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Mass spectrometry in cancer biomarker research: a case for immunodepletion of abundant blood-derived proteins from clinical tissue specimens

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Abstract

The discovery of clinically relevant cancer biomarkers using mass spectrometry (MS)-based proteomics has proven difficult, primarily because of the enormous dynamic range of blood-derived protein concentrations and the fact that the 22 most abundant blood-derived proteins constitute approximately 99% of the total plasma protein mass. Immunodepletion of clinical body fluid specimens (e.g., serum/plasma) for the removal of highly abundant proteins is a reasonable and reproducible solution. Often overlooked, clinical tissue specimens also contain a formidable amount of highly abundant blood-derived proteins present in tissue-embedded networks of blood/lymph capillaries and interstitial fluid. Hence, the dynamic range impediment to biomarker discovery remains a formidable obstacle, regardless of clinical sample type (solid tissue and/or body fluid). Thus, we optimized and applied simultaneous immunodepletion of blood-derived proteins from solid tissue and peripheral blood, using clear cell renal cell carcinoma as a model disease. Integrative analysis of data from this approach and genomic data obtained from the same type of tumor revealed concordant key pathways and protein targets germane to clear cell renal cell carcinoma. This includes the activation of the lipogenic pathway characterized by increased expression of adipophilin (PLIN2) along with 'cadherin switching', a phenomenon indicative of transcriptional reprogramming linked to renal epithelial dedifferentiation. We also applied immunodepletion of abundant blood-derived proteins to various tissue types (e.g., adipose tissue and breast tissue) showing unambiguously that the removal of abundant blood-derived proteins represents a powerful tool for the reproducible profiling of tissue proteomes. Herein, we show that the removal of abundant blood-derived proteins from solid tissue specimens is of equal importance to depletion of body fluids and recommend its routine use in the context of biological discovery and/or cancer biomarker research. Finally, this perspective presents the background, rationale and strategy for using tissue-directed high-resolution/accuracy MS-based shotgun proteomics to detect genuine tumor proteins in the peripheral blood of a patient diagnosed with nonmetastatic cancer, employing concurrent liquid chromatography–MS analysis of immunodepleted clinical tissue and blood specimens.

Keywords

blood; cancer biomarker discovery; clinical proteomics; clinical specimens; high-resolution/accurate LC-MS; immunoaffinity depletion; tissue

Mass spectrometry-based clinical proteomics in cancer biomarker research

Mass spectrometry (MS)-based clinical proteomics is defined as the application of proteomic technologies for molecular profiling of tissues or body fluids in all aspects of clinical studies, including biomarker research [1]. Often, data obtained from cancer cell lines [2] or animal models [3] are not predictive of what is actually happening in human cancers *in vivo*. Therefore, clinical proteomics is expected to remain of critical importance in cancer biomarker research since it measures gene end products, namely proteins, as direct effectors of biological function [4,5]. Unlike transcriptomics, expression proteomics has the ability to measure the level of gene products in isolated subcellular compartments/ organelles, within the extracellular environment (e.g., biofluids) and measure changes in post-translational modifications (i.e., phosphorylation). For that reason, innovative MS-based approaches capable of in-depth profiling of clinical specimens are essential to capture differences in protein expression between tumor and normal adjacent tissue, and to facilitate elucidation of human tumor molecular signatures [6]. Proteins act as ultimate bioeffectors of the malignant phenotype. Thus, proteomic profiling of human tumors may aid the understanding of how the changes at the transcriptional level translate to the protein level, including signaling pathways/networks nurturing the malignant phenotype under study.

Despite recent advances in MS-based clinical proteomics [7,8], the translation of proteomic findings to applicable clinical tests has proved difficult. The reasons are primarily due to the extensive dynamic range of protein concentrations in tissue/blood, which exceeds six orders of magnitude in solid tissue (cells) and ten orders of magnitude in plasma/serum [9].

Numerous studies have clearly demonstrated that current MS platforms still struggle to identify/discover low-abundance cancer-associated markers in peripheral blood, primarily owing to volume dilution effects and significant mismatches between the dynamic range capabilities of current MS versus the dynamic range of proteins in plasma/serum [10].

Finally, the poor experimental design of early 'proof-of-principle' studies, which focused exclusively on plasma/serum specimens, along with the use of low-resolution MS and other relatively unsophisticated analytical methods, were not beneficial to the very young field of clinical proteomics [11].

While it is not yet clear if the analysis of plasma/serum is an adequate choice for the cancer biomarker discovery phase, the capture of disease-associated biomarkers in tissues is clearly becoming possible, primarily owing to higher concentrations of disease-associated proteins at the site of the pathological process, use of high-resolution MS and the ability to remove abundant blood-derived proteins from tissue homogenates [12]. On the other hand, it is certain that current MS technology permits measurements/validation of known proteins/targets in plasma/serum using the type of instruments (e.g., triple quadrupole) capable of targeted detection and absolute quantitation (i.e., multiple reaction monitoring [MRM] and immuno-MRM) [13,14]. For these reasons, clinical proteomics experimental design, sample

preparation methodologies and data processing approaches are intricate, challenging and different from basic science research, where experiments are characterized by the use of *in vitro*-cultured cells or transgenic animal models within well-controlled settings [15].

Liquid chromatography-MS analysis of human clinical specimens: challenges/solutions

MS-based profiling of clinical specimens (e.g., tissues and biofluids) has been increasingly used in cancer research. Characterizing changes in protein expression between tumor and healthy tissue or between the blood of diseased and healthy individuals is a common approach. These molecular profiles may lead to distinct insights that are not readily evident using *in vitro* cultured cells or animal models, and thus may facilitate discovery of cancer biomarkers.

The MS-based profiling of clinical specimens, including fresh frozen (FF) tissue [16–18], formalin-fixed, paraffin-embedded (FFPE) tissue [19,20] and biofluids, has been an active scientific focus for many years in our laboratory [19,21,22]. Experimental design and sample preparation remains the most challenging component of any clinical proteomic workflow. Here, the goal was to develop a reproducible, high-throughput MS-based pipeline amenable to cancer biomarker discovery or targeted quantitative assays [5,11,23,24].

