Towards an Animal Model of Ovarian Cancer: Cataloging Chicken Blood Proteins Using Combinatorial Peptide Ligand Libraries Coupled with Shotgun Proteomic Analysis for Translational Research

Yingying Ma,^{1,*} Zeyu Sun,^{1-3,*} Ricardo de Matos,⁴ Jing Zhang,¹ Kunle Odunsi,⁵ and Biaoyang Lin^{1-3,6,7}

Abstract

Epithelial ovarian cancer is the most deadly gynecological cancer around the world, with high morbidity in industrialized countries. Early diagnosis is key in reducing its morbidity rate. Yet, robust biomarkers, diagnostics, and animal models are still limited for ovarian cancer. This calls for broader omics and systems science oriented diagnostics strategies. In this vein, the domestic chicken has been used as an ovarian cancer animal model, owing to its high rate of developing spontaneous epithelial ovarian tumors. Chicken blood has thus been considered a surrogate reservoir from which cancer biomarkers can be identified. However, the presence of highly abundant proteins in chicken blood has compromised the applicability of proteomics tools to study chicken blood owing to a lack of immunodepletion methods. Here, we demonstrate that a combinatorial peptide ligand library (CPLL) can efficiently remove highly abundant proteins from chicken blood samples, consequently doubling the number of identified proteins. Using an integrated CPLL-1DGE-LC-MSMS workflow, we identified a catalog of 264 unique proteins. Functional analyses further suggested that most proteins were coagulation and complement factors, blood transport and binding proteins, immune- and defense-related proteins, proteases, protease inhibitors, cellular enzymes, or cell structure and adhesion proteins. Semiquantitative spectral counting analysis identified 10 potential biomarkers from the present chicken ovarian cancer model. Additionally, many human homologs of chicken blood proteins we have identified have been independently suggested as diagnostic biomarkers for ovarian cancer, further triangulating our novel observations reported here. In conclusion, the CPLL-assisted proteomic workflow using the chicken ovarian cancer model provides a feasible platform for translational research to identify ovarian cancer biomarkers and understand ovarian cancer biology. To the best of our knowledge, we report here the most comprehensive survey of the chicken blood proteome to date.

Introduction

EPITHELIAL OVARIAN CANCER (EOC) is the most deadly gynecological cancer around the world, with high morbidity in industrialized countries (Siegel et al., 2012). Early diagnosis is key in reducing its morbidity rate. Serum tumor markers such as cancer antigen 125 (CA-125 or mucin 16) have been widely used in diagnosing EOC, along with other diagnostic methods (Maggino and Gadducci, 2000; Bast et al., 2005; Suh et al., 2010). However, CA-125 has limited

value in early-stage EOC screening owing to its poor sensitivity and specificity (Maggino and Gadducci, 2000; Nolen and Lokshin, 2012). Although several novel EOC biomarkers have been proposed in recent years (Zhang et al., 2004; Lin et al., 2009; Amon et al., 2010; Andersen et al., 2010; Lorkova et al., 2011; Tang et al., 2011; Shield-Artin et al., 2012; Wang et al., 2012a), none is approved for clinical applications. Hence, there still exists a need to identify markers for early-onset EOC. Compared with direct analysis of human clinical samples, outbred animal models with well-defined

¹System Biology Division, Zhejiang-California International Nanosystem Institute (ZCNI), and ²State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China.

³Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, China.

⁴Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York.

⁵Department of Gynecological Oncology, Roswell Park Cancer Institute, Buffalo, New York.

⁶Swedish Neuroscience Institute, Swedish Medical Center, Seattle, Washington.

⁷Department of Urology, University of Washington, Seattle, Washington.

*These authors contributed equally to this work.

cancer progression patterns are considered valuable surrogates for identifying potential biomarkers prior to human validation. Nevertheless, the decades-long search for a suitable EOC animal model has not been successful. Rodents rarely develop EOC, and even transgenic mice with induced EOC do not pathologically resemble the human counterpart (Orsulic et al., 2002). Compared to rodents, the aged domestic chicken (*Gallus domesticus*) has a high rate of developing spontaneous epithelial ovarian cancer (Wilson, 1953; Fredrickson, 1987; Johnson and Giles, 2006). A reduced egg-laying rate in chickens correlates with a lower EOC rate (Hakim et al., 2009), which is consistent with a previous postulation that extravagant incessant ovulation contributes to high risk of developing EOC in humans (Fathalla, 1971). The chicken EOC model has similarities to human EOC at the molecular level, including similar mutation patterns in p53 and overexpression of Her-2/neu (Hakim et al., 2009). Most importantly, overexpression of CA-125, a hallmark of EOC in human, was also reported in chicken EOC tissue (Jackson et al., 2007). Therefore, we hypothesize that chicken serum or plasma can be used as a potential surrogate reservoir from which cancer biomarkers can be identified via proteomic approaches.

Unfortunately, proteomic surveys of blood biomarkers are often hindered by the existence of highly abundant proteins in the blood. Removal of abundant proteins commonly relies on immunoaffinity-based methods (Huang et al., 2005; Qian et al., 2008; Zhou et al., 2009), which are not available for the chicken model owing to the lack of suitable antibodies. An alternative approach is to enrich low-abundance proteins using a combinatorial peptide ligand library (CPLL) prior to proteomic analysis. CPLL beads provide diversified peptide–protein interactions to specifically enrich different binding partners from complex samples (Thulasiraman et al., 2005; Guerrier et al., 2006). When incubated with a limited amount of immobilized CPLL ligands, high-abundance proteins readily saturate available binding sites, while low-abundance proteins are likely to be fully enriched (Thulasiraman et al., 2005; Guerrier et al., 2006). CPLL has been successfully applied to reduce the abundance differences between protein species for deep proteome coverage in various sample types (Righetti and Boschetti, 2008; Boschetti and Righetti, 2009;

Righetti et al., 2010), including serum and plasma (Sennels et al., 2007; Beseme et al., 2010; Fertin et al., 2010a, 2010b; Tu et al., 2011; Pinet, 2013).

In this report, we first demonstrated the effectiveness of an integrated shotgun proteomic workflow based on CPLL method coupled with 1DGE-LC-MS/MS to characterize serum or plasma of chickens with EOC. Compared with conventional protein precipitation by acetonitrile (ACN), the CPLL method significantly improved the protein identification rate by approximately two-fold for both chicken plasma and serum samples. We next conducted a larger-scale proteomic cataloging study for chicken blood samples using the CPLL approach. From this cataloging study, 232 and 173 nonredundant proteins were identified from chicken serum and plasma samples, respectively, resulting in a total of 264 chicken blood proteins. To the best of our knowledge, this is the largest blood protein dataset reported to date on the chicken blood proteome. Differential analysis based on spectral counting identified 10 potential EOC markers, levels of which were significantly altered in blood. In addition, our approach and the chicken blood proteome catalog can benefit the research community in the avian veterinary and poultry industries.

