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Multidimensional Liquid Chromatography Platform for Profiling Alterations of Clusterin N-Glycosylation in the Plasma of Patients with Renal Cell Carcinoma

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Abstract

Identification of potential changes in the glycosylation of existing cancer biomarkers can result in a higher level of diagnostic sensitivity and specificity. Clusterin (Apolipoprotein J) has been implicated in renal cell carcinoma (RCC) and other types of malignancy as potential biomarker. In the present work, an automated multidimensional HPLC platform enabling high throughput affinity enrichment of clusterin from plasma samples was developed. Integrated with two dimensional gel electrophoresis, high purity clusterin in microgram quantities suitable for glycan characterization was isolated. The analytical platform was applied to study clusterin glycosylation in a small group of RCC patients before and after nephrectomy as a pilot study to evaluate the performance of the platform. A statistically significant decrease was observed in the levels of a bi-antennary digalactosyl disialylated (A2G2S(3)2) glycans while the levels of a core fucosylated bi-antennary digalactosyl disialylated glycan (FA2G2S(6)2) and a tri-antennary trigalactosyl disialylated glycan (A3G3S(6)2) were increased in the post-surgery plasma samples.

Keywords

Multi-dimensional HPLC; clusterin; two dimensional gel electrophoresis (2-DE); UPLC-HILIC; N-glycan; renal cell carcinoma

1. Introduction

Proteomics based biomarker discovery research targeting cancer cell lines and tissues has nominated a broad range of diagnostic and prognostic biomarker candidates, many of which

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are *N*- and *O*-glycosylated. Glycosylation is a common post translational modification that plays a pivotal role in many aspects of the host protein function [1]. It has been long known that aberrant protein glycosylation is associated with cancer development and thus a great deal of effort has been devoted to research on glycan-based biomarkers [2,3]. Considering the association between changes in protein glycosylation and cancer development [1], characterization of differences in glycosylation of a target glycoprotein biomarker in disease and healthy state may provide an additional level of marker specificity or sensitivity [4–6].

Renal Cell Carcinoma (RCC) is the most common subtype of renal cancer which accounts for 92% of renal cancer cases[7]. Due to the lack of early stage symptoms and the diagnosis of the disease at a late advanced stage, treatment options become limited [8]. A further complication associated with diagnosis is the cellular or pathological heterogeneity of the disease [9]. To date, only few efforts to identify body fluid based biomarkers for RCC have been reported and they have met with limited success [10,11]. This is due to the pathological complexity of the RCC and associated challenges in analyzing complex body fluids such as urine and plasma. Given its intimate contact with the kidneys, urine is an attractive source for RCC biomarkers due to the specificity of urinary protein content. However low protein concentration with associated high variation, high salt [12] and difficulty in standardizing urine collection has hindered the widespread application of urine as a biofluid for biomarker discovery [13].

Clusterin (Apolipoprotein J) expression level has been shown to change in different types of RCC cell lines and tissue [14,15]. Clusterin has also been suggested to contribute to anti-tumor activity of pVHL [16–18]. Our understanding of RCC biology has been expanded by unveiling the strong correlation between germline mutations in the von Hippel-Lindau (VHL) tumor suppressor gene and susceptibility to RCC. pVHL plays an important role in poly-ubiquitination and removal of the transcription factor; hypoxia-inducible factor (HIF). Clusterin is highly expressed in VHL target organs such as kidney, brain, liver, and adrenal medulla [19,20]. Clusterin has been shown to be involved in a number of carcinogenesis related cell events including DNA repair, apoptosis, cell adhesion, tissue remodeling, and lipid transportation [20,21]. Clusterin expression has been associated with progression of several human malignancies [22,23]. Nevertheless, there has been no study on the status of clusterin glycosylation in RCC. Given the strong association between glycosylation aberration and cancer development, and the significance of this particular protein in RCC, a pilot study focusing on the development and application of a platform for the extraction of plasma clusterin and subsequent profiling of the N-glycans present on the enriched protein is described.