To establish a pipeline for effective analysis of clinical specimens in the context of cancer biomarker research, we focused our efforts on addressing technological issues interfering with MS-based profiling of tissue and blood specimens. Using nonmetastatic clear cell renal cell carcinoma (ccRCC) as a model disease, we developed a tissue-directed approach for the detection of true tumor proteins in the peripheral blood, employing high-resolution MS-based shotgun proteomics (Figure 1) [12]. This approach/pipeline relies on the combined molecular profiling of solid tumor, normal adjacent tissue and pre-operative blood/plasma. The successful detection of tumor proteins in peripheral blood of a patient newly diagnosed with stage I ccRCC was accomplished (Figure 1) [12]. The experimental design employed in our proof-of-principle study coherently integrates innovative sample preparation, high-performance multidimensional liquid chromatography (LC)–MS along with subtractive and label-free quantitative proteomics. This approach allowed identification of a panel of tumor/disease-related proteins at the site of the pathological process, followed by their detection in peripheral blood [15]. In essence, a less complex space (i.e., solid tumor and normal adjacent tissue exhibiting a dynamic range of $\sim 10^{6-8}$) was studied via molecular profiling to facilitate detection of tumor-specific proteins in a more complex space (i.e., peripheral blood that had a dynamic range of $\sim 10^{10-12}$).

The identities of these proteins were cross-validated using antibody-based assays that are amenable for further evaluation in large patient cohorts using custom immunoassays (e.g., multiplex ELISA) [25] or targeted MS assays (e.g., multiplex MRM or immuno-MRM) [13,26]. This strategy/pipeline offers an effective solution for technological barriers interfering with MS-based profiling of clinical specimens and may accelerate translation of proteomic findings into clinical tests. Finally, it is compatible with the workflow of National Cancer Institute's Clinical Proteomic Technologies for Cancer (NCI-CPTC) initiative,

focused on development of assays against proteins prioritized in the discovery phase as valid cancer biomarker candidates [6,23].

Herein, we discuss in detail the challenges associated with MS-based profiling of clinical specimens and offer adequate solutions for issues related to experimental design, sample preparation, LC–MS analysis and raw data processing/interpretation. The proposed solutions were tested in our proof-of-principle study, which shows the ability of the present methodology to unambiguously identify genuine tumor proteins in tissue and to subsequently detect them in peripheral blood [12].

Prospective versus retrospective profiling of clinical specimens

An important decision related to the experimental design of any study analyzing clinical specimens is the selection of the mode of sample procurement. Samples may be collected prospectively or procured retrospectively from tissue repositories. Retrospective collection of clinical specimens that were stored for an extended period of time and were typically collected using different protocols will certainly increase preanalytical variability and may negatively affect the overall reproducibility of MS measurements. This may create measurements/biases reflective of differences related to the collection/storage circumstances rather than true changes associated with the pathology under study [27,28]. To avoid such biases we advocate for the prospective collection of clinical samples.

Thus, in our study, we collected tissue and blood specimens prospectively via the Cooperative Human Tissue Network (CHTN). However, the retrospective use of clinical samples may be justified if the selected specimens were collected using the same protocol and stored under identical conditions, or if the low incidence of a certain type of disease/cancer in general population is a limiting factor [29]. We also recommend that matched patient blood should be collected immediately prior to any surgical procedure to eliminate potential biases caused by leaking of tissue proteins into the vascular system during the surgical procedure [12].

Human population heterogeneity & protein variability

Variation in protein expression is reflected in diverse human populations along with the existence of many well-defined molecular cancer subphenotypes (e.g., breast cancer). These phenomena may introduce considerable systemic biases in case–control studies and may render the results of proteomic profiling difficult to interpret. To minimize preanalytical variation emanating from natural proteome differences found in the human population or distinct tumor subphenotypes, we propose the collection of clinical specimens (i.e., tumor tissue), matched controls (i.e., normal adjacent tissue) and peripheral blood from the same patient [12]. In this experimental setting, patient samples serve as their own controls in a manner similar to the genomic approach described by Von Hoff in the molecular profiling of solid tumors to identify/select therapeutic targets in patients with refractory cancers [30]. In 27% of patients, the molecular profiling approach resulted in a longer progression-free survival on a molecular profiling-suggested regimen than on a regimen in which the patient had just experienced progression. The Von Hoff study showed that molecular profiling of patients' tumors may provide valid targets that would improve the clinical outcome for an

individual with refractory cancer. It also suggests that the ‘N of one’ approach is of value in the context of personalized medicine and may be employed in proteomics as well [31]. Importantly, individually obtained molecular profiles at the proteome level may facilitate creation of a publicly accessible Cancer Proteome Atlas, complementary to the existing Cancer Genome Atlas [101] and may improve our ability to diagnose and treat human cancers via integration of genomic and proteomic data obtained from individually profiled solid tumors [32].

Blood versus tissue in biomarker research

Should blood or solid tissue be used for biomarker discovery? This dilemma still exists. The utility of peripheral blood as a starting point is unmatched by any other clinical specimen given its ease of acquisition, the diversity of analytes it contains and its practicality [33]. However, there are a number of items to consider. First, the dynamic range of protein expression in human plasma/serum still represents a significant analytical challenge for MS-based proteomics [33]. Second, the strategy focused on peripheral blood profiling makes it very difficult to trace the origin of the detected differences in protein expression back to the tumor. Third, the majority of disease-relevant biomarkers are expected to be at their highest concentration at the site of the pathological process (e.g., organ/tissue). Thus, we proposed that both tissue and blood should be profiled in parallel within the single cancer-specific platform [15]. Our results show that the combined profiling of tissue and blood permits identification of genuine tumor proteins at the site of the pathological process, followed by their detection in peripheral blood [12]. Hence, we argue that, in the biomarker discovery phase, matched tissue and blood specimens should be obtained from a single patient and analyzed simultaneously [12].

FF tissue versus FFPE tissue in biomarker research

There is a significant amount of interest in using FFPE tissue in biomarker research, primarily owing to its abundance and simple storage requirements [19,34]. However, the overall impact of uneven protein crosslinking and the variable duration of formalin fixation on the reproducibility of protein identification and/or quantitation accuracy is not well understood [35]. For these reasons, there is a consensus among clinical pathologists that FF tissue remains the sample of choice for molecular profiling of biomolecules, since proteins and/or nucleic acids in FF tissue remain unmodified and preserved in their natural environment [36]. Importantly, for antibody-based immunoaffinity depletion to be incorporated into the clinical specimen preparation workflow, it is of paramount importance that high-abundant proteins remain in their natural conformation. Therefore, we argue that snap FF tissue should be the sample of choice in MS-based clinical proteomics, as described in our proof-of-principle study [12].