Materials and Methods

Chemicals

Acetonitrile (HPLC grade) was purchased from Burdick & Jackson (SK Chemie, Ulson, Korea). Formic acid (FA) was obtained from Tedia Company, Inc (USA). Ammonium bicarbonate (ABC), Tris [2-carboxyethyl] phosphine (TCEP), and iodoacetamide (IAA) were purchased from Sigma (St. Louis, MO). Sodium dodecyl sulfate (SDS) and bis-acrylamide were produced by BIO BASIC Inc. (Hamilton, Canada). In all experiments, ultrapure water with the resistance of 18.2 MΩ/cm was obtained from ELGA Lab Water purification system (High Wycombe, UK). Trypsin was obtained from Promega Corporation (Madison, WI).

Chicken ovarian cancer models

Babcock B-300 single-comb White Leghorn hens aged 2.5 years were individually caged with free access to water and

TABLE 1. SAMPLE INFORMATION OF CHICKEN MODELS

Sample ID	Age (months)	Group	Description
Serum 37	37	cancer	Ultrasound: ovary with no pre-ovulatory follicles, nodular and hyperechoic
Serum 41	44	cancer	Ultrasound: ovary with no pre-ovulatory follicles, enlarged, nodular, cystic and hyperechoic
Serum 43	38	cancer	Ultrasound: ovary with no pre-ovulatory follicles, enlarged, nodular, cystic and hyperechoic
Serum 51	38	cancer	Ultrasound: ovary with no pre-ovulatory follicles, small, cystic
Serum 70	38	cancer	Ultrasound: ovary with 3 pre-ovulatory follicles, enlarged, cystic (different size cysts)
Plasma 17	48	Normal	Ultrasound: ovary with 4 pre-ovulatory follicles; normal parenchyma
Plasma 22	45	Normal	Ultrasound: ovary with 5 pre-ovulatory follicles; normal parenchyma; one postovulatory follicle in the oviduct
Plasma 29	48	Normal	Ultrasound: ovary with 3 pre-ovulatory follicles; normal parenchyma
Plasma 35	45	Normal	Ultrasound: ovary with 4 pre-ovulatory follicles; normal parenchyma
Serum 52	41	Normal	Ultrasound: ovary with 4 pre-ovulatory follicles; normal parenchyma; one postovulatory follicle in the oviduct

food (standard layer diet). The birds were maintained on a 16 h light and 8 h dark schedule. Each bird was identified with a standard tab wingband. The birds were subjected monthly to physical exams, body weight measurement, blood collection, and transcutaneous ultrasonography of the ovary and oviduct. Cancer progression was determined based on ultrasonography findings. In domestic chickens, only the left ovary and oviduct are fully developed and functional, so a left lateral approach can be used for transcutaneous ultrasound evaluation of the ovary and oviduct. The ultrasound probe (5–8 MHz curved array attached to Titan ultrasound system, Sonosite, Bothell, WA) was placed in contact with the skin caudal to the last rib and ventral to the ilium and angled in a cranial, medial, and dorsal direction to visualize the ovary. The oviduct was examined by moving the probe from the ovary area in a caudal direction ventral to the ilium and pubis and oriented in a dorsal and medial direction. Both cranial-caudal and transverse B-mode gray ultrasonographic exams were performed. Necropsy was performed in all birds, including birds that died or were euthanized during the study or at the end of the study. Diagnosis of ovarian cancer was based on ultrasonography and/or postmortem evaluation. The study was approved by the Institutional Animal Care and Use Committee at Cornell University.

Chicken blood collection

Blood was collected from the right jugular vein [approximately 3 mL into refrigerated BD® Vacutainer serum tubes (serum samples) or lithium heparin tubes (plasma samples)], and spun at 4°C at 5,000 rpm for 5 min. For this study,

six serum and four plasma samples were collected from 10 birds as summarized in Table 1 and were immediately stored at –80°C for later proteomic analysis.

CPLL enrichment and ACN precipitation

Each sample was processed either by conventional ACN precipitation or by CPLL enrichment. For CPLL enrichment, protein of serum or plasma was extracted according to the instructions of ProteoMiner Protein Enrichment kits (catalog No. 163-3006, Bio-Rad, Hercules, CA). Briefly, the column was conditioned by 200 µL wash buffer (150 mM NaCl, 10 mM NaH₂PO₄, pH 7.4) three times. Two hundred microliters of serum or plasma were loaded onto the column, and then the column was washed three times with 200 µL wash buffer. Finally, the bound proteins were eluted from the column using 20 µL elution reagent (8 M urea, 2% CHAPS, 5% acetic acid) three times. The eluent was stored at –80°C.

In the ACN precipitation protocol, 200 µL serum or plasma sample was added to nine times volume of pre-cooled ACN and incubated at –20°C for 12 h. The precipitation was then collected by centrifugation at 15,000 g for 10 min. The concentration of all protein samples was determined by the standard BCA protein assay (Thermo Fisher, San Jose, CA) according to the manufacturer’s manual.

SDS polyacrylamide gel electrophoresis (PAGE) and in-gel trypsin digestion

Sixty micrograms of protein from each sample were separated by 12% SDS polyacrylamide gel electrophoresis (PAGE).

FIG. 1. Number of protein IDs identified from three serum and three plasma samples. CPLL enrichment method (*black*) significantly improves protein identification rate compared with the ACN precipitation method (*gray*).

FIG. 2. Number of proteins commonly or uniquely identified from the CPLL-enriched (*gray*) or the ACN-precipitated (*white*) serum and plasma samples.

The gels were stained with Coomassie Brilliant Blue (CBB) G (Sigma). Each CBB-stained gel lane was manually cut into 10 strips according to the molecular weights (MWs) of the proteins. Each strip was diced into 1–2 mm³ gel pieces and then destained completely by 25 mM ABC in 50% ACN at 37°C. The protein was reduced by 50 mM TCEP in 25 mM ABC at 60°C for 10 min and alkylated by 100 mM IAA in 25 mM ABC for 1 h at room temperature in the dark. Subsequently, the supernatant was discarded, and the gel pieces were washed using 50 μ L of ACN before they were dried by a SpeedVac. Sequencing-grade trypsin

was prepared in 25 mM ABC buffer to a concentration of 0.01 μ g/ μ L, and 10 μ L was added to the dried gel pieces in each fraction. Each gel fraction was allowed to rehydrate for 15 min at room temperature. Tryptic digestion was carried out overnight at 37°C. The resulting tryptic peptides mixture was extracted 3X from the gel pieces with 50 μ L 5% FA in 50% ACN solution. The pooled extracts were concentrated by a SpeedVac. All peptide samples were resuspended in 15 μ L of water with 0.1% formic acid and centrifuged at 17,000 g for 10 min before mass spectrometric analysis.

