected in the range from 500 to 3,500 m/z values, positive mode, single protonated, and with a collection of a minimum of 10,000 laser shots for every single spectrum. For calibration, tryptic autodigestion peptides were used (842.51, 1045.56, and 2211.10 Da). Spectra were collected by using FlexControl software (Bruker Daltonics, Bremen, Germany). Analysis of spectra was performed with FlexAnalysis software (Bruker Daltonics, Bremen, Germany). Collected mass lists were used for identification by using ProFound and Mascot search engines. NCBIr database (version by October 2021) was used in the searches, and the significance of identification was set to $p<0.5$. Mass tolerance was +/-0.1 Da for peptide mass fingerprinting, the database was set to "human", with no restriction of pI, and molecular mass was set to +/- 50 kDa of the experimental migration position. For identification, the number of matched peptides and coverage of the identified protein with detected peptides were considered.

2.5. Prediction of PTMs

For the identification of modified peptides, we used FindMod tool that predicts PTMs in the experimentally measured peptide masses (https://www.expasy.org/resources/findmod) [21]. We included only PTMs that confirmed to the rules of PTMs identification by mass spectrometry, including statistical significance of identification set by FindMod to $p<0.5$ [21]. Manual curation of assigned PTMs was also performed to exclude false assignments. For example, if a PTM would interfere with the tryptic digestion and generation of a predicted peptide, such a PTM would be excluded. Mass spectrometry and PTMs assignment data were collected after 3 independent collections of cell culture experiments (see sections 2.1, 2.2, and 2.3) and analysis of mass spectra from independent samples from 2 mass spectrometry acquisitions and assignment of PTMs (see sections 2.4 and 2.5). Only reproducible data were used. Note that the mass spectrometry experiments, e.g., mass list generation and analysis, were set to statistical significance $p<0.5$.

2.6. Three-dimensional analysis of PTMs location

Protein Data Bank (PDB) was used to retrieve a 3-dimensional structure of human albumin. The structure deposited at PDB with accession number 1E78 for human serum albumin (doi: 10.2210 /pdb1E78/pdb) was used as a template for the analysis of identified PTMs. ChimeraX tool was used for allocation of the identified PTMs in the 3D structure of albumin (https://www.rbvi.ucsf.edu/chimerax) [22].

3. RESULTS

3.1. Preparation of albumin samples.

Presented here and our previously published [9] data, show that solubilization of HSA in water or in a cell culture medium DMEM did not affect the PTMs profile of albumin purified from the serum of healthy individuals. This allows efficient detection of PTMs in albumin incubated with human cancer cells, and comparison of its PTM profile with HSA that was kept solubilized in water or in