Presence of highly abundant proteins in human plasma/serum

The immense complexity of the human plasma proteome and the proteolytic background created by tryptic digestion of highly abundant proteins (the 22 most abundant blood-derived proteins constitute 99% of the total plasma protein mass) [37] make the MS-based identification of tumor-derived proteins in peripheral blood a daunting task [33]. An excess of highly abundant proteins/ peptides limits the dynamic range of MS-analysis and creates a

bias toward the identification of highly abundant proteins. To address this complexity, numerous techniques were developed for removal of highly abundant proteins from plasma/serum [38], including chicken-derived IgY antibodies [39] or hexapeptide combinatorial ligand libraries (i.e., ProteoMiner™; Bio-Rad Laboratories, CA, USA) [40,41]. Methods for removal of highly abundant proteins from other biofluids (e.g., amniotic fluid and synovial fluid) were also described [42,43]. Arguably, immuno-depletion is considered the most effective, reliable and reproducible technique [38,44–47], and is widely used in contemporary biomarker research [48–50]. This increases the identification rate of lower-abundant proteins by an average of two-to four-fold [45] and has been consistently used for removal of abundant proteins from peripheral blood specimens (i.e., plasma and serum). Furthermore, the Human Proteome Organization (HUPO) recommends removal of highly abundant proteins from plasma/serum as a mandatory step in MS-based molecular profiling of peripheral blood specimens [51]. Consequently, in our proof-of-principle study, we have incorporated immunodepletion for the removal of highly abundant proteins from patients' blood using Agilent's (CA, USA) Multiple Affinity Removal System (MARS) [12].

Presence of highly abundant blood-derived proteins in tissue homogenates

Often overlooked, tissue-embedded networks of blood and lymph capillaries (Figure 2), along with interstitial fluid, contain substantial amounts of highly abundant blood-derived proteins that may interfere with proteomic analysis. Blood, plasma, lymph and interstitial fluid are major constituents of tissue extracellular space/fluid. Specifically, water accounts for approximately 60% of the human body/tissue mass where approximately 55% of total body water is found inside the cells (i.e., intracellular fluid) and approximately 45% of water is found in the extracellular tissue compartment (i.e., extracellular fluid) [52]. The distance between any single functional cell and the nearest capillary in any human tissue is approximately 25 μm [52]. Perfusion is a fundamental process of continuous blood delivery to the tissue via microcirculation within closely packed capillaries facilitating the exchange of water, oxygen, carbon dioxide, nutrients and metabolites. Additionally, a formidable amount of blood, lymph and interstitial fluid is found in dense networks of arterioles and venules, as well as in lymph capillaries involved in tissue perfusion (Figure 2).

Furthermore, a major prerequisite for effective/constant tissue perfusion is tightly regulated colloidal osmotic pressure (i.e., oncotic pressure). The oncotic pressure exerted by proteins in tissue capillaries (i.e., microcirculation) tends to pull water into the circulatory system. Oncotic pressure is the opposing force to hydrostatic pressure. The principal role of albumin, which accounts for approximately 60% of the total plasma protein mass, is the regulation of the oncotic pressure to prevent nonphysiologic extravasation of the intravascular fluid into the interstitial space. Albumin creates approximately 80% of the total oncotic pressure exerted by blood plasma on interstitial fluid [52]. Importantly, only one-third of total body albumin is found intravascularly. The remaining two-thirds of albumin is distributed in the extravascular tissue compartment [53,54].

These principles of tissue physiology served as a rationale to investigate the presence of highly abundant blood-derived proteins in our proof-of-principle study and to assess the need for their removal from FF tissue homogenates. As anticipated, SDS-PAGE analysis of

the renal cell carcinoma (RCC) tissue homogenate (line 1) and the adjacent normal kidney tissue (line 2), visualized using silver stain, revealed the existence of an excess of highly abundant blood-derived proteins (albumin) in crude tissue homogenates (Figure 3A). These results were consistent with the role of albumin in tissue perfusion and maintenance of colloidal oncotic pressure [53,54]. The same electrophoretic separation indicated successful immunodepletion of highly-abundant blood-derived tissue proteins using Agilent's MARS Human 14 cartridges (i.e., detailed protocol depicted in Figure 4), exemplified by an absence of the albumin and other highly abundant blood-derived proteins bands in immunodepleted flow-through fractions (lines 3 and 4; Figure 3a). For comparison purposes, the immunodepletion of highly-abundant proteins from patients' blood is shown in Figure 3B. Taken together, these results indicated the need for removal of highly abundant blood-derived proteins from tissue homogenates exemplified by the unmasking effect of tissue immunodepletion, the absence of the high-abundant blood-derived proteins (albumin) and the appearance of bands previously not seen in the crude tissue homogenates (lines 3 and 4; Figure 3a). Notably, the SDS-PAGE pattern of tissue depletion (Figure 3A) mirrors the immunodepletion pattern of peripheral blood (Figure 3B). To the best of our knowledge, apart from our study [12], there are no peer-reviewed publications on the use of immunoaffinity depletion of abundant blood-derived proteins from clinical tissue specimens in the context of cancer biomarker research/discovery.

Reducing the complexity of clinical specimen digestates

Even after immunodepletion, tissue and plasma/serum digests need further fractionation to reduce the immense complexity of clinical sample peptide mixtures. Strong cation exchange (SCX)-based fractionation has been proven effective in reducing complex peptide mixtures for MS-based bottom-up proteomics [55]. It increases the dynamic range of proteome coverage by creating peptide fractions of lower complexity that can be expansively sampled/analyzed by LC-MS. Instead of using the original MudPIT approach where SCX and reversed phases are packed on-line in a single column, we developed a sensitive laser-based off-line SCX fractionation coupled to a fluorescent detection system [56–58]. This modification permits a prescreening run, using solid-state UV laser technology, where only 10 µg of peptide is sufficient to visualize the SCX separation as it occurs [56]. Our system enables fine tuning of the elution gradient and allows for evidence-based pooling of peptide fractions, taking into account peak distributions and intensities. This sample-tailored SCX fractionation improves substantially the dynamic range of subsequent LC-MS analysis, resulting in wider proteome coverage [57]. Hence, we argue that off-line SCX (if available) should be preferentially used in shotgun clinical proteomics to maximize the coverage of clinical sample proteomes.

Concomitant analysis of tissue & blood is critical for detection of tumor-derived proteins in blood

The primary goal of our proof-of-principle study was to develop a method for detection of tumor proteins by MS in the peripheral blood of a patient diagnosed with nonmetastatic RCC. Toward this goal, the lists of identified proteins were filtered to discover proteins found in tumor but not normal tissue, identified in matching plasma and whose spectral count was higher in tumor than in plasma (Figure 1). For accurate measurement/comparison

of relative differences in protein concentration between two samples using spectral counting or any other quantitative proteomic approach, it is critical that the normalization of protein content is carried out before the digestion step and that the compared samples are processed using identical workflows. Therefore, tissue immunodepletion and normalization of protein mass were of critical importance to reveal genuine tumor-derived protein species at the site of the malignant process and to detect a panel of biomarker candidates in peripheral blood of a patient diagnosed with nonmetastatic ccRCC (Figure 1) [12].