To facilitate complete characterization of the *N*-glycosylation present on clusterin it must be completely isolated from complex biological samples, *i.e.* blood plasma in this instance. In plasma, clusterin is associated with high density lipoproteins (HDL) [24]. Also it forms complexes with numerous proteins including immunoglobulins [25], heparin [26], paraoxanase [27], and complement components [28]. Therefore, immunoprecipitation and immuno-affinity chromatography approaches generally result in an enriched clusterin fraction that carries other co-purified plasma (glyco)proteins which may or may not be revealed by low resolution techniques such as SDS-PAGE [29,30]. Even trace quantities of

co-extracted glycoprotein impurities will interfere in the quantitative oligosaccharide profiling of clusterin. Therefore additional degrees of orthogonality are required to minimize co-enrichment of impurities.

In the present work, a semi-automated platform for the highly selective immunoaffinity based isolation of clusterin from plasma followed by quantitative analysis of the protein *N*-glycome has been developed. The methodology utilizes an automated multi dimensional HPLC platform for fast and high throughput immuno-affinity and desalting of clusterin, followed by complete isolation of the enriched clusterin from all co-extracted specific and non-specific interactors within the two dimensional gel electrophoresis (2-DE) separation space. The developed platform was subsequently applied to study the *N*-glycosylation of plasma clusterin in RCC facilitating the evaluation of alterations in the oligosaccharides as potential companion biomarker targets. Ultra performance hydrophilic interaction liquid chromatography (UPLC) combined with fluorescence was employed for the quantitative *N*-glycomic profiling of isolated clusterin from the plasma of patient diagnosed with RCC both before and after surgical intervention.

2.0 Material and Methods

2.1. Plasma Samples

Twelve plasma samples from six patients with clear cell renal cell carcinoma before (referred to as Pre) and after (referred to as Post) curative nephrectomy were provided by Massachusetts General Hospital cancer center. The clinical information of the patients are summarized in table 1. The samples were collected according to protocol 01–130 and approved by the Institutional Review Board at Massachusetts General Hospital. All plasma samples were stored at – 80 °C and did not undergo more than two freeze-thaw cycles. For method development, pooled normal female plasma were purchased from Bioreclamation (Jericho, NY) and stored at – 80 °C.

2.2. Clusterin ELISA

Plasma clusterin levels before and after surgery was measured by using a commercial ELISA kit following the manufacturer's instruction (R&D Systems, Inc., Minneapolis, MN).

2.3. Multi-dimensional HPLC Platform for Immuno-affinity Enrichment of Clusterin

Clusterin was immuno-affinity purified from plasma samples using a 3-column multidimensional method. The removal of albumin and IgG (depletion columns) was coupled on-line with clusterin capture and on-line desalting using a reversed-phase “trap” column.

For depletion of albumin (anti-HSA), CaptureSelect affinity ligand with specificity for albumin (BAC B.V., The Netherlands) was immobilized on POROS AL 20 µm Self Pack media (Life Technologies, Inc., Carlsbad, CA) and packed into a 4.6 mm × 100 mm PEEK column (Isolation Technologies, Hopedale, MA). Protein G media (POROS MabCapture A, Life Technologies, Inc. Carlsbad, CA) was packed into a 4.6 mm × 50 mm PEEK column and used for depletion of IgG. For the clusterin Affinity column, CaptureSelect anti-

clusterin ligand immobilized on agarose based media (BAC B.V., The Netherlands) was gravity packed into a 20 mm × 6.6 mm Omnifit glass column (Biochem Fluidics, Boonton, New Jersey). For The RP Trap, R1 POROS bulk media (Life Technologies, Inc. Carlsbad, CA) packed into a PEEK column (4.6 mm × 50 mm). Chromatography was performed using a Prominence 2D HPLC (Shimadzu, Columbia, MD) equipped with two pumps, a sample injection valve, and three auxiliary valves which allowed switching between multiple columns during the HPLC run. The anti-HSA and protein G columns, connected together with PEEK tubing, were placed on valve position 1. The anti-clusterin column was in valve position 2, and the RP trap was positioned on valve 3. Columns were switched on- or off-line for sample binding, column washing, and elution purposes; each valve was controlled independently from the software through contact closures.