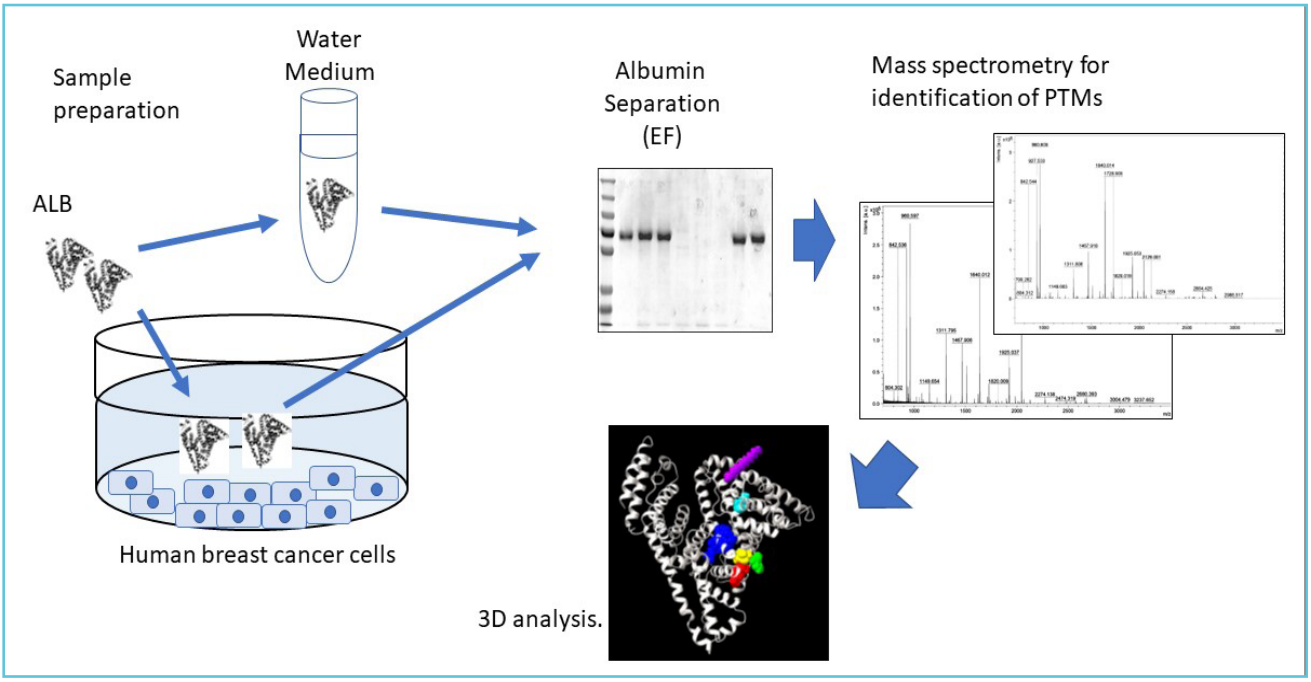
a cell culture medium (Figure 1). This comparison of PTMs profiles leads to the identification of PTMs affected by an exposure to cells.

We did not observe differences in migration in SDS-PAGE for HSA solubilized in water, medium, or exposed to different breast cancer cells (Figure 2A). This indicated the absence of PTMs affecting the molecular mass of HSA more than for a few thousands Da. The recovery of HSA incubated with the cells was estimated to be more than 95% of the initially added to the cells, and as compared to HSA prepared and kept in the medium without incubation with the cells. The low loss of HSA is due to adding HSA at a concentration that is similar to the concentration in human serum (i.e., 5%) [12, 13], and due to a relatively low albumin adhesion to the surface of the plates and cells.

As was expected, MCF7, MDA-MB-231, and MDA-MB-468 cells did not change their morphology, neither showed signs of cell death during incubation with 5% HSA in DMEM. These cells are extensively used in breast cancer research, and are known to tolerate incubation conditions that are similar to the used in this work. Incubation in a serum-free culturing medium for 24 hrs could inhibit cell proliferation but does not affect cellular viability and cellular tumorigenic properties.

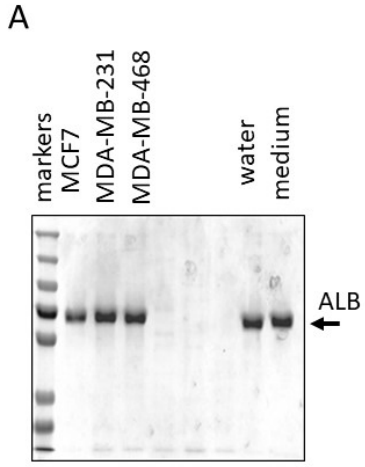
Large quantities of recovered HSA after its incubation with cells, e.g., 30 to 50 µg in a single electrophoresis lane, allowed robust mass spectrometry analysis. The mass spectra provided confirmatory identification of human serum albumin (accession number P02768). The collected mass spectra of albumin samples showed a similar pattern of main peaks for all 5 experimental conditions (Supplementary Figure S1, this figure can be retrieved at https://doi.org/10.6084/m9.figshare.21552372.v1).