FIG. 3. Compressed dynamic ranges of the CPLL method compared with the ACN method. Each bar represents the total number of PSM counts of a given protein ID from three serum or plasma samples. (A) and (B) show a clear dominance of PSM counts for highly abundant proteins (e.g., chicken serum albumin and vitamin-D binding protein) for the ACN method. (C) and (D) show a reduced dynamic range of protein abundance by the CPLL method in both chicken serum and plasma samples.

Mass spectrometry

All digested peptide mixtures were separated by online reversed-phase (RP) nanoLC using the Ettan MDLC nano-flow/capillary LC system (GE Healthcare, Pittsburgh, PA). Peptides were loaded into a trap column (PepMap100 C18, 5 μm , 100 $\text{\AA}$, 300 μm I.D. $\times$ 5 mm, Dionex, USA) at 8 $\mu\text{L}/\text{min}$ flow rate for peptide enrichment and desalting. Then the peptides were separated on an analytical nanocolumn (PepMap100 C18, 3 μm , 100 $\text{\AA}$, 75 μm I.D. $\times$ 15 cm, Dionex, USA) at a flow rate of 300 nL/min. The chromatographic

separation started with 95% buffer A (5% ACN, 0.1% FA) and 5% buffer B (95% ACN, 0.1% FA) with 60-min gradients from 5% to 35% of buffer B and 10-min gradients from 35% to 85% of buffer B. The column was regenerated for 20 min with buffer A before the next loading.

The nanoLC separation was coupled with an LTQ-Orbitrap (Thermo Fisher, San Jose, CA) mass spectrometry with a nanospray ion source. The temperature of the ion transfer capillary was set at 200°C. The spray voltage was 1.8 kV, and the normalized collision energy was set at 35.0% for MS/MS. A data-dependent acquisition LC-MS/MS scheme was performed, with

FIG. 3. (Continued).

full-scan MS spectra (m/z 300–2,000) acquired in the Orbitrap at 60,000 FWHM (m/z 400), each followed by MS/MS LTQ scans on the five most intense ions with the dynamic exclusion period of 180 sec. All data were stored in centroid mode.

Mass spectrometry analysis

Thermo Finnegan's Xcalibur Qual Browser (2.0) was used to generate the MS peak list. For MS/MS spectra interpretation, a consensus database searching strategy using both Sequest and X!tandem was performed against an ipi.CHICK.v3.81.fasta protein database (25,992 entries, <http://www.ebi.ac.uk/IPI/IPIchick.html>). A decoy database was generated within TPP by reversing the target sequences and concatenated to the target database to control the false-positive discovery rate during searches. Sequest search was performed by BioWorks 3.3.1 (Thermo Fisher, San Jose, CA) on a local server, and the X!Tandem search was performed within the Trans-Proteomic Pipeline (TPP, v4.5 JETSTREAM rev 2, <http://tools.proteomecenter.org>). For both searches, the monoisotopic precursors and fragments were selected. The peptide mass tolerance was 10 ppm, and the fragment ions tolerance was 1.0 Da. Two missed cleavages were allowed for tryptic digestion. Fixed modification of carbamidomethylation on cysteine and variable modification of oxidation on methionine were set. All search results were converted to .pepXML format. Data from all fractions and injection replicates from the same sample and the same search algorithm were compiled by PeptideProphet (Keller et al., 2002) with a peptide probability threshold of 0.9. The compiled data from Sequest and X!tandem searches of the same sample were combined by the interProphet algorithm (Shteynberg et al., 2011) with the default settings. Finally, protein lists were generated by ProteinProphet (Nesvizhskii et al., 2003) with an iProbability of 0.9 and proteinProbability of 0.95. The false discovery rate (FDR) was calculated by dividing the number of false hits by the number of target hits.

Spectral counting analysis

Semiquantitative comparison between cancer and normal samples was performed using the spectral counting method (Balbuena et al., 2013). Proteins with significant differences between groups were identified using the quasi-likelihood algorithm (Ming et al., 2010). The analysis was conducted using QuasiTel script in the R software environment. Two-fold changes in the spectral counting ratio and p values (<0.05) adjusted for the false discovery rate were used to filter the QuasiTel results. Additional criteria applied for selecting differential expressed protein included more than 5 counts in at least one sample, at least 4 non-zero counts across all 10 samples, and at least 20 total counts across the whole experiment.

Results

The CPLL protocol significantly increases the number of proteins identified from chicken blood samples

In a preliminary test, we compared the number of proteins identified from three serum and three plasma samples from healthy chickens, each processed by either conventional ACN precipitation or CPLL enrichment. For all samples, 60 μ g of CPLL or ACN purified proteins were separated by

1D-SDS-PAGE, and 8 gel fractions were excised for in-gel digestion coupled with LC-MS/MS. Two replicate injections were performed for each peptide fraction. We used a threshold iProbability >0.95 as the peptide identification criterion using iProphet to process Sequest and X!Tandem searches in parallel, and a threshold ProteinProbability >0.95 with two distinct peptide sequences (iProbability >0.95) for confident protein assignment. Such stringent criteria led to an overall FDR of 0.09% at the unique peptide level (see all 11,487 unique peptide IDs from preliminary test in Supplementary Table S1; supplementary material is available online at www.liebertpub.com) and resulted in only 1–2 decoy hits in only a few samples, while searches on most samples gave the target sequence only. Our results (Fig. 1) suggest that compared with those identified in the ACN-precipitated samples, the CPLL protocol significantly improved the discovery power of shotgun proteomics by providing 83.3%–96.2% and 70.7%–148.3% more protein IDs for chicken plasma and serum samples, respectively.

Combining all protein IDs together, 196 and 190 proteins were identified from those three serum and three plasma samples, respectively. As shown in Figure 2, 66 and 46 IDs from serum and plasma, respectively, were identified by both sample preparation methods, while 109 (55.6%) IDs in serum samples and 100 (52.5%) IDs in plasma samples were identified only with the CPLL method.

The CPLL method empowers the proteomic workflow by retaining low-abundance proteins while limiting the binding of high-abundance species due to the limited binding capacities of peptide ligands. To assess how well this mechanism worked for the chicken blood samples, we compared the number of peptide spectrum matches (PSMs) assigned to each protein ID in the CPLL-treated samples to that of the ACN-treated samples, assuming the PSM spectral counts represented the relative abundance of proteins in the plasma and serum. We combined unique protein IDs from three serum datasets and counted only confident PSMs with an iProbability >0.95 . In the end, we obtained 12,462 and 24,159 PSMs for 87 and 175 protein IDs from the ACN- and CPLL-treated serum, respectively, and 18,344 and 35,649 PSMs for 90 and 146 protein IDs from the ACN- and CPLL-treated plasma, respectively.