Phosphate buffer saline (1x PBS) was used as binding buffer whereas 0.1 M glycine (pH 2.5) was the elution buffer; 100 µL plasma was diluted with 400 µL binding buffer and then introduced to the anti-HSA, protein G and anti-clusterin columns at the flow rate of 0.5 mL/min. 8.75 mL of the binding buffer was passed through columns for complete transfer of the depleted plasma to the anti-clusterin column. The flow-through of the three affinity columns was collected for further analysis. At this point the RP trap was brought on-line and the captured proteins were then eluted using 8 mL of the elution buffer and transferred to the RP trap while the depletion columns were off-line. The RP trap column was then washed with 4 mL of 0.1% (v/v) trifluoroacetic acid in 2% (v/v) acetonitrile/water for desalting. The bound proteins were then eluted using 6 mL of 0.1% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile/water. This fraction contained clusterin and was collected and lyophilized for subsequent analysis. The RP trap was then re-equilibrated with 4 mL 0.1% (v/v) trifluoroacetic acid in 2% (v/v) acetonitrile/water for the next run. Subsequently, the albumin and IgGs depletion columns were eluted using 8 mL of the elution buffer. The albumin-IgGs fraction was collected for further analysis. The last step was neutralization of all three affinity columns with 14 mL of the binding buffer. The whole method duration was 44 minutes.

2.4. 1-D SDS-PAGE and 2-D Gel Electrophoresis

Affinity purified clusterin was loaded on SDS-PAGE gels (NuPAGE® Novex 4–12% Bis-Tris) and separated in a XCell SureLock™ Mini-Cell (Invitrogen, Carlsbad, CA) for 45 min at 200 V using a MES running buffer. 5 µg of total protein was loaded in each lane of the gel. Following completion of the electrophoretic separation proteins were visualized by immersion of the gel in SimplyBlue Safestain followed by destaining with multiple changes of ultra pure water.

Reagents and instrumentation used for 2-D gel electrophoresis separation were purchased from Bio-RAD Laboratories, Inc. (Hercules, CA). IEF separation was performed using ReadyPrep™ 2-D starter kit. Briefly, a volume of desalted clusterin corresponding to 150 uL plasma was dissolved in rehydration buffer (8M urea, 4% CHAPS, 50 mM dithiothreitol (DTT), 0.2% (w/v) Bio-Lyte® 3/10 ampholytes and trace amount of Bromophenol Blue) and loaded on an 11 cm IPG strip pH 4–7 via overnight passive rehydration. Isoelectric focusing was performed using the PROTEAN IEF cell according to the manufacturer's instructions.

Once focused, the strips were equilibrated in reducing buffer (6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8) and 20% glycerol and 2% (w/v) DTT) for 10 min followed by immersion in an alkylating buffer (6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8) and 20% glycerol and 2.5% (w/v) Iodoacetamide), each incubation was 10 minutes at room temperature. The IEF strip was then placed on top of a 1mm Criterion™ precast 12% Bis-Tris gel and sealed with agarose. Second dimension SDS-PAGE separation was achieved at a constant voltage of 200 V for 1 hr using a MES running buffer. Visualization of protein spots was performed using Coomassie Brilliant Blue staining.

2.5. Enzymatic N-Glycan Release and Fluorescent Labeling

2-D gel spots of interest were excised and washed with acetonitrile and 20 mM sodium bicarbonate pH 7 buffer in alternating fashion up to three cycles. N-glycans were enzymatically released through overnight incubation at 37 °C with PNGase F (New England Biolabs, Ipswich, MA). The released N-glycans were eluted from gel pieces by successive washing with acetonitrile and 20 mM sodium bicarbonate buffer, pH 7.0 and dried using vacuum centrifugation. Once dry, 20 μ L of 1% formic acid solution was added to the glycans to promote hydrolysis of the released glycosylamines to the corresponding reducing sugar and they were subsequently evaporated to dryness via vacuum centrifugation. The liberated glycans were labeled with 2-aminobenzamide *via* reductive amination in the presence of sodium cyanoborohydride in 70:30 DMSO/acetic acid solution at 65 °C for 2 hr. Following the incubation, 90 μ L water and 900 μ L acetonitrile was added to the reaction mixture. Unreacted fluorescent label was removed by PhyNexus Normal Phase PhyTips (San Jose, CA, USA) using an adaptation of the method of Olajos et al. [31].