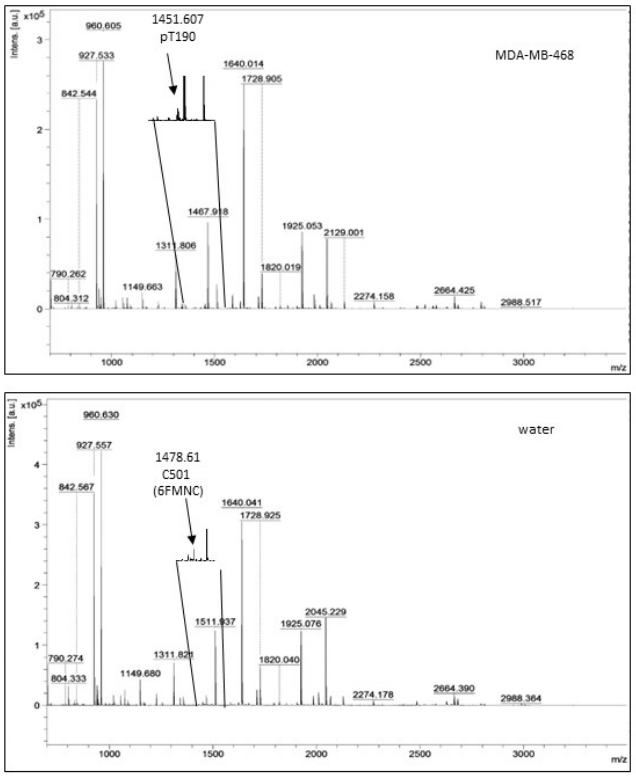
Figure 1 **Workflow of the project: the main steps of this study are shown**



Samples were prepared by incubation of human serum albumin with human breast cancer cells, or by keeping albumin solubilized in water or a cell culture medium. The recovered albumin was separated by SDS-PAGE, and subjected to mass spectrometry for identification of post-translational modifications. Modeling of the location of the identified PTMs in the 3-dimensional model of human serum albumin was performed.

Figure 2 Separation of HSA and mass spectrometry analysis





A) Separation of human serum albumin (ALB) samples in SDS-PAGE gel is shown. The arrow and annotation "ALB" indicate the migration position of HSA. The annotation of samples is indicated at the top of the gel. Migration of molecular mass markers is indicated as "markers". B) Two mass spectra show representative spectra of albumin recovered after exposure to MDA-MB-468 cells and water solubilized HSA. Representative mass spectra of all 5 types of albumin samples are shown in Supplementary Figure S1. The intensity of peaks and m/z values are indicated. Inserts show examples of peaks with assigned PTMs. See Table 1 for annotation of all PTMs.

After optimization experiments, the experiments with the established protocol were repeated 3 times and are presented here. Thus, the protocol of this study allowed the generation of reproducible and representative mass spectra of HSA of 5 tested conditions, e.g., albumin exposed to MDA-MB-231, MDA-MB-468, and MCF7 cells, or kept in water or in the cell culture medium.

3.2. Exposure of albumin to cancer cells altered PTMs in 14 peptides of albumin

The mass spectra of HSA exposed to human breast cancer cells, solubilized in water or in the cell culture medium were used to search for post-translational modification of HSA. We applied FindMod tool which is one of the most used tools for PTMs assignment with mass spectrometry data [21]. FindMod is known for the application of stringent criteria for PTMs prediction. We generated PTMs profiles for HSA retrieved in each experimental condition. Then, we removed all PTMs that were not affected by exposure of HSA to the cells in comparison to HSA in water or the cell culture medium. These removed PTMs were not altered by exposure to cells. We repeated mass spectrometry experiments with independent samples from 2 different experiments, and only reproducibly detected PTMs were considered for analysis.

The analysis showed 14 modified peptides of 8 types of PTMs (Table 1; Supplementary Table S1, this table can be retrieved at https://doi.org/10.6084/m9.figshare.21552414.v1).