As shown in the top panels of Figure 3, a significant portion of PSMs in the ACN-treated samples (both serum and plasma) were assigned to the most abundant proteins. Notably, the four most abundant proteins in serum, albumin (ALB), vitamin D-binding protein (VTDB), and apolipoproteins A1 and A4 (APOA1 and APOA4), accounted for up to 7036 PSMs in total, representing 56.5% of the total PSMs assigned to all proteins in the serum samples. In plasma, the same four proteins together with vitellogenin-2 (or phosvitin, VIT2) constituted 18,344 PSMs, which accounted for 66.8% of the total PSMs assigned to all proteins from the plasma samples. In contrast, the dominant PSM counts for the abundant proteins were greatly reduced in the CPLL method (Fig. 3, lower panels).

Another interesting observation was that the effectiveness of highly abundant proteins removed by CPLL was protein- and sample dependent. As shown by Figure 4, for the five most abundant proteins in serum, the PSM counts for ALB, VTDB and ovotransferrin (TRFE) were significantly reduced by CPLL, while APOA1 and APOA4 were largely

FIG. 4. Comparison of the relative PSM counts (relative abundance) of the five most abundant protein species in three serum (A) and three plasma samples (B) with the CPLL (black) or the ACN precipitation method (gray). The relative PSM counts across three serum or plasma samples were averaged. Error bars indicate standard deviation.

unaffected. In plasma, the PSM counts for ALB, VTDB, APOA1, and APOA4 were significantly lower, but that for VTG2 was unchanged.

Cataloging of the chicken blood proteins

Given the effectiveness of the CPLL method to increase protein identification, we applied the CPLL enrichment approach to six chicken serum and four plasma samples that we had previously collected, and analyzed the peptides by nanocapillary LC-MS/MS followed by X!tandem and Sequest database searches. These samples were obtained from five healthy and five ovarian tumor-bearing chickens, respectively

(Table 1). A representative 1D gel electrophoretogram of the serum proteome from both normal and diseased chicken are shown in Figure 5. To increase identification of the shotgun proteomics workflow, each sample was prefractionated into 10 gel fractions after 1D PAGE electrophoresis. Three replicate LC-MS/MS injections were performed for each fraction. Using PeptideProphet with a filter of iProbability >0.95, we collected a total of 74,085 PSMs for 2317 unique peptides from six serum samples, and a total of 58,145 PSMs for 1637 unique peptides from four plasma samples. Compiling peptide IDs using ProteinProphet with a ProteinProbability >0.95, we obtained 232 and 158 IPI IDs (see full list of unique peptide IDs in Supplementary Table S2) from serum and plasma

samples with two distinct peptides (AA > 6) across the whole study. As some proteins have multiple IPI IDs, the protein lists had only 221 and 151 proteins, respectively. Combined with the protein IDs identified exclusively from our preliminary study, we identified 235 and 174 non-redundant protein IDs from serum and plasma at a FDR of 1.3% and 1.1%, respectively. Combining the lists from serum and plasma, we obtained 264 proteins for the chicken blood proteome (Table 2), which to our knowledge is the largest catalog of the chicken blood proteome to date. In addition, we report a list of 176 “single-hit wonder” proteins, defined as proteins with a ProteinProbability > 0.95 but with only one unique peptide sequence across all samples (Supplementary Table S3). This single peptide-protein list might represent true identification, in particular when evidence from other publications or studies provides a second peptide hit.

The proteins identified from this study have a wide array of functions. It should be borne in mind that the functional annotation of chicken protein databases is still incomplete. Therefore, we took the liberty of using the functional annotations of human homologs via pBLAST for uncharacterized and hypothetical protein IDs. Within this list of 264 proteins, most were coagulation and complement factors (13.6%), blood transport and binding proteins (12.5%), immune- and defense-related proteins (13.3%), proteases or protease inhibitors (14.4%), cellular enzymes (11.4%), or cell structure and adhesion proteins (13.6%). We also identified a small fraction of hormones and signaling proteins (3.4%), cell membrane proteins or channels or receptors (3.0%) and nuclear proteins (3.0%). As expected, GO analysis suggested that most proteins were extracellular (48.1%) or membrane proteins (27.7%).

Identifying differential expressed blood proteins in chicken with ovarian cancer versus healthy chicken using spectral counting

To identify circulating biomarkers relevant to ovarian cancer onset in our chicken model, the spectral count of each protein in the disease group was compared to that in the control group using the quasi-likelihood model. A total of 10 proteins displayed significantly alteration of expression (Table 3). Among those, IPI00577279 (inter-trypsin alpha inhibitor heavy chain H2) and IPI00680258 (SERPINF2 similar to alpha-2-plasmin inhibitor) showed more than 5-fold upregulation in diseased chicken compared with healthy

chicken. In contrast, IPI00584356 (C1R hypothetical protein) and IPI00575264 (ZP3 Zona pellucida sperm-binding protein 3) showed more than 5-fold downregulation in diseased chicken compared with healthy chicken. Table 3 also lists the most relevant human homolog for each of the differentially expressed proteins retrieved from pBLAST so that their relevance to human ovarian cancer can be assessed.

Discussion

Using an integrated CPLL-1DGE-LC-MSMS workflow, we identified a catalog of 264 unique proteins, making this study the most comprehensive survey of chicken blood proteome to date. Huang et al. (2006) performed a 2DE of chicken serum and identified a total of 119 CBB-stained and 315 silver-stained protein spots. These spots were excised and analyzed by mass spectrometry. In the end, Huang et al. (2006) were only able to identify 30 protein IDs. In another report, a total of 116 proteins were identified from undepleted chicken plasma using a 1DGE-LC-MS/MS protocol (Hawkrige et al., 2010). In comparison, the number of protein IDs obtained in this study is far greater. It is worth noting that although all enrichment methods, including CPLL, help to lower the abundance of dominant protein species in the complex sample, the amount of enriched low-abundance proteins is still hard to detect. In a previous report using CPLL enrichment of 1.2 mL chicken serum, fewer than 50 protein spots on 2DE gels were visualized by Coomassie blue (Rasaputra et al., 2012).

Another unexpected observation deserves particular notice: some highly abundant proteins, such as APOA1 and APOA4 in serum and VTG2 in plasma, were not removed efficiently by the CPLL beads. Interestingly, a previous report also found that APOA1 from chicken serum could not be removed efficiently by CPLL (Rasaputra et al., 2012). This could be caused by nonspecific retention of some highly abundant proteins or different affinities of peptide ligands in the CPLL library for different proteins.

We noted that a few proteins were only identified from the ACN-precipitated samples but missed by the CPLL method. This was potentially caused by 1) the stochastic nature of LC-MS/MS analysis; 2) low-abundance species bound and detached when removing the high-abundance proteins; or 3) the lack of binding peptides in the CPLL library. Therefore, the ACN precipitation can be used as a complementary

FIG. 5. A representative 1D gel electrophoretogram of serum samples from a normal and a cancerous chicken.