2.6. UPLC-HILIC Profiling of N-Glycans

Fluorescently labeled N-glycans were separated on a Waters BEH Glycan chromatography column (100 mm $\times$ 2.1 mm), 1.7 μ m BEH particles (Waters, Milford, MA). Ultra performance liquid chromatography system used was Waters Acquity UPLC instrument consisting of a binary solvent manager, sample manager, column manager, and a FLR fluorescence detector. The system was controlled by Empower 3 Chromatography workstation software. A linear gradient of 30–47% 50 mM ammonium formate pH 4.5 over 16.5 min at the flow rate of 0.56 mL/min, followed by 2 min re-equilibration with 70% acetonitrile was employed for oligosaccharide separation. The total run time was 19 min. The volume of 10 μ L of each sample prepared in 80:20 acetonitrile/water and maintained at 5 °C in the thermostated autosampler prior to injection on column. The separation temperature was 40 °C. The fluorescence detection setting were λ_{ex} =330 nm and λ_{em} = 420 nm with a data collection rate of 20 Hz.

2.7. Glycan Characterization Using Weak Anion Exchange (WAX) Chromatography and Exoglycosidase Digestion

Weak anion exchange separation of fluorescently labeled N-glycan pool was performed on a GlycoSep C (Prozyme, Hayward, CA, 75 mm $\times$ 7.5 mm), 10 μ m particle packed DEAE functionalized styrene divinyl benzene column using above mentioned chromatography system. Charge-based separation of glycans was achieved by a linear gradient of 0–100%

100 mM ammonium acetate pH 7 in 20% v/v acetonitrile over 20 min following a 5 minute isocratic hold at 100% buffer A (20% v/v acetonitrile), at the flow rate of 0.75 mL/min and the total run time of 30 min. The separation was carried out at room temperature.

Exoglycosidase digestions were performed as previously described by Royle et al (Royle, L. et al. *Methods Mol. Biol.* 2006). Structural annotation of the resulting HILIC peaks was performed by comparison of retention time data expressed as glucose unit (GU) values with GlycoBase, a HPLC relational database of GU values and exoglycosidase digestion data freely available at www.glycobase.nibrt.ie [32]. *N*-glycan nomenclature and symbolic representations used throughout is as previously described by Harvey et al. [33].

2.8. In-gel and In-solution Trypsin Digestion

For in-solution digestion, protein samples were denatured in 6M guanidine hydrochloride buffered at pH 8. Denatured protein was then reduced by adding Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) to the final concentration of 5 mM. The sample was incubated at room temperature for 30 min. The mixture was then alkylated by adding iodoacetamide to the final concentration of 20 mM followed by incubation at room temperature for 1 hr in the dark. Excess reagents were subsequently removed by injecting the sample on to a POROS R1 reversed-phase HPLC column. 0.1% (v/v) trifluoroacetic acid (TFA) in water and 0.1% (v/v) TFA in acetonitrile was used as the mobile phases A and B respectively. The sample was loaded on the column in 2% mobile phase B and washed for 3 minutes at the flow rate of 3 mL/min to remove the salts and other reagents. The bound proteins were then eluted with 70% mobile phase B at the same flow rate for 3 min. The bound fraction was then collected, reduced in volume *via* vacuum centrifugation and reconstituted in 50 mM ammonium bicarbonate at the concentration of ~ 0.2 mg/mL. Trypsin (sequencing grade, Promega, Madison, WI) was added to the proteins at the ratio of 1:40 (w/w) followed by overnight incubation at 37°C. Resulting peptides were lyophilized and reconstituted in 0.1% (v/v) solution of formic acid in water prior to LC-MS analysis. In-gel trypsin digestion of the gel bands/spots of interest was performed by the previously described method [34].