We observed PTMs that discriminated HSA exposed to MDA-MB-231 and MDA-MB-468 from albumin exposed to MCF7 cells, or kept in water or in the cell culture medium. MDA-MB-231 and MDA-MB-468 cells represent aggressive breast cancer and form tumors in animal studies, while MCF7 cells are conditionally tumorigenic and much less aggressive [23, 24]. PTMs identified in HSA solubilized in water represent important controls of PTMs present in HSA from healthy individuals. The cell culture medium contains chemicals that theoretically may affect PTMs, e.g., by oxidation and chemical addition reactions. The Table 1 shows that the PTMs in HSA that originated from the healthy individuals were also present in the albumin solubilized in the cell culture medium. It means that the cell culture medium did not interfere with PTMs described in Table 1. However, the exposure of HSA to the cells affected these PTMs.

Eight PTMs were observed after exposure to aggressive cells, e.g., phosphorylation, palmitoylation, and cysteine persulfide modification. Other 6 PTMs were observed in albumin exposed to non-aggressive MCF7 cells and for HSA non-exposed to cells, e.g., deamidation followed by methylation, acetylation, and S-6-FMN cysteine modification (Table 1).

The distribution of the identified PTMs in the sequence of albumin showed localization of PTMs mostly in the IB and IIA domains of HSA (Figure 3).

Table 1 **Post-translational modifications of human serum albumin detected after exposure of HSA to breast cancer cells**

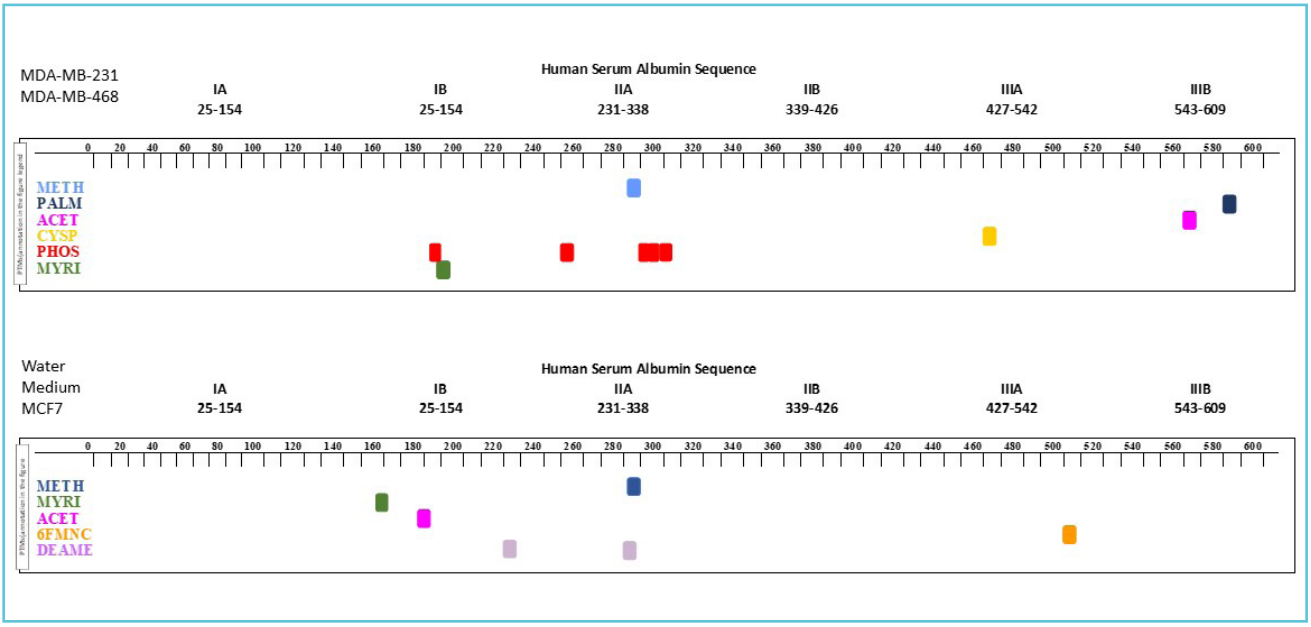
PTM*	Peptide	Position (peptide)	Position (site)	MCF7	MDA-MB-231	MDA-MB-468	Water	Medium
6FMNC	C**C**TESLVNR	500-508	C501	Yes	-	-	Yes	Yes
DEAME	CASL**Q**K	224-229	Q228	Yes	-	-	Yes	Yes
METH	YI**C**ENQDSISSK	287-298	C289	Yes	-	-	Yes	Yes
DEAME	ADLAKYICEN**Q**DSISSK	282-298	Q292	-	-	-	Yes	Yes
ACET	HPYFYAPELLFFA**K**R	169-183	K182	Yes	-	-	-	-
MYRI	LVRPEVDVMCTAFHDNEETFL**K**K	139-161	K160	Yes	-	-	-	-
PHOS	AEFAEV**S**K	250-257	S256	-	Yes	Yes	-	-
PHOS	YICENQD**S**I**SS**KLK	287-300	S294, S296, S297	-	Yes	-	-	-