TABLE 2. MERGED LIST OF PROTEINS FROM CHICKEN SERUM/PLASMA CHARACTERIZED BY 1Dgel-nLC-MS/MS*

<i>IPI ID</i>	<i>Protein name</i>	<i>Plasma</i>	<i>Serum</i>
<i>Category</i>	<i>Coagulation and complement factors</i>		
IPI00573007	F2 Thrombin	80.00	255.67
IPI00571323	Fibrinogen alpha chain	137.25	321.00
IPI00588322	FGB fibrinogen beta chain precursor	129.75	330.50
IPI00592072	FGG Uncharacterized protein (68% to FIBG_human, Isoform gamma-A of fibrinogen gamma chain)	225.25	394.33
IPI00683006	F5 Uncharacterized protein (Fragment) (56% to FA5_human, Coagulation factor V)	33.25	13.67
IPI00590880	F5 similar to coagulation factor V	NA	3.00
IPI00573955	F7 Coagulation factor VII	0.75	0.50
IPI00574534	F9 Coagulation factor IX	22.00	6.33
IPI00684997	Coagulation factor IX	NA	12.33
IPI00811641	LOC771012 similar to Coagulation factor X precursor	NA	1.33
IPI00594898	PROC Uncharacterized protein (Fragment) (32% to FA10_human, Coagulation factor X)	5.25	NA
IPI00588151	F13A1 Coagulation factor XIIIa	5.50	5.83
IPI00586326	F13B similar to Coagulation factor XIII, B polypeptide (47% to F13B_HUMAN, Coagulation factor XIII B chain)	57.75	35.50
IPI00586458	PROC Anticoagulant protein C	4.00	1.50
IPI00577678	CFI hypothetical protein (51% to CFAI_human, Complement factor I)	NA	0.67
IPI00683977	C1QB hypothetical protein (56% to C1QB_human, Complement C1q subcomponent subunit B)	69.50	18.33
IPI00586968	C1QC similar to Complement component 1, q subcomponent, gamma polypeptide	87.25	37.33
IPI00584356	C1R hypothetical protein (61% to C1R_human, Complement C1r subcomponent)	111.50	29.33
IPI00602550	C1S Uncharacterized protein (55% to C1S_human, Complement C1s subcomponent)	123.75	30.50
IPI00581479	LOC426719 similar to C2, partial (43% to CO2_HUMAN, Isoform 2 of Complement C2)	1.50	1.17
IPI00581158	C3 Complement C3	668.00	510.83
IPI00683214	C3 Complement component 3d (Fragment)	39.75	20.00
IPI00823324	LOC768475 Complement C4	353.50	162.83
IPI00681096	LOC418892 similar to complement C4-1	382.75	316.83
IPI00807214	C4 MHC-linked complement C4	503.25	415.17
IPI00572510	C4BPA Complement regulatory soluble protein	417.00	302.83
IPI00591736	C5 Uncharacterized protein (55% to CO5_human, Complement C5)	124.25	55.66
IPI00588843	Sixth complement component	188.75	94.83
IPI00600048	C7 Complement component 7	200.25	144.33
IPI00584072	C8A similar to Complement component 8, alpha polypeptide	65.25	33.00
IPI00595351	C8B Uncharacterized protein (61% to CO8B_human, Complement component C8 beta chain)	125.00	94.17
IPI00810881	C8G similar to Complement component 8, gamma polypeptide (47% to CO8G_HUMAN, Complement component C8 gamma chain)	48.50	20.00
IPI00577650	CFH Uncharacterized protein (38% to CFAH_human, Complement factor H)	759.25	411.33
IPI00588708	Complement factor B-like protease (Fragment)	27.00	25.17
IPI00819356	CLU Clusterin	41.75	38.34
IPI00821912	A2M Uncharacterized protein (57% to A2MG_human, alpha-2-macroglobulin)	34.75	44.00
<i>Category</i>	<i>Blood transport and binding proteins</i>		
IPI00574195	ALB Serum albumin	694.50	731.33
IPI00577903	AMBP similar to alpha 1 microglobulin/bikunin	3.50	4.00
IPI00575926	HBAA Hemoglobin subunit alpha-A	17.75	35.33
IPI00819976	Hemoglobin subunit alpha-D	4.50	13.83
IPI00590350	HBG2;HBG1 Hemoglobin subunit beta	51.25	105.67
IPI00578012	LTF Uncharacterized protein (48% to TRFL_human, lactotransferrin)	2.25	61.00

(continued)

TABLE 2. (CONTINUED)

<i>IPI ID</i> <i>Category</i>	<i>Protein name</i> <i>Coagulation and complement factors</i>	<i>Plasma</i>	<i>Serum</i>
IPI00683271	LTF Ovotransferrin	46.00	48.67
IPI00579242	Apolipoprotein AIV	785.00	548.17
IPI00580765	APOA1 Apolipoprotein A-I	502.50	555.17
IPI00602907	APOA5 similar to apolipoprotein A-V	28.75	22.83
IPI00819590	APOB Uncharacterized protein (Fragment) (33% to APOB_human, Apolipoprotein B-100)	22.75	9.67
IPI00775749	APOB Apolipoprotein B	872.50	500.33
IPI00600265	Apolipoprotein D	NA	1.17
IPI00599712	APOH Uncharacterized protein (45% to APOH_human, Beta-2-glycoprotein 1)	22.50	19.33
IPI00580509	APOVLDLII Apovitellenin-1	708.50	415.83
IPI00819050	CP similar to ceruloplasmin precursor	93.75	88.50
IPI00573327	GC Vitamin-D binding protein	268.50	290.67
IPI00596756	PROZ Uncharacterized protein (47% to PROZ_human, vitamin K-dependent protein Z)	2.50	9.67
IPI00578086	PLTP Phospholipid transfer protein	NA	5.17
IPI00581297	HRG hypothetical protein (34% to human HRG_human, histidine-rich glycoprotein)	29.75	20.67
IPI00602226	Phosvitin, vitellogenin-2, vitellogenin	1280.50	464.33
IPI00600069	P20K Extracellular fatty acid-binding protein	NA	1.00
IPI00573473	Hemopexin	24.00	20.83
IPI00600795	HEPH similar to hephaestin	NA	1.33
IPI00579585	TCN2 similar to LOC496768 protein, partial (38% to TCO2_human, transcobalamin-2)	15.00	10.83
IPI00601238	LOC421091 Uncharacterized protein (61.0% to TTHY_Human, transthyretin)	11.75	4.00
IPI00591282	TTR Transthyretin	105.25	104.00
IPI00591843	TPRXL Vitellogenin-1	NA	27.00
IPI00820086	TPRXL Uncharacterized protein	371.75	176.17
IPI00594746	LOC396449 Riboflavin-binding protein	18.00	4.33
IPI00866805	SEPP1 selenoprotein P precursor	3.25	0.67
IPI00575546*	Hemoglobin subunit epsilon (epsilon-globin)	NA NA	0.33 0.33
IPI00971372*	Ovotransferrin CC type, ovotransferrin BB type, ovotransferrin BC type	NA NA	NA 0.33
<i>Category</i>	<i>Immune and defense system</i>		
IPI00598113	Alpha 1-acid glycoprotein	NA	4.50
IPI00596284	IL6ST interleukin-6 receptor subunit beta precursor/ glycoprotein 130	NA	0.67
IPI00813394	CRP similar to C-reactive protein, partial (48% to CRP_HUMAN, C-reactive protein)	2.50	2.33
IPI00601419	CRP C-reactive protein precursor	3.50	NA
IPI00572206	LOC430862 similar to IgG Fc binding protein, partial	5.75	4.00
IPI00598402	LOC429249 similar to IgG Fc binding protein, partial	4.75	6.83
IPI00813132	LOC769397 similar to IgG Fc binding protein	9.50	8.17
IPI00683203	Uncharacterized protein (Fragment) (59% to LV104_human, Ig lambda chain V-I region NIG-64)	2.25	5.83
IPI00812585	LOC769327 similar to immunoglobulin lambda chain (60% to LV104_HUMAN, Ig lambda chain V-I region NIG-64)	NA	1.33
IPI00811463	LOC769305 similar to immunoglobulin lambda chain (56% to LV104_HUMAN, Ig lambda chain V-I region NIG-64)	NA	0.33
IPI00574703	Uncharacterized protein (Fragment) (56% to LV107_human, Ig lambda chain V-I region BL2)	14.50	12.33
IPI00810959	LOC769283 similar to immunoglobulin lambda chain (56% to LV107_HUMAN, Ig lambda chain V-I region BL2)	4.50	NA
IPI00679743	Uncharacterized protein (Fragment) (60% to LV302_HUMAN, Ig lambda chain V-III region LOI)	NA	1.33
IPI00577021	IGLL1 Ig lambda chain C region	96.75	71.50
IPI00591488	Ig mu chain C region	14.00	21.17
IPI00585276	LOC772174 13 kDa protein (67% to HV303_human, Ig heavy chain V-III region VH26)	2.00	25.34