2.9. LC-MS Analysis

Tryptic peptides from in-gel or in-solution digestion were analyzed by LC-MS/MS on a LTQ mass spectrometer (ThermoFisher Scientific, Waltham, MA). The nano-LC separation was performed using an Eksigent Nano-LC.2D system (Dublin, CA). 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile were the mobile phases A and B. The sample was loaded onto a Cap Trap column (MICHROM Bioresources, Inc., Auburn, CA) with 5% mobile phase B at flow rate of 5 µL/min using an autosampler. The trapped sample was then separated on a C₁₈ capillary column (150 mm × 75 mm i.d.) (New Objective, Woburn, MA) packed in-house with Magic C₁₈ 200 Å 5 µm stationary phase (MICHROM Bioresources, Inc., Auburn, CA). The flow rate was 300 nL/min and the gradient was from 5% to 40% mobile phase B over 45 min, then from 40% to 80% over 7 min, then was kept at 80% mobile phase B for 6 min. For complex samples a longer gradient consisting of a gradient of 5% to 40% B over 90 min, followed by a sharp gradient of 40% to 80% over 9 min and finally 9 min of 80% B was used. The mass spectrometer was operated in the data

dependent mode; each full MS scan over the range of 400–2000 m/z was followed by data-dependant MS/MS fragmentation events of the eight most intense precursor ions present in the MS spectrum. Dynamic exclusion was employed for duration of 2 min.

2.10. Bioinformatics

MS/MS spectra were searched against the human protein database Uniprot (release 2011_03, total number of entries of 20234) using the SEQUEST search engine (Thermo Fischer scientific) contained within the Bioworks software suite (Thermo Fischer Scientific). The search parameters were the following: monoisotopic precursor and fragment ion mass with the tolerance of 1Da. Carbamidomethylation on Cysteine was considered as fixed modification and up to 4 modifications were allowed per peptide. C_n 0.1 and peptide probability <0.001, cross correlation (XCorr) 1.9, 2.5, and 3.8 for singly, doubly, and triply charged ions, respectively were used for peptide identification.

2.11. Statistical Analysis

Multivariate statistical analysis of HILIC profiling data was performed using Umetrics Simca-P+ software version 11.5 (Umetrics Inc, San Jose, USA). Non-parametric Wilcoxon (signed rank) test were performed and box plots were generated using PASW Statistics 18 (IBM, NY, USA) to evaluate the differences in each experimental parameter for patients before and after surgery. P values <0.05 were considered statistically significant.

3.0 Results and Discussion

Clusterin or Apolipoprotein J is expressed in different organs such as liver, brain, and kidney and is found in blood plasma in the range of 50–150 µg/mL quantity. The secreted form of the protein is 75 to 80 kDa and consists of α and β subunits with nearly identical molecular weights. Clusterin has seven sites of N-glycosylation distributed on both α and β subunits and several heparin binding domains. The exact function of clusterin is still a matter of great uncertainty; however a number of different functions including anti-apoptotic activities have been attributed to this protein [20]. A study showed clusterin secretion was decreased in p-VHL defective RCC cell lines [16–18]. This observation and other reports on clusterin link with pVHL make it conceivable that clusterin contributes to anti-tumor activity of pVHL [15]. On the other hand, it has been reported that clusterin was over expressed in 52% of patients with nonpapillary renal cell carcinoma and correlated with the incidence of tumor recurrence [15]. However VHL status was not examined in this study.

3.1. Automated Multi-dimensional HPLC Platform for Affinity Enrichment of Clusterin

To purify clusterin from patient plasma samples, a multi-dimensional HPLC platform shown in figure 1 was developed. Albumin and IgGs are two most abundant plasma proteins which account for nearly 70% of total plasma proteins. Because of the hydrophobic nature of albumin and IgGs, as well as albumin overall net negative charge at physiological pH, these two abundant proteins tend to bind non-specifically to many other plasma/serum proteins. Depletion of these two proteins significantly reduces the complexity of plasma and decreases the chance of nonspecific binding in the subsequent clusterin affinity enrichment.