Integrative meta-analysis of proteomic & gene-expression data

A rather weak correlation has previously existed between data obtained from genomic investigations and data obtained from corresponding early proteomic studies. This weak correlation was primarily due to nonstandardized genomic technologies and low-resolution MS [59]. Nevertheless, recent advances in gene sequencing technologies and high-resolution/accuracy MS instrumentation has contributed to an increasing number of proteogenomic studies showing significantly higher correlation/congruence between proteomic and genomic data [31,60,61].

To gauge the efficacy of our proteomic pipeline, we performed a joint meta-analysis of our previously published proteomic data reported in our proof-of-principle study [12] (also see the supplementary data from our previous study [102]) with the genomic data reported by Tun *et al.* [62] investigating the same type of ccRCC using the Affymetrix GeneChip® Human Genome HG-U133a Array and GeneChip® HT Human Genome U133B Array Plate. The objective of this genomic study was to reveal and characterize pathways driving ccRCC biology by analyzing ten nonmetastatic early stage RCC tissue specimens and patient-matched normal adjacent kidney tissue. The gene data revealed three striking biological alterations in ccRCC that included deregulation of metabolism, epithelial-mesenchymal transition (EMT) and adipogenic transdifferentiation. These results were further validated using quantitative real-time PCR (qPCR) and immunohistochemistry (IHC) on two independent RCC tissue sets [62].

Herein, by means of comparative analysis of two data types, we extended our previous efforts to analyze molecular profiles obtained from the same type of FF ccRCC clinical specimens. For this joint analysis, we focused on a subset of overlapping protein species identified in both ccRCC and normal adjacent tissue (accessible as supplemental data in our proof-of-principle study) [12]. We used spectral counting to calculate relative differences in protein concentration and to correlate/compare proteomic and genomic data. Only proteins identified by 2 protein specific (unique) peptides were included in the quantitative analysis. These criteria permitted the association of a single protein identifier with a single gene symbol and aided in improving relative protein quantitation accuracy.

Joint analysis of proteomic & genomic data revealed deregulation of carbohydrate metabolic pathways

The sigPathway analysis of genomic data employed by Tun *et al.* revealed deregulation of carbohydrate metabolic pathways that included fructose and mannose metabolism along with the pentose phosphate pathway [62]. Accordingly, increased expression of γ -enolase

(ENO2) and J-polypeptide 2 (CYP2J2), along with decreased expression of fructose-1,6-bisphosphate aldolase (ALDOB), were detected in the tumor as part of carbohydrate metabolic alteration, subsequently validated by qPCR and IHC.

Relative quantitation of protein expression via spectral counting also revealed changes in carbohydrate metabolism similar to ones observed by Tun *et al.* (Table 1). Evidently, the coverage at the protein level was less extensive since the proteomic workflow lacked technology capable of amplifying protein species in the manner similar to nucleic acid amplification (i.e., PCR). Proteomic analysis also revealed the upregulation of γ -enolase (ENO2) and adrenomedullin (ADM) in parallel with downregulation of fructose-bisphosphate aldolase B (ALDOB) in the tumor (Table 1). In addition to a higher concentration of γ -enolase, proteomic analysis revealed >3-fold higher relative concentrations of the α -enolase (ENO1) isozyme in the tumor. α -enolase was recently proposed as a diagnostic follow-up marker for RCC recurrence [63]. In addition to the virtual absence of fructose-bisphosphate aldolase B observed in the genomic and proteomic study, we also observed an increased relative concentration of aldolases C and A (ALDOC and ALDOA) in the tumor (Table 1).

Our measurements of increased expression of aldolases C and A in the tumor are in agreement with the findings reported by Takshi *et al.*, using IHC and an enzyme-based immunoassay [64,65]. The upregulation of these isozymes in the tumor also concurs with results of a study by Zhu *et al.*, showing a strong signal of aldolases A and C in all analyzed RCC tissues (n = 10) and only weakly stained aldolase B in RCC tissues [66]. This is indicative of the prevalence of aldolase B in the normal differentiated kidney tissue while isozymes A and C are prevalent in the tumor. Taken together, the differences in expression levels of aldolases between tumor and normal kidney observed at the gene and protein level are consistent with substantial alterations in carbohydrate metabolism in ccRCC. These findings allowed us to hypothesize that the switch from isozyme B prevalent in normal kidney to isozymes A/C predominant in ccRCC is consistent with simultaneous glycolysis pathway activation and gluconeogenesis pathway deactivation in ccRCC demonstrated by the virtual absence of aldolase B in tumor homogenates. This is also consistent with the role of these enzymes in kidney carbohydrate metabolism, as previously reported [67].

Both genomic & proteomic analyses exposed EMT as a major process driving RCC tumorigenesis

Kidneys are of mesenchymal origin. Thus, mesenchymal–epithelial transition is accepted as the principal biological process driving kidney development/differentiation. On the other hand, EMT is increasingly recognized as an important oncogenic dedifferentiation process responsible for the invasive/metastatic phenotype of human carcinomas [68]. Correspondingly, genomic analysis revealed upregulation of EMT markers in tumor that included fibronectin (*FN1*), vimentin (*VIM*) and cadherin-2 (*CDH2*), indicating an important role of EMT in ccRCC tumorigenesis [69–71].

In a similar manner, our proteomic analysis revealed increased relative concentrations of fibronectin [69] and vimentin [70] in the tumor (Table 1). While our proteomic analysis did not show upregulation of cadherin-2, it did uncover increased relative concentrations of

cadherin-6 and cadherin-13 in the tumor. In addition, cadherin-5 and cadherin-11 were identified in tumorous tissue only and were detected in the blood of the patient diagnosed with ccRCC. The overall upregulation of these ‘mesenchymal cadherins in the tumor is indicative of their complex role in ccRCC carcinogenesis and neoangiogenesis [72,73]. This finding at the proteome level is consistent with ‘cadherin switching’, a phenomenon indicative of transcriptional reprogramming linked to renal epithelia dedifferentiation [74].