(continued)

TABLE 2. (CONTINUED)

<i>IPI ID</i> <i>Category</i>	<i>Protein name</i> <i>Coagulation and complement factors</i>	<i>Plasma</i>	<i>Serum</i>
IPI00823165	12 kDa protein (62% to HV303_human, Ig heavy chain V-III region VH26)	7.50	NA
IPI00819112	15 kDa protein (69% to HV303_HUMAN, Ig heavy chain V-III region VH26)	0.5	0.17
IPI00592803	VH1 protein (Fragment) (64% to HV303_human, Ig heavy chain V-III region VH26)	NA	1.33
IPI00812005	VH1 protein	0.75	NA
IPI00685019	IGLL1 Uncharacterized protein (59% to Q6GMX4_human, IGL@protein)	45.50	108.17
IPI00581589	PIT 54 135 kDa protein (51.0% to DMBT1_human, deleted in malignant brain tumors 1 protein)	24.75	15.83
IPI00593580	LOC426824 similar to deleted in malignant brain tumors 1 (53% to DMBT1_HUMAN, deleted in malignant brain tumors 1 protein)	NA	1.50
IPI00866802	PIGR polymeric immunoglobulin receptor (38% to PIGR_HUMAN, polymeric immunoglobulin receptor)	NA	11.83
IPI00596947	BPI hypothetical protein (50.0% to BPI_human, bactericidal permeability increasing protein)	1.50	1.50
IPI00595959	Gallinacin-7 (Gal 7) (Beta-defensin 7) (Gallinacin-5) (Gal-5),Beta-defensin 7 preproprotein,gallinacin-7, beta-defensin 7 antimicrobial peptide	NA	2.17
IPI00680520	VNN1 Uncharacterized protein (68.0% to VNN1_human, pantetheinase)	2.00	21.67
IPI00600859	LYZ Lysozyme C	98.00	95.50
IPI00573323	LYG2 Lysozyme g	NA	9.00
IPI00590040	LY86 Lymphocyte antigen 86	NA	1.17
IPI00593199*	Beta-2 microglobulin	12.00 NA	NA NA
IPI00619886*	MHC class II antigen	2.00 NA	NA NA
IPI00598054*	Uncharacterized protein (54% to IGJ_human, immunoglobulin J chain)	NA NA	2.00 NA
IPI00597129*	Ovomucoid (Allergen Gal d I)	8.33 NA	NA NA
IPI00577213*	Lymphocyte antigen 6E (Ly-6E) (stem cell antigen 2) (SCA-2), stem cell antigen 2	2.33 NA	NA NA
<i>Category</i>	<i>Proteases, protease inhibitors</i>		
IPI00589759	AHSG Uncharacterized protein (38% to FETUA_human, alpha-2-HS-glycoprotein)	9.75	6.83
IPI00587313	Ovoinhibitor	129.50	80.16
IPI00584472	FETUB similar to APO AI promoter B-region binding protein	1.50	NA
IPI00581798	CPB2 similar to thrombin-activatable fibrinolysis inhibitor	43.25	18.00
IPI00576782	CST3 cystatin precursor	2.25	4.33
IPI00684448	LOC417848 Uncharacterized protein (51% to CATE_human, isoform 1 of Cathepsin E)	460.00	105.83
IPI00600003	CTSD Cathepsin D	NA	9.17
IPI00583070	Calpain small subunit	NA	0.50
IPI00577279	ITIH2 Inter-alpha inhibitor heavy chain 2	5.00	64.17
IPI00588436	ITIH3 similar to inter-alpha (globulin) inhibitor H3	NA	32.67
IPI00571779	KNG1 similar to KNG protein	34.50	26.83
IPI00602043	LOC428141 similar to Trappin-6 (48% to ELAF_HUMAN, elafin)	2.25	8.00
IPI00680258	SERPINF2 similar to alpha-2-plasmin inhibitor	7.00	63.67
IPI00591427	SERPINA1 Uncharacterized protein (44% to A1AT_human, alpha-1-antitrypsin)	NA	5.33
IPI00811716	SERPINA3 similar to alpha-1-antitrypsin (46% to AACT_HUMAN, alpha-1-antichymotrypsin)	NA	1.50
IPI00588591	SERPINA9 similar to alpha-1-antitrypsin	15.50	10.67
IPI00582505	SERPINA10 similar to Serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10	6.25	2.67
IPI00583974	SERPINB14 Ovalbumin	2.75	5.67
IPI00604394	SERPINC1 Antithrombin	121.75	95.50

(continued)

TABLE 2. (CONTINUED)