This automated platform enables affinity purification of plasma clusterin (or the target protein of choice) with minimal sample handling in a short amount of time (25 min). The platform is versatile and can be adapted to immuno-enrichment of any target protein from human plasma or serum. The performance of the platform was evaluated by LC-MS proteomic analysis of three fractions: the flow-through enriched clusterin fraction, and Ig-Albumin fraction. As indicated by the LC-MS analysis in table 2 almost all clusterin present in plasma was captured by the clusterin affinity column used in our platform, indicated by only one clusterin peptide identified in the LC-MS/MS analysis of the flow-through fraction. Also rechromatography of the flow-through fraction did not result in any binding to the clusterin affinity column; indicating the specificity of the platform (data not shown). IgG depletion of plasma raises a concern in regards with loss of some clusterin isoforms as clusterin has been shown to bind to IgG in vivo [25]. To address this concern we analyzed the albumin + IgG fraction with LC-MS/MS (data shown in table 2). No clusterin peptides were identified in this fraction; indicating high selectivity of our HPLC platform.

3.2. Isolation of Clusterin by Two Dimensional Gel Electrophoresis (2-DE)

Although almost all plasma clusterin was captured by the clusterin affinity column, as can be seen in the SDS-PAGE analysis (figure 2.a.) and LC-MS/MS analysis of the captured clusterin, there is still a significant amount of abundant plasma proteins present, predominantly fibronectin (detected by MS). The observed association of clusterin and fibronectin is potentially mediated via heparin, a sulfated oligosaccharide that binds through heparin binding domains present on both clusterin and fibronectin. Moreover, the in-gel digestion and LC-MS/MS proteomic analysis of the clusterin band shown in figure 2.a. shows co-migration of other glycoproteins such as Apolipoprotein E and Complement C3. Due to the presence of these glycosylated contaminants, 2-D gel electrophoresis (2-DE) was used to isolate clusterin from immuno-affinity enriched fraction. Figure 2.b shows 2-DE separation of affinity purified clusterin from a control plasma sample. The IEF range chosen was pH 4–7 which covers the isoelectric point of clusterin (pI 5.89). The identity of the excised spots was verified by proteomic analysis with LC-MS/MS, as shown in table 3. 2-DE resulted in high purity isolation of clusterin isoforms indicated by clusterin being the only non-keratin protein identified in the LC-MS/MS analysis of each gel spot. This illustrates the need for multidimensionality for high performance affinity isolation of target glycoproteins and the performance of orthogonal strategies employed here.

3.3. Application of the Platform to the Characterization of Plasma Clusterin *N*-Glycans in RCC

To investigate the presence of cancer related alterations in clusterin *N*-glycans, HILIC profiling of the clusterin glycan from RCC patients (total number of 6), here named as 'Pre' and their corresponding controls (after curative nephrectomy), named as 'Post' was performed. Having each patient as their own control significantly reduces the effect of inter-individual biological variability generally associated with multiple cohort studies. HILIC-based separation of glycans is a well recognized method which takes advantage of highly reproducible and predictable retention time to annotate *N*-glycan structures [35]. Combined with fluorescence labeling, HILIC offers low femtomole sensitivity [36]. In the present work we used the 1.7 μ m BEH Glycan stationary phase for quantitative profiling of *N*-glycans

released from clusterin. Figure 3 shows an example UPLC chromatogram for the glycans released from clusterin isolated from one of the control plasma samples. Combination of weak anion exchange (WAX) (figure S1, supplementary information) separation for simplification of sialylated *N*-glycans into charge based sub-pools and exoglycosidase digestion, followed by HILIC profiling with conversion of retention time values to corresponding glucose unit values (GU) enabled structural annotation of the peaks in HILIC profile, as previously described [37,38]. The identified glycans were predominantly biantennary type sugars with varying core and antennary fucosylation and varying degrees of sialylation. Tri-antennary sugars were also detected but at lower quantities. Two tri-antennary isomers A3 and A3' were detected. As was noted with the bi-antennary isomers sugars, a variable degree of sialylation on the tri-antennary structures was also observed. A tri-antennary structure with Sialyl Lewis X (SLe^X) epitope, a cancer associated antigen was also detected [39].