PHOS	AAF**T**ECCQAADK	187-198	T190	-	-	Yes	-	-
PALM	AVMDDFAAFVEK**CC**K	570-584	C582, C583	-	Yes	Yes	-	-
ACET	EQL**K**AVMDDFAAFVEK	566-581	K569	-	Yes	Yes	-	-
CYSP	C**C**KHPEAK	461-468	C462	-	Yes	Yes	-	-
METH	ADLA**K**YICENQDSISSK	282-298	K286	-	Yes	Yes	-	-
MYRI	AAFTE**CC**QAADK	187-198	C192, C193	-	Yes	Yes	-	-

MCF7 are conditionally tumorigenic, and less aggressive as compared to invasive and highly tumorigenic MDA-MB-231 and MDA-MB-468 cells. PTMs in HSA that was solubilized in water or medium are shown to indicate the presence of these PTMs in HSA from healthy individuals (Water), and no impact on these PTMs of the chemicals in the cell culture medium (Medium). In bold and underlined are indicated modified amino acids. "Yes" indicates detection of a peptide, "-" indicates absence of a peptide. A peptide position in HSA sequence is indicated.

** 6FMNC, S-6-FMN cysteine modification; DEAME, deamidation followed by methylation; METH, methylation; ACET, acetylation; MYRI, myristoylation; PHOS, phosphorylation; PALM, palmytoylation; CYSP, cysteine persulfide modification.*

Figure 3 **Distribution of the identified 14 PTMs in the sequence of human serum albumin**