<i>IPI ID</i> <i>Category</i>	<i>Protein name</i> <i>Coagulation and complement factors</i>	<i>Plasma</i>	<i>Serum</i>
IPI00583396	SERPIND1 similar to heparin cofactor II	114.50	219.83
IPI00587267	SERPINF1 Uncharacterized protein (62% to PEDF_human, pigment epithelium-derived factor)	152.00	157.00
IPI00593872	PSMA2 proteasome subunit alpha type-2	NA	1.33
IPI00604354	PSMA4 proteasome subunit alpha type-4	NA	0.83
IPI00592949	PSMA5 Proteasome subunit alpha type	NA	1.00
IPI00600823	PSMA7 Proteasome subunit alpha type-7	NA	1.17
IPI00591682	LOC415690 similar to Chymotrypsinogen A (64% to CTRB2_HUMAN, Chymotrypsinogen B2)	NA	0.67
IPI00604334	PRSS2 Trypsinogen	NA	5.33
IPI00574250	PLG similar to plasminogen	19.50	19.67
IPI00580408	LOC428593 similar to plasminogen precursor (52% to PLMN_HUMAN, plasminogen)	53.25	23.33
IPI00599918	LOC427942 similar to alpha-2-macroglobulin	32.50	42.33
IPI00589043	Uncharacterized protein (Fragment) (44.0% to A2ML1_human, alpha-2-macroglobulin-like protein 1)	54.00	116.67
IPI00597455	HABP2 similar to HGF activator like protein	14.50	56.33
IPI00598803	HGFAC similar to hepatocyte growth factor activator	10.50	10.67
IPI00574984	LTA4H Putative uncharacterized protein (76% to LKHA4_human, leukotriene A-4 hydrolase)	NA	0.83
IPI00597109*	Aminopeptidase N (EC 3.4.11.2)	NA NA	1.00 NA
IPI00603678*	Hepatocyte growth factor-like/macrophage stimulating protein	15.33 NA	NA NA
IPI00600825*	Uncharacterized protein (55% to CYTB_human, Cystatin-B)	5.33 NA	4.67 NA
IPI00573738*	Ovalbumin-related protein Y	6.33 NA	NA NA
<i>Category</i>	<i>Cytokines, hormones and signaling proteins</i>		
IPI00585118	Angiotensin-3	3.00	2.67
IPI00598292	ANG Angiogenin	NA	2.33
IPI00586548	ANGPTL3 Angiopoietin-like protein 3	10.75	1.00
IPI00575874	SPARC SPARC (86% to SPRC_human, SPARC, Secreted protein acidic and rich in cysteine)	NA	1.50
IPI00578016	DKK3 Dickkopf-related protein 3	6.25	5.83
IPI00579092	YWHAE 14-3-3 protein epsilon	NA	2.83
IPI00587444*	14-3-3 protein gamma	NA NA	NA 0.67
IPI00591817*	Uncharacterized protein (67% to NKAP_human, NF-kappa-B-activating protein)	NA NA	0.67 NA
IPI00586369*	Protein Wnt	NA NA	1.00 NA
<i>Category</i>	<i>Enzymes</i>		
IPI00598809	IDH1 similar to cytosolic NADP-dependent isocitrate dehydrogenase	NA	4.50
IPI00574506	ACAT2 acetyl-CoA acetyltransferase, cytosolic	NA	1.67
IPI00594653	GAPDH Glyceraldehyde-3-phosphate dehydrogenase	3.50	15.50
IPI00586105	ENO3 Beta-enolase	NA	2.17
IPI00583368	PLOD2 similar to procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	9.00	NA
IPI00601713	FBP1 similar to fructose 1,6-bisphosphatase	NA	3.67
IPI00604086	AMY2A pancreatic alpha-amylase	NA	10.00
IPI00585486	PGAM1 Phosphoglycerate mutase 1	NA	3.00
IPI00588756	ADA Adenosine deaminase	NA	2.83
IPI00579936	ASL2 Argininosuccinate lyase	NA	1.67
IPI00598749	GPLD1 Uncharacterized protein (62% to PHLD_human, phosphatidylinositol-glycan-specific phospholipase D)	253.50	103.50
IPI00581958	Phosphatidylcholine-sterol acyltransferase	NA	4.00
IPI00586371	Cytochrome b (Fragment)	1.50	0.33
IPI00602021	SOD3 similar to extracellular-superoxide dismutase	1.00	0.83
IPI00580059	PRDX1 Peroxiredoxin-1	2.00	NA
IPI00589360	GPX3 glutathione peroxidase 3	153.00	92.17
IPI00582664	CHIA CBPch04 (74.0% to CHIA_Human, acidic mammalian chitinase)	NA	1.67
IPI00735160	VNN1 Putative uncharacterized protein (64% to VNN2_human, vascular non-inflammatory molecule 2)	10.25	68.33

(continued)

TABLE 2. (CONTINUED)