3.4. Alteration in Clusterin *N*-Glycan in RCC

To evaluate the capability of clusterin *N*-glycans to differentiate between pre and post samples, principle component analysis (PCA) was performed using the integrated HILIC-fluorescence chromatograms for clusterin *N*-glycans from the six pre-surgery patient plasma samples and their corresponding post surgery controls. 16 peaks in the HILIC-fluorescence profile were integrated for each sample. Integration data for all 12 samples were aligned and were subjected to multivariate PCA analysis. As depicted in figure 4, the PCA loading plot shows complete segregation of the Pre and Post patients. Partial least squares differential analysis (PLS-DA) was also performed. The resulting variable importance plot (VIP) which lists the peaks in order of contribution to the PCA separation is included as figure S2 in the supplementary information. Moreover, an intra class separation was noted for the post operative samples. The observed sub grouping may have arisen from treatment or environmental factors as these have been recently shown to cause alterations in the glycome [40]. Overall, PCA clustering shows the utility of the clusterin *N*-glycosylation pattern to differentiate between these two clinical states.

To pinpoint individual glycan structures with statistically different levels in RCC and controls, a non-parametric Wilcoxon (signed rank) test was performed. Pair wise comparison using the Wilcoxon test returned three glycan structures with statistically significant difference. Example chromatograms for a pre and post surgery plasma pair are included in figure S3 in the supplementary information.. Our study shows a significant increase in the level of a core-fucosylated bi-antennary structure (FA2G2S(6)2) (peak 7 in figure 3) in the post surgery samples with a p-value of 0.028. In addition, an increase in the level of a tri-antennary glycan isomer (A3'G3S(6)2) (peak 12 in figure 3) and decrease in the level of a bi-antennary structure (A2G2S(3)2) (peak 3 in figure 3) in post surgery samples with p-values approaching the 0.05 significance threshold were observed. Overall, our results show clusterin oligosaccharides shift towards smaller glycans through a decrease in branching and core-fucosylation in the event of RCC.

The observed shift from tri-antennary to bi-antennary glycans on clusterin in RCC has been reported previously in other types of malignancies. Decreased level of tri-antennary

oligosaccharides were noted in hepatocellular carcinoma [41]. In a glycomic/glycoproteomic analysis of stomach cancer, clusterin glycosylation was found to shift from trisialylated tri-antennary to disialylated bi-antennary oligosaccharides with cancer pathogenesis [42]. Decrease in the level core-fucosylated bi-antennary glycans have been indicated in a number of other cancers. Arnold *et al.* [43] reported a decrease in the level of FA2G2S2 in the serum glycomic analysis of lung cancer patients. In another study by Sarrats *et al.* [44] it was found that level of core fucosylated glycans on PSA (prostate specific antigen) was decreased in prostate cancer. More interestingly, a study of RCC tissue has showed significant decrease in the activity of *N*-acetylglucosaminyltransferases III and IV leading to a decrease in bisecting *N*-acetylglucosamine (GlcNAc) and antennary branching of complex-type *N*-glycans in 7-glutamyltranspeptidase (7-GT) purified from RCCs [45]. These observations combine to strengthen the hypothesis concerning alterations in glycosylation are a hallmark of cancer pathogenesis and therefore, the development of enrichment and analytical platforms as described herein are necessary to facilitate further understanding of the role of these glycoproteins in cancer.

3.5. Level of plasma clusterin in RCC

To further investigate the status of clusterin in the current study, we performed an ELISA to determine the levels of this protein in Pre and Post plasma samples. The levels of clusterin as determined by ELISA are shown in the box plot displayed in figure 5. Wilcoxon (signed rank) test of the data showed no statistically significant change (p-value of 0.753) in the level of plasma clusterin for the RCC patients before and after curative nephrectomy. This result further supports the hypothesis that alterations in glycosylation on pathologically relevant target proteins may serve as potential target markers for providing diagnostic or prognostic information. However, it should be noted that the ELISA assay, as well as the immuno-affinity enrichment performed in this study does not discriminate on the tissue of clusterin origin. Further studies of RCC Pre-surgery biopsies will be necessary in order to correlate the glycosylation changes observed in circulating clusterin and renal derived clusterin.