Joint analysis of proteomic & genomic data revealed activation of a lipogenic pathway in RCC

Cancer is characterized by increased levels of anaerobic glycolysis (i.e., Warburg effect), even in the presence of normal levels of oxygen, as well as by increased synthesis of proteins, DNA and fatty acids [75,76]. Consistent with this concept, the accumulation of lipid droplets characteristic of ccRCC cells suggests an activation of the lipogenic pathway and its prominent role in ccRCC biology. Indeed, altered gene expression of fatty acid-binding proteins (FABPs) and adipose differentiation-related protein/adipophilin (*ADFP/PLIN2*) were consistent with the adipogenic transdifferentiation that is taking place in ccRCC [77]. Specifically, downregulation of *FABP1* and *FABP3* along with upregulation of *FABP5*, *FABP7* and *PLIN2* were revealed at the gene level [62]. Altered expression of FABPs was confirmed by qPCR followed by validation of adipophilin upregulation in ccRCC by IHC [62]. Notably, Oil Red O staining revealed lipid-laden clear cell morphology of ccRCC [62]. Subsequently, Tun *et al.* showed that KIJ-308 and KIJ-265 ccRCC cells grown in adipogenic media undergo adipogenic differentiation, as indicated by Oil Red O staining. Notably, normal patient-matched cells were unable to undergo adipogenic differentiation when grown using identical adipogenic conditions [62]. This lipogenic molecular signature is consistent with the ‘clear cell’ ccRCC morphology characterized by cytoplasmic lipid accumulation [78], indicating an important role of neosteatogenesis in ccRCC biology [77].

Proteomic analysis produced similar results depicting decreased relative concentrations of FABP1 and FABP3 [79] along with increased relative concentrations of FABP5 and adipophilin in the tumor (Table 1). Recent studies revealed an important role of FABPs and adipophilin in ccRCC biology [80,81]. Importantly, adipophilin was recently proposed as a biomarker for early diagnosis of ccRCC [82] and as a potential therapeutic target in RCC vaccine development [83]. Owing to this we carried out validation of adipophilin expression via western blot analysis in commercially available ccRCC homogenates and matched normal kidney tissues on two additional specimens (Figure 5). Consistent with proteomic and genomic analyses, western blotting confirmed upregulation of adipophilin in ccRCC tissue. Therefore, changes in expression levels of FABPs and adipophilin may be considered ccRCC specific.

Taken together, the results of our present proteogenomic comparison suggest a higher degree of correlation between protein and gene-expression data than previously thought. These findings validate the utility of our approach, at least for classes of proteins implicated in ccRCC tumorigenesis. While this analysis was focused on concordant gene-/protein-

expression patterns, the opposite patterns were also observed and will be discussed elsewhere.

These results also suggest that spectral counting-based measurements of relative protein concentrations obtained using clinical MS-based shotgun proteomics in the context of tissue immunodepletion are comparable to microarray-based intensity measures of gene expression in the same tissue type. Also, other label-free strategies (e.g., integrated peptide ion chromatograms extracted from the MS¹ spectra) can be evaluated using gene-expression intensities as an indirect benchmark for similar protein-expression levels.

Further application of immunodepletion to different tissue types

We subsequently carried out the optimization of immunodepletion of various solid tumor tissue homogenates (i.e., Ewing's sarcoma and breast cancer) and lipid tissue using the MARS Human 14 cartridges, as depicted in Figure 4. As anticipated, SDS-PAGE analysis confirmed a substantial presence of highly abundant blood-derived proteins, warranting their removal prior to LC-MS analysis (Figures 6 & 7). The optimization of our immunodepletion workflow for these tissues was straightforward, as exemplified by effective depletion of abundant proteins from crude tissue homogenates shown in Figures 4 & 5.

Unlike in other tissues, lipids in adipose tissue are stored in monovacuolar or polyvacuolar structures within fat cells (adipocytes) surrounded by thin layer of cytoplasm. For this reason, the extraction of proteins from adipose tissue specimens has generally been challenging because lipids comprise approximately 87% of the total adipose tissue mass. In view of that, we anticipated that the removal of lipids prior to abundant protein depletion may be a necessary step. Indeed, crude lipids clogged 0.22- μ m immunodepletion prefilters, indicating the necessity of delipidation prior to immunoaffinity removal of abundant blood-derived proteins from lipid tissue homogenates. While there are a plethora of methods for lipid removal from biological specimens, most of them utilize denaturing reagents (e.g., organic solvents, detergents or chaotropes) rendering subsequent immunodepletion unfeasible. After a series of optimization experiments, we found that centrifugation of the adipose tissue homogenate at 4°C for 20 min at 12,000 $\times$ g separates the upper crude lipid phase from the lower protein-containing phase, rendering the adipose tissue homogenate suitable for immunodepletion. Figure 8A depicts successful immunodepletion of a normal human adipose tissue homogenate after mechanical delipidation. For quality control purposes, Figure 8B shows immunodepletion of standard human serum obtained from Sigma-Aldrich Co., LLC (MO, USA). Immunodepletion of body fluids other than blood may be required and optimized. Therefore, we modified the protocol depicted in Figure 4 and applied it to both amniotic and blister fluids, as shown in Figure 9.

To investigate the benefits of delipidation coupled with immunodepletion, we performed a series of simple 1D-LC-MS experiments, in order to assess repeatability and reproducibility of protein identification in different experimental settings. For comparative LC-MS analyses, we used high-resolution hybrid linear ion trap (LIT) Fourier-transform ion cyclotron (FTICR)-MS (LT-FT, Thermo-Scientific, CA, USA) as well as a low-resolution

LIT MS (LTQ, Thermo-Scientific) to analyze the lipid tissue proteome solubilized and digested in various buffers (organic solvent based [i.e., methanol]; and chaotrope-based [i.e., urea]), employing identical LC systems and separation methods. Both instruments were operated in a data-dependent mode in which each full MS scan was followed by seven tandem mass spectrometry (MS/MS) scans where the most abundant peptide molecular ions were dynamically selected for collision-induced dissociation using a normalized collision energy of 36%. All MS/MS spectra from clinical tissue specimens were searched independently against a nonredundant human protein database (UniProt Human [103]) using SEQUEST. The sequence database searches were carried out on a Beowulf 18-node parallel virtual machine cluster-computer. Dynamic modifications were added for the detection of carboxyamidomethylated cysteine (+57 Da) and oxidized methionine (+16 Da). For the LIT-FTICR-MS spectra, the monoisotopic precursor ion mass tolerance was set at 5 ppm. For the MS/MS spectra, the fragment ion tolerance was set at 0.5 Da. For the LIT-MS spectra, the monoisotopic precursor ion mass tolerance was set at 1.5 Da and the fragment ion tolerance was set at 0.5 Da. The collision-induced dissociation spectra were searched against the same nonredundant human protein database by SEQUEST (Thermo-Scientific) using identical thresholds. Peptide identifications were considered legitimate based on the following thresholds: correlation 0.08 and crosscorrelation 2.0 for 1+, 2.2 for 2+ and 3.3 for 3+ peptides.