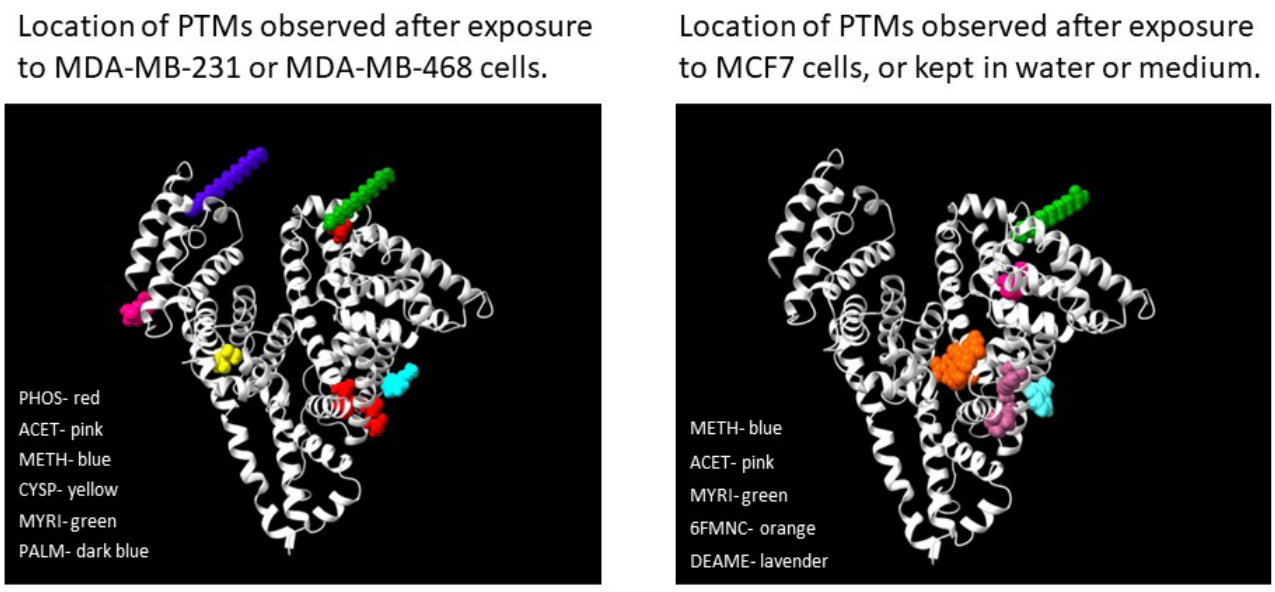
Panels show the distribution of PTMs annotated in Table 1. The upper panel shows 8 PTMs observed in HSA exposed to aggressive breast cancer cells MDA-MB-231 or MDA-MB-468. The lower panel shows 6 PTMs identified in HSA exposed to conditionally tumorigenic MCF7 cells, or kept in water, or in the cell culture medium. The sequence of HSA (accession number P02768) corresponds to a pro-form of albumin of 609 amino acids length. PTMs are colour coded, the same colour coding is used in Figures 3 and 4.

This contrasts with the relatively uniform distribution of PTMs in HSA from the serum of healthy individuals [4, 9, 18-20]. The focus on IB and IIA domains indicates functional implication of this region in modifications by cancer cells, by promotion of PTMs upon exposure to aggressive cancer cells, or by removal of PTMs. The 8 PTMs that appeared in HSA after its exposure to aggressive cancer cells have not been detected in HSA from healthy individuals [4, 9, 18-20].

3.3. Three-dimensional modeling shows location of PTMs at the Sudlow's site 1

The 3-dimensional model of human serum albumin [25, 26] is an excellent tool for the evaluation of the potential impact of identified PTMs on albumin functions. Structural regions involved in interactions with fatty acids, drugs, and metals have been identified in HSA [25-28]. We observed that the location of identified PTMs may affect lipid-binding sites in HSA by cysteine persulfide modification (CYSP), methylation (METH), and phosphorylation (PHOS) after exposure to MDA-MB-231 and MDA-MB-468 cells (Figure 4). In HSA incubated with MCF7 cells or kept in water or cell culture medium, one of the lipid-binding sites (Sudlow's site 1) is also affected by deamidation followed by methylation (DEAME), methylation (METH), and S-6-flavine mononucleotide (FMN) conjugation to cysteine (6FMNC) (Figure 4). 3D model shows the location of myristylation (MYRI) and palmitoylation (PALM) on the top of the 'heart' structure of HSA.

Figure 4 3-dimensional modelling of location of the 14 identified PTMs



The left panel shows 3D modelling of the location of PTMs at 8 modified peptides that were observed in HSA exposed to MDA-MB-231 and MDA-MB-468 cells but not in HSA exposed to conditionally tumorigenic MCF7, or solubilized in water or in the cell culture media. The right panel shows 3D modelling of the location of PTMs at 6 modified peptides that were observed in HSA exposed to conditionally tumorigenic MCF7 cells or were kept in water and in the cell culture medium. PTMs are colour coded as indicated in the images. The 3D model of non-PTM modified human serum albumin, accession number 1E78 (doi: 10.2210/pdb1E78/pdb; 585 amino acids representing 25 to 609 amino acids of HAS, was retrieved from Protein Data Bank (PDB) under open access permit. ChimeraX tool was used to locate reported here PTMs in the 3D model of HSA.