4.0 Conclusion

In the current study, an automated multi-dimensional HPLC platform for high throughput affinity purification of clusterin from plasma samples for downstream targeted glycosylation analysis was developed. The HPLC platform was integrated with two dimensional gel electrophoresis for complete isolation of the target protein, a requirement for accurate annotation of *N*-glycan structures and quantitative analysis in any subsequent biomarker verification study. Using the developed platform, we isolated clusterin from plasma samples collected from RCC patients before and after nephrectomy and investigated the glycosylation status of clusterin. Our results show there is a statistically significant decrease in the level of A2G2S(3)2 glycan while the level of FA2G2S(6)2 and A3'G3S(6)2 was increased in the post-surgery plasma samples. However, no significant change in clusterin concentration in blood plasma before and after surgery was detected. The observed changes in this pilot study will require transfer into a more clinically friendly assay format to facilitate verification and validation prior to utilization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Multidimensional HPLC platform for high efficiency enrichment of clusterin from plasma
2. Combined with 2-DE, high purity clusterin in μg quantity needed for glycomic analysis was obtained
3. Application of the platform to *N*-glycomic characterization of enriched clusterin described
4. Alterations in clusterin glycosylation associated with the presence of the cancer were observed
5. Observed alterations in glycosylation may serve as potential markers with further verification

Figure 1. Schematic representation of the multi-dimensional HPLC platform for affinity purification of clustrein. Columns were independently switched on- or off-line for sample binding, column washing, and elution purposes.

Figure 2.

(a) SDS-PAGE analysis of immune-affinity enriched clusterin from control plasma and (b) Commassie stained 2-DE separation of affinity purified clusterin from 150 ug plasma, first dimension: IEF pH range 4 to 7 and second dimension: 10% SDS-PAGE. The spots highlighted represent different isoforms of clusterin confirmed by LC-MS analysis (data shown in table.1). clusterin spots were excised and pooled for glycomic analysis.

Figure 3.

Representative UPLC-HILIC chromatogram of clusterin N-glycan displaying 16 integrated peaks used for multivariate statistical analysis and Wilcoxon signed rank test

Figure 4.

PCA plot generated using multivariate statistical analysis of HILIC-fluorescence glycan profiles of clusterin isolated from RCC patients plasma before (♦) and after (◆) surgery showing Pre samples clustering together and controls (post surgery) dividing into a group of 4 and a group of 2.

Figure 5.
ELISA measurement of the level of clusterin in plasma of RCC patients before and after surgery.

Table 1

Clinical Information of the patients

PATIENT #	SEX and AGE (Age recorded at time of diagnosis)	Histology	Tumor size (in cm)	FURHMAN GRADE
1	Female, 71yo	Clear Cell	6 × 4.8 × 3.5	Grade 2/4
2	Male, 74yo	Clear Cell	7×6.5×5.8	Grade 3–4/4
3	Male, 53yo	Clear Cell	3 × 3 × 3	Grade 2/4
4	Female, 53yo	Clear Cell	8.8 × 5.0 × 5.2	Grade 2/4
5	Female, 62yo	Clear Cell	8.5 × 7.5 × 6.5	Grade 4/4 (possible small met to liver)
6	Female, 60yo	Clear Cell	7.5 × 4.5 × 3.2	Grade 2/4

Table 2

LC-MS/MS identification of clusterin in each HPLC fraction of reference plasma

HPLC fraction	Sf score	% sequence coverage	Number of identified peptides	total number of identified peptides in LC-MS/MS run
Flow-through	10.17	2.7	1	1182
Bound Clusterin	180.30	30.70	281	1995
Bound IgG and Albumin	-	0	0	566

Table 3

LC-MS/MS verification of reference plasma clusterin spots on 2-D gel

2-DE spot	Protein identified	XC score	% sequence coverage	Number of identified peptides
1	clusterin	220.28	38.3	131
2	clusterin	240.23	35.9	264
3	clusterin	210.24	31.60	241
4	clusterin	200.25	25.20	105
5	clusterin	180.22	30.10	46
6	clusterin	130.23	25.60	24