Results shown in Table 2 indicate that regardless of the instrument platform used, delipidation coupled with immunodepletion produced the best results exemplified by the highest number of total protein identifications by 2 peptides. These findings suggest that delipidation is required prior to immunodepletion of adipose tissue homogenates. Also, a remarkably lower number of albumin peptide identifications in depleted specimens demonstrated effective removal of highly abundant blood-derived proteins via immunodepletion. Importantly, results shown in Table 2 substantiate the benefit of using high-resolution/accuracy MS (i.e., LT-FT) in the context of clinical proteomics as indicated by the highest number of a total protein identifications ($n = 86$) by 2 peptides versus the low mass accuracy (LTQ) instrument ($n = 52$). Notably, the percentage (18%) of proteins identified by 2 peptides using low mass accuracy MS is indicative of a high false-discovery rate, which is consistent with a 'precision proteomics' paradigm outlined elsewhere [32,84].

To quantify the removal rate of abundant blood-derived proteins from lipid tissue homogenates, we analyzed immunodepleted and nondepleted adipose tissue specimens using a 2D-LC-MS analysis that relies on SCX LC fractionation and reversed-phase LC-MS using high-resolution hybrid LIT Fourier-transform (i.e., LT-FT) MS. We used spectral counting-based quantitation to estimate the removal rate of abundant blood-derived proteins from lipid tissue homogenates. The observed changes in the relative concentration of overlapping nontargeted protein species identified in both specimens showed an average enrichment of twofold, as previously reported [45]. These results are similar to previously reported findings for human plasma/serum [85]. Our analysis also revealed a 28% increase in identified proteins in the immunodepleted tissue specimen. Importantly, adipose-specific fragment 2 was identified by multiple peptides only in the immunodepleted sample, thus confirming the adipose tissue phenotype under study. Collectively, these results are consistent with findings depicted in Figure 8, signifying the prerequisite for delipidation of

lipid tissue homogenate prior to immunodepletion to increase the depth of the adipose tissue proteome coverage.

Conclusion

The results of our proof-of-principle study along with the findings presented in this perspective indicate that solid tissue specimens contain an excess of highly abundant blood-derived proteins and that their removal is required to improve the dynamic range of MS-analysis and permit accurate measurement of changes in protein concentration in tissue and blood specimens. We argue that the immunodepletion of abundant blood-derived proteins from tissue homogenates is of equal importance as the immunodepletion of plasma/serum and recommend that it should be routinely employed in MS-based biomarker research. We also propose concomitant analysis of tissue and peripheral blood to increase the chance of identifying genuine tumor proteins in peripheral blood.

Future perspective

Cancer is the second leading cause of death in the western world. It is estimated to become the leading cause of death within the next decade. Concomitant molecular profiling of tissues and biological fluids (e.g., blood) using MS-based clinical proteomics will be increasingly used to enhance cancer biomarker research to facilitate detection of genuine tumor proteins/markers in the peripheral blood of the same patient diagnosed with cancer. These molecular profiles will allow for deeper insights into cancer biology that are not readily attainable using *in vitro* cultured cells, which lack a tumor/tissue microenvironment.

To avoid the shortcomings of early proof-of-principle studies and to increase the dynamic range of MS-analysis, as well as to allow accurate measurement of changes in protein concentration in tissue and blood, we anticipate that the removal of highly abundant blood-derived proteins from tissue homogenates will be routinely used to increase the chance of identifying cancer biomarkers at the site of malignancy and peripheral blood.

We also anticipate that an increasing number of integrative proteogenomic investigations will be carried out to facilitate better characterization of the tumor phenotype coupling high-resolution MS with next-generation transcriptome sequencing of clinical specimens.

The results from our proof-of-principle study [12], as well as the results from similar succeeding investigations [31,48], suggest that a small number of clinical samples may be sufficient in the discovery phase, providing that the initial finding can be cross-validated using orthogonal immunoassays (e.g., western blotting) or MS-based assays [13,14,48].

Subsequently, cross-validated biomarker candidates will be further investigated/tested on large cohorts of clinical specimens/patients using high-throughput assays (e.g., multiplex ELISA and MRM).

We believe that, in the next 5–10 years, concomitant immunodepletion of tissue and blood specimens using present proteomic pipeline or similar proteomic workflow will fully

complement the advances in genomics and metabolomics, in the quest for cancer biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Executive summary

Mass spectrometry-based clinical proteomics in cancer biomarker research

- Mass spectrometry (MS)-based profiling of clinical tissue specimens has increasingly been used in cancer biomarker research to characterize changes in protein expression between tumor and healthy tissue, or between the blood of diseased and healthy individuals, because it is capable of capturing post-transcriptional and post-translational changes at the protein/phenotype level.
- The discovery of valid cancer biomarkers using MS-based proteomics has proven difficult, primarily owing to the analytical challenges caused by the huge dynamic range of human plasma protein levels (i.e., >10 orders of magnitude) and the fact that the 22 most abundant proteins constitute approximately 99% of the total plasma protein mass.
- The poor experimental design of early ‘proof-of-principle’ studies, which focused exclusively on plasma/serum specimens, and the use of low-resolution MS instrumentation and other relatively unsophisticated analytical methods have not been beneficial to the field of clinical proteomics.

Addressing issues/challenges related to the analysis of clinical specimens in the context of MS-based biomarker research

- Innovative tissue-directed proteomic platforms are needed to increase the dynamic range of proteomic profiling and to bridge the gap between *in vitro* systems/models and *in vivo* human cancers in order to facilitate the discovery of sorely needed clinically applicable cancer biomarkers.
- Often overlooked, tissue-embedded networks of blood and lymph capillaries, along with intestinal fluid, contain substantial amounts of highly abundant blood-derived proteins. These proteins interfere with proteomic analysis and their removal is of equal importance to the immunodepletion of peripheral plasma/serum clinical specimens.
- To increase the chance of identifying tumor proteins/markers in peripheral blood, it is critical to use tissue-directed approaches and concomitantly analyze clinical tissue and blood specimens collected prospectively from a single patient diagnosed with cancer.