These modifications (MYRI and PALM) are considered as a mechanism for tethering proteins to a membrane. It remains to be explored whether these modifications affect the distribution of albumin.

4. DISCUSSION

Our data show that HSA exposed to cancer cells acquires novel PTMs. Our identification of enhanced phosphorylation of HSA upon exposure to aggressive cancer cells (Figure 3; Table 1) is in an agreement with the enhanced activities of many kinases in growing tumors [29]. The reported here phosphorylation of HSA at T190, S256, S294, and S297 was observed in non-small cell lung cancer tumors [18]. Phosphorylation at S256, S294, and S296 was detected in pancreatic cancer tumors [19]. Phosphorylation at T190, S256, S294, and S296 were observed in samples of breast cancer tumors [20]. However, none of the published reports [18-20] showed a dependency of the phosphorylation on tumor cells, or in comparison to HSA from healthy individuals. Our data demonstrate that the phosphorylation mentioned in Table 1, was observed only after exposure of HSA to aggressive cancer cells, and no phosphorylation was observed in HSA exposed to conditionally tumorigenic MCF7 cells or in HSA kept in water or in the cell culture medium (Table 1). Our data suggest that the phosphorylation at T190, S256, S294, S296, and S297 may serve as a marker of advanced breast cancer.

The sequences surrounding the phosphorylated residia (Table 1, Figure 3, Supplementary Table S1) differ from consensus sequences reported for SerPro-targeting kinases. Only 1% of reported phosphorylation sites have functional information, e.g., information about a kinase and/or its functional role (www.phosphonet.ca). Therefore, the prediction of the kinase(s) of HSA and/or functional impact of the 3 identified phosphorylated peptides await further studies.

We observed HSA acetylation at K182 and K569 only after its exposure to cells (Table 1, Figure 3). Acetylation at K182 was observed in humans [16, 17] and at K569 in rats [30]. In humans, K182 acetylation was described in datasets of mass spectrometry studies of liver and colorectal cancer [16, 17]. These reports did not explore the dependency of K182 and K569 acetylation on tumorigenesis. Our data show that this acetylation is detected only after HSA contact with cancer cells.

Modifications with lipids were described for HSA [9]. Our finding of myristylation at C192 or C193 has not been described earlier. A tripeptide CCK with C582 and C583 was identified as palmitoylated, with C583 as the probable site of palmitoylation, due to unlikely trypsin cut with adjacent palmitoylated cysteine [9]. Detection of the peptide consisting of 15 amino acids, with a lysine before C582 and possible no-cut by trypsin after lysine followed by the palmitoylated C582, indicates that the C582 is the most probable palmitoylation site in HSA exposed to cancer cells. Both S-palmitoylation and S-myristylation were detected for HSA that was exposed to aggressive cancer cells MDA-MB-231 and MDA-MB-468 (Table 1, Figure 3). We also observed a lysine-myristylation at K160 in HSA exposed to conditionally tumorigenic MCF7 cells. N-myristylation is associated with cancer progression. The enzyme lysine myristoyltransferease NMT1 was associated with the aggressive development of human breast cancer [31]. The location of the modifications at the top of the “heart” structure of albumin (Figure 4) may indicate their potential interactions with membranes.