Evaluation of the proof-of-principle study using the integrative meta-analysis of proteomic data & gene-expression data obtained from the same type of ccRCC

- Integrative analysis of protein- and gene-expression data obtained from the same type of ccRCC showed a high degree of correlation between proteomic and microarray genomic data for classes of proteins and pathways implicated in ccRCC tumorigenesis.
- Spectral counting-based measurements of changes in relative protein concentrations in the context of tissue immunodepletion and high-resolution

MS are comparable to intensity-based measures of gene-expression microarray data in the same tissue type.

- The results obtained from proteogenomic analysis indicate that a small number of samples showing large size effect may be sufficient in the discovery phase, providing that initial findings can be cross-validated using orthogonal immunoassays (e.g., western) or MS-based assays.

Further application of immunodepletion to different tissue types

- Immunodepletion of tissue homogenates is readily amenable for different tissue types, as well as biofluids (i.e., plasma, blister fluid and amniotic fluid).

Conclusion

- Immunodepletion of abundant blood-derived proteins from human tissue homogenates is of equal importance as immunodepletion of plasma or serum specimens to decrease tissue homogenate's dynamic range and allow accurate measurements of changes in protein levels between tissue and blood specimens.
- Tissue immunodepletion should be routinely used in MS-based clinical proteomics for biological discovery or cancer biomarker research.
- Concomitant profiling of tissue and peripheral blood is critical for better characterization of tumor phenotype and detection of tumor proteins in peripheral blood by MS.
- The biomarker discovery phase using MS-based proteomics does not necessarily require a large number of samples, especially when combined with genomic analysis of matching clinical tissue/blood specimens.

Figure 1. Experimental design

LC: Liquid chromatography; LC-LIT-FTICR-MS: Liquid chromatography linear ion trap Fourier transform ion cyclotron mass spectrometer; SCX: Strong cation exchange.

Figure 2. Distribution of blood, lymph and interstitial fluid in human tissue

Tissue-embedded networks of blood and lymph capillaries along with interstitial fluid compartment contain significant amounts of abundant blood-derived proteins. Reproduced with permission from [104].

Figure 3. SDS-PAGE analysis of immunoaffinity-depleted renal cell carcinoma tissue homogenate and matched patient serum

(A) Renal cell carcinoma tissue homogenate; (B) matched patient serum. Samples were resolved on a 4–12% Bis-Tris gel (Invitrogen Life Technologies, CA, USA) and stained with a SilverQuest™ Silver Staining Kit (Invitrogen Life Technologies). (A) Lane designations: lane 1, normal kidney tissue homogenate (5 μ g); lane 2, RCC tissue homogenate (5 μ g); lane 3, depleted normal kidney tissue homogenate (5 μ g); and lane 4, depleted RCC tissue homogenate (5 μ g). (B) Lane designations: lane 1, renal cell carcinoma patient serum (5 μ g); lane 2, depleted matched patient serum (5 μ g); and lane 3: high-abundant fraction (15 μ l).

Figure 4. Clinical tissue immunodepletion workflow

Each surgically collected fresh frozen tissue block was sectioned into 8-µm-thick slices using a cryostat followed by tip sonication and bicinchoninic acid assay. Particulates were removed from the homogenate using a 0.22-µm spin filter. A total of 1000 µg of tissue protein was immunodepleted using MARS Human 14 immunoaffinity cartridges (Agilent, CA, USA). The resulting low-abundant protein fractions were pooled and concentrated using 5 kDa molecular weight cutoff spin concentrators (Agilent, CA, USA). Immunodepleted tissue homogenate (200 µg each) was then digested with trypsin. Final

clean-up of the tryptic digest was achieved using a C18 column (Waters, MA, USA). Peptide fractions were collected using off-line SCX chromatography and analyzed by μ RPLC tandem mass spectrometry. QC measures were employed to assess MARS Human 14 column (Agilent) integrity by removing an aliquot of both the low- and high-abundant pool and subsequently resolving the proteins on an SDS-PAGE gel.

μ RPLC: Microflow reversed-phase liquid chromatography; LC: Liquid chromatography; MARS: Multiple Affinity Removal System; MS/MS: Tandem mass spectrometry; QC: Quality control; SCX: Strong cation exchange.

Figure 5. Detecting adipophilin in renal cell carcinoma patient-derived tissue lysates using western blotting

Paired tissue lysates from two patients (lane 1: renal cell carcinoma [RCC], patient 1; lane 2: normal renal tissue, patient 1; lane 3: RCC, patient 2; lane 4: normal renal tissue, patient 2) were used. Quantitative analysis of the blot as fold change is shown in the lower panel. The blot intensity from the RCC patient was normalized to the blot intensity from the respective normal renal tissue. Elevated adipophilin was detected in RCC compared with the respective normal renal tissues. Tissue lysates were purchased from Abcam (MA, USA). A total of 10 μ g of tissue lysates were separated on 4–20% Tris-glycine gradient gels (Invitrogen Life Technologies, CA, USA), transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, CA, USA) and blotted with anti-adipophilin antibody (Epitomics, CA, USA) followed by a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, PA, USA). Image of the blot was quantitated using Image J (NIH, MD, USA).

Figure 6. SDS-PAGE analysis of immunoaffinity-depleted Ewing's sarcoma tissue homogenate and matched patient serum

(**A**) Ewing's sarcoma (EWS) tissue homogenate; (**B**) matched patient serum. Samples were resolved on a 4–12% Bis-Tris gel (Invitrogen Life Technologies, CA, USA) and stained with SimplyBlue™ Coomassie® G-250 SafeStain (Invitrogen Life Technologies). (**A**) Lane designations: lane 1, EWS tissue homogenate (10 µg); lane 2, depleted EWS tissue homogenate (10 µg); and lane 3, high-abundant fraction (20 µl). (**B**) Lane designations: lane

1, matched patient serum (10 μ g); lane 2, depleted patient serum (20 μ l); and lane 3, high-abundant fraction (20 μ l).

Figure 7. SDS-PAGE analysis of immunoaffinity-depleted breast tissue homogenate and matched patient serum

(A) Breast tissue homogenate; (B) matched patient serum. Samples were resolved on a 4–12% Bis-Tris gel (Invitrogen Life Technologies, CA, USA) and stained with SimplyBlue™ Coomassie® G-250 SafeStain (Invitrogen Life Technologies). (A) Lane designations: lane 1, SeeBlue® Plus2 (Invitrogen Life Technologies) prestained protein molecular weight standard (kDa); lane 2, breast tissue homogenate (15 µg); lane 3, depleted breast tissue homogenate, flow-through fraction #1 (20 µl); lane 4, depleted breast tissue homogenate, flow-through fraction #2 (20 µl); and lane 5, high-abundant fraction (20 µl). (B) Lane designations: lane 1, SeeBlue Plus2 prestained protein molecular weight standard (kDa); lane 2, matched patient serum (10 µg); lane 3, depleted patient serum, flow-through fraction #1 (20 µl); lane 4, depleted patient serum, flow-through fraction #2 (20 µl); and lane 5, high-abundant fraction (20 µl).

Figure 8. SDS-PAGE analysis of immunoaffinity-depleted adipose tissue homogenate and matched patient serum

(**A**) Adipose tissue homogenate; (**B**) matched patient serum. Samples were resolved on a 4–12% Bis-Tris gel (Invitrogen Life Technologies, CA, USA) and stained with SimplyBlue™ Coomassie® G-250 SafeStain (Invitrogen Life Technologies). (**A**) Lane designations: lane 1, SeeBlue® Plus2 (Invitrogen Life Technologies) prestained protein molecular weight standard (kDa); lane 2, adipose tissue homogenate (10 µg); lane 3, depleted adipose tissue homogenate (10 µg); and lane 4, high-abundant fraction (20 µl). (**B**) Lane designations: lane 1, SeeBlue Plus2 prestained protein molecular weight standard (kDa); lane 2, matched patient serum (10 µg); lane 3, depleted matched patient serum (20 µl); and lane 4, high-abundant fraction (30 µl).

Figure 9. SDS-PAGE analysis of immunoaffinity-depleted amniotic fluid patient specimen and blister fluid patient specimen

(A) Amniotic fluid patient specimen; (B) blister fluid patient specimen. Samples were resolved on a 4–12% Bis-Tris gel (Invitrogen Life Technologies, CA, USA) and stained with SimplyBlue™ Coomassie® G-250 SafeStain (Invitrogen Life Technologies). (A) Lane designations: lane 1, amniotic fluid patient specimen (10 µg); lane 2, depleted amniotic fluid patient specimen (30 µl); and lane 3, high-abundant fraction (30 µl). (B) Lane designations: lane 1, SeeBlue® Plus2 (Invitrogen Life Technologies) prestained protein molecular weight standard (kDa); lane 2, blister fluid patient specimen (10 µg); and lane 3, depleted blister fluid patient specimen (10 µg).

Proteogenomic analysis/comparison of expression levels for selected protein species implicated in clear cell renal cell carcinoma biology.

Table 1

Gene	Protein	UniProt accession number [†]	Johann <i>et al.</i> [12] SC values			TT:NT ratio		Validated	
			TT	NT		Johann <i>et al.</i> [12]	Tun <i>et al.</i> [62]	Johann <i>et al.</i> [12]	Tun <i>et al.</i> [62]
<i>ADFP</i>	Adipophilin	Q99541	37	3		Increased	Increased	Western	qPCR/IHC
<i>ADM</i>	Adrenomedullin	P35318	4	2		Increased	Increased	NA	qPCR
<i>CDH2</i>	Cadherin-2	P19022	13	28		Decreased	Increased	NA	qPCR
<i>CDH5</i>	Cadherin-5	P33151	12	0		TT only	NA	Western	NA
<i>CDH6</i>	Cadherin-6	P55285	37	26		Increased	NA	NA	NA
<i>CDH11</i>	Cadherin-11	P55287	4	0		TT only	NA	Western	NA
<i>CGH13</i>	Cadherin-13	P55290	24	8		Increased	NA	NA	NA
<i>ALDOA</i>	Fructose-bisphosphate aldolase A	P04075	19	4		Increased	Increased	NA	qPCR
<i>ALDOB</i>	Fructose-bisphosphate aldolase B	P05062	0	15		NT only (kidney)	Decreased	NA	qPCR/IHC
<i>ALDOC</i>	Fructose-bisphosphate aldolase C	P09972	10	1		Increased	Increased	NA	qPCR
<i>ENO1</i>	Alpha-enolase	P06733	11	3		Increased	Increased	NA	qPCR
<i>ENO2</i>	Gamma-enolase	P09104	2	0		TT only	Increased	NA	qPCR/IHC
<i>FABP1</i>	Fatty acid-binding protein 1	Q05CP7	3	56		Decreased	Decreased	NA	qPCR
<i>FABP3</i>	Fatty acid-binding protein 3	P05413	2	32		Decreased	Decreased	NA	qPCR
<i>FABP5</i>	Fatty acid-binding protein 5	Q01469	9	1		Increased	Increased	NA	qPCR
<i>FIN1</i>	Fibronectin	P02751	8	2		Increased	Increased	NA	qPCR
<i>VIM</i>	Vimentin	P08670	506	187		Increased	Increased	NA	qPCR/IHC

Accession numbers from [103].

IHC: Immunohistochemistry; NA: Not applicable; NT: Normal tissue; qPCR: Quantitative real-time PCR; SC: Spectral count; TT: Tumor tissue.

Table 2
Comparative liquid chromatography–mass spectroscopy analyses of adipose tissue homogenate.

Identification	1D-LC-LIT/FTICR-MS		1D-LC-LIT-MS		2D-LC-LIT/FTICR-MS	
	Dpl-M	N-Dpl-M	Dpl-M	N-Dpl-M	Dpl-M	N-Dpl-M
Σ peptide IDs (n)	997	1031	959	745	14,931	15,187
Σ protein IDs (n)	137	110	290	231	1,569	1,249
Σ protein IDs 2 peptides (n)	86	76	52	25	1,249	869
Σ protein IDs 2 peptides (%)	63	69	18	11	71	69
Σ albumin peptide IDs (n)	3	148	1	97	104	700
Albumin ranking	53rd	3rd	54th	3rd	3rd	427th

Σ: Total; 1D-LC-LIT/FTICR-MS: 1D liquid chromatography linear ion trap Fourier transform ion cyclotron mass spectrometer (high resolution/accuracy mass spectroscopy); 2D-LC-LIT-MS: 1D liquid chromatography linear ion trap mass spectrometer (low resolution/accuracy mass spectroscopy); 2D-LC-LIT/FTICR-MS: 2D liquid chromatography linear ion trap Fourier transform ion cyclotron mass spectrometer (high resolution/accuracy mass spectroscopy); Dpl-M: Depleted, digested in methanol-based buffer; N-Dpl-M: Nondepleted, digested in methanol-based buffer; N-Dpl-U: Nondepleted, digested in urea-based buffer.