Ten methylation sites were reported for HSA [32], however, without a confirmed dependency on cancer. We observed methylation at C289,

and methylation after deamidation at Q228 and Q292 in HSA that was kept in water or the cell culture medium, or was incubated with MCF7 cells, but no detection of these PTMs in HSA that was incubated with aggressive cancer cells. (Table 1, Figure 3). Deamidation followed by methylation at Q292 was observed by us earlier in HSA [9]. This suggests that demethylation and/or inhibition of deamidation at Q292 could be associated with the exposure to aggressive cancer cells. K286 was found methylated in HSA incubated with MDA-MB-231 or MDA-MB-468 cells (Table 1, Figure 3). The detected cancer cell-relevant differences in methylation are in the Sudlow's site 1 in HSA (Figure 4), suggesting modifications of interactions via this site.

Cysteine persulfide modification is the result of the action of reactive sulfur species, and is one of the oxidation mechanisms of cysteine. Our data predicted the formation of a cysteine persulfide at C462 in HSA exposed to aggressive cancer cells (Table 1, Figure 3). No data about this PTM in HSA was reported earlier. However, Fukuoka *et al.* observed an increased level of cysteine persulfide in colon tumors, as compared to a normal colon tissue [33], which may indicate the relevance of this PTM to cancer. Doka *et al.* showed that cysteine persulfide modification is involved in the regulation of protein functions and growth factor responsiveness [34]. Our detection of cysteine persulfide modification in albumin exposed to aggressive cancer cells but not in HSA exposed to MCF7 cells, suggests that this modification may be explored as a marker of aggressive development of breast cancer.

S-6-flavine mononucleotide (FMN) conjugation to cysteine (6FMNC) at C501 in albumin is an example of another oxidation-dependent PTM. We observed this modification earlier in HAS from healthy individuals [9]. 6FMNC at C501 was absent in HSA after exposure to the aggressive cancer cells. Flavine mononucleotide is involved in the regulation of the redox potential in cells, and this modification may indicate 6FMNC modification of HSA due to higher oxidation potential in tumors or in the tumor microenvironment.

Hypoalbuminemia is a marker of aggressive development of cancer [12, 13, 35]. End glycation of HSA serves as a marker of diabetes [6, 8]. Oxidation of Cys34 in HSA is considered a sensor of oxidative stress-related diseases [10, 11]. These observations are sufficient proof of the clinical value of HSA PTMs. The 14 PTMs reported here show that HSA is engaged by the microenvironment of cancer cells. We show that the cells can affect PTMs of albumin. This is an important finding that opens for further study of PTMs clinical marker value.

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Conflict of interest

Authors declare that they have no competing interests.

Ethical approval

This work was performed under an IBC permit from Qatar University (QU-IBC-2019/023).

Availability of data and materials

Original files are freely available upon request. All data generated and analyzed during the study are included in this published article.

The Supplementary Figure S1 can be retrieved at: https://doi.org/10.6084/m9.figshare.21552372.v1.

Representative mass spectra of human serum albumin recovered in the 5 tested conditions are shown. The intensity of peaks and m/z values are annotated. The tested conditions are exposure of HSA to human breast cancer cells MDA-MB-231 (A), MDA-MB-468 (B), MCF7 (C), or HSA kept solubilized in water (D), or in the cell culture medium (E).

The Supplementary Table S1 can be retrieved at: https://doi.org/10.6084/m9.figshare.21552414.v1.

The Supplementary Table S1 shows expanded information about identified PTMs presented in Table 1. The information about the user mass, database mass, mass difference, modification mass difference, PTM name, miscut or not, peptide sequence, and position of modified peptides are indicated.

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Authors' contributions

1. Study Design: Serhiy Souchelnytskyi;
2. Data Collection: Surya Kannan;
3. Statistical Analysis: Surya Kannan, Serhiy Souchelnytskyi;
4. Data Interpretation: Surya Kannan, Serhiy Souchelnytskyi;
5. Manuscript Preparation: Surya Kannan, Serhiy Souchelnytskyi;
6. Literature Search: Surya Kannan, Serhiy Souchelnytskyi;
7. Funds Collection: Serhiy Souchelnytskyi.

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