<i>IPI ID</i> <i>Category</i>	<i>Protein name</i> <i>Coagulation and complement factors</i>	<i>Plasma</i>	<i>Serum</i>
IPI00819636	Heparanase	NA	9.50
IPI00578632	YWHAZ 14-3-3 protein zeta, puromycin-sensitive aminopeptidase isozyme I	NA	4.50
IPI00599522	Creatine kinase B-type	NA	1.67
IPI00573166	CKB Isoform Bb-CK-3 of creatine kinase B-type	NA	3.00
IPI00592568	Creatine kinase M-type (EC 2.7.3.2) (creatine kinase M chain) (M-CK),B-creatine kinase	NA	25.50
IPI00587710	GDA similar to mKIAA1258 protein	NA	2.17
IPI00600353*	Chondrogenesis associated lipocalin (lipocalin-type prostaglandin D synthase)	19.33 NA	3.67 NA
IPI00655487*	Peptidoglycan recognition protein L	7.00 NA	NA NA
IPI00584379*	ADP-ribosyl cyclase CD157 (EC 3.2.2.5)	3.00 NA	NA NA
IPI00597798*	Carbonic anhydrase 2 (EC 4.2.1.1)	2.33 NA	5.00 NA
IPI00602351*	Hepatic alpha-amylase	NA NA	0.67 NA
IPI00600818*	EXTL2 similar to EXTR2 (76% to EXTL2_human, exostosin-like 2)	3.33 NA	NA NA
<i>Category</i>	<i>Cell membrane proteins, channels, receptors</i>		
IPI00819536	LOC421125 Uncharacterized protein (53% to TMM68_human, TransMEMBRANE protein 68)	NA	0.50
IPI00587210	IGFBP1 Insulin-like growth factor binding protein 1	NA	5.00
IPI00593541	ATP6AP2 Renin/prorenin receptor	2.00	NA
IPI00571731	RBP4 Retinol-binding protein 4	2.50	14.83
IPI00575515	TFRC transferrin receptor protein 1	51.75	39.33
IPI00580787	12K serum protein, beta-2-m cross-reactive (60% to FREM2_HUMAN, FRAS1-related extracellular matrix protein 2)	NA	7.33
IPI00597095	WFDC8 similar to putative porin	14.00	9.00
IPI00684336	PIT 54 PIT 54 (40% to SRCRL_human, Soluble scavenger receptor cysteine-rich domain-containing protein)	48.25	79.67
<i>Category</i>	<i>Nuclear proteins</i>		
IPI00595186	Liver ribonuclease A	22.00	24.67
IPI00597439	ANG;RSFR ribonuclease homolog	NA	12.50
IPI00575164	PHF2 similar to PHD finger protein 2	NA	0.50
IPI00591777	CHD1 Chromo-helicase-DNA-binding on the Z chromosome protein	NA	1.00
IPI00580239	Histone H2A-IV	3.75	14.67
IPI00584144	H2B-VIII Histone H2B 8	NA	5.83
IPI00572919	Histone H4	2.00	12.50
IPI00591274	H1F0 Histone H5	NA	4.50
<i>Category</i>	<i>Cell structure, adhesion</i>		
IPI00572084	ACTG1 Actin, cytoplasmic type 5	66.00	85.33
IPI00578082	ACTA2 Actin, aortic smooth muscle	NA	4.40
IPI00593455	CDH1 Cadherin-1	NA	0.83
IPI00582056	GSN Gelsolin	184.25	117.67
IPI00571680	GP1BA similar to glycoprotein Ib	4.50	8.50
IPI00572382	Nidogen-1	3.00	NA
IPI00590535	FN1 fibronectin	127.50	183.00
IPI00576764	IGFALS similar to insulin-like growth factor binding protein complex acid-labile subunit (59% to ALS_HUMAN, Insulin-like growth factor-binding protein complex acid labile subunit)	31.75	6.33
IPI00599012	KRT12 similar to K12 keratin	NA	0.83
IPI00597243	KRT19 Keratin, type I cytoskeletal 19	NA	1.17
IPI00584075	KRT75 Type II alpha-keratin IIC	NA	9.17
IPI00814313	KRT79 similar to keratin 5	NA	16.67
IPI00575205	FBLN1 Isoform C of fibulin-1	9.75	3.50
IPI00570704	FBLN1 Isoform D of fibulin-1	177.25	107.83
IPI00578026	FBLN5 Uncharacterized protein (84.0% to FBLN5_human, fibulin-5)	9.25	5.83

(continued)

TABLE 2. (CONTINUED)

<i>IPI ID</i> <i>Category</i>	<i>Protein name</i> <i>Coagulation and complement factors</i>	<i>Plasma</i>	<i>Serum</i>
IPI00603974	EFEMP1 similar to EGF-containing fibulin-like extracellular matrix protein 1	51.00	35.67
IPI00592361	LUM Lumican	5.25	17.00
IPI00586709	TLN1 Talin-1	NA	8.17
IPI00575989	TUBA1C Tubulin alpha-1 chain (Fragment)	7.75	40.83
IPI00602786	TUBB1 Tubulin beta-6 chain	4.75	20.17
IPI00604330	Titin isoform b11, Connectin	NA	0.50
IPI00583488	Tetranectin	NA	8.17
IPI00572343	TPM1 Isoform 2 of Tropomyosin alpha-1 chain	NA	0.83
IPI00574198	TPM3 Isoform 3 of Tropomyosin beta chain	NA	1.00
IPI00581566	TINAG Uncharacterized protein (53.0% to TINAG_human, tubulointerstitial nephritis antigen)	3.50	NA
IPI00587900	SV2B Uncharacterized protein (81% to SV2B_human, Synaptic vesicle glycoprotein 2B)	NA	0.50
IPI00595980	POSTN Periostin	NA	4.50
IPI00590719	VTN Uncharacterized protein (56.0% to VTNC_human, vitronectin)	148.75	184.33
IPI00578082*	Actin	NA 0.67	NA NA
IPI00597846*	Basement MEMBRANE-specific heparan sulfate proteoglycan core protein	4.00 NA	NA NA
IPI00570820*	Uncharacterized protein (58% to LAMB4_HUMAN, Laminin subunit beta-4)	1.00 NA	NA NA
IPI00579755*	LOC431301 similar to Krt6b protein (48% to K2C3_human, keratin, type II cytoskeletal 3)	5.67 NA	NA NA
IPI00822585*	KRT4 hypothetical protein (62% to K2C5_human, keratin, type II cytoskeletal 5)	3.00 NA	NA NA
IPI00578649*	Uncharacterized protein (53% to KRT35_human, keratin, type I cuticular Ha5)	22.00 NA	NA NA
IPI00589873*	Type I alpha-keratin 15	3.67 NA	NA NA
IPI00811462*	Uncharacterized protein (94% to TEN3_human, teneurin-3)	1.67 NA	NA NA
<i>Category</i>	<i>Miscellaneous and uncharacterized</i>		
IPI00601265	Avidin	3.00	11.83
IPI00579589	LOC426220 similar to avidin, partial	1.25	NA
IPI00573162	SPP2 Secreted phosphoprotein 24	18.00	NA
IPI00812787	LOC776594 hypothetical protein, partial (41% to RPH3L_human, isoform 2 of Rab effector Noc2)	NA	1.50
IPI00577587	LOC421956 similar to MGC86501 protein	0.75	NA
IPI00574331	GKN2 Uncharacterized protein (39% to GKN2_Human, gastrophilic-2)	2.00	2.50
IPI00576444	Uncharacterized protein (TSC2, 80.348% to TSC2_human or tuberlin)	NA	2.67
IPI00584371	PSAP Proactivator polypeptide (57.0% to SAP_human, Isoform Sap-mu-9 of proactivator polypeptide)	NA	0.67
IPI00602099	Myeloid protein 1	18.25	40.17
IPI00572720	LOC428531 Uncharacterized protein (43% to CC178_human, Coiled-coil domain-containing protein 178)	NA	0.33
IPI00588555	LOC425727 similar to natterin 3	40.00	17.17
IPI00580810	RARRES2 similar to tazarotene-induced gene 2	47.75	40.33
IPI00601492	LTBP1 similar to latent transforming growth factor beta binding protein 1 isoform LTBP-1L	NA	1.67
IPI00596154	GKN1 similar to 18 kDa antrum mucosa protein	NA	4.17
IPI00603114	OAF Out at first protein homolog	NA	0.33
IPI00599218	LOC425324 similar to RanBP2 (51% to RGPD3_human, RanBP2-like and GRIP domain-containing protein 3)	NA	0.50
IPI00598179	von Willebrand factor	12.50	6.17
IPI00811116	LOC777576 similar to CDNA sequence BC052055, partial (42% to VW5B1_human, von Willebrand factor A domain-containing protein 5B2)	NA	0.33
IPI00578517	ZP1 Zona pellucida protein 1	275.00	119.00

(continued)

TABLE 2. (CONTINUED)

<i>IPI ID</i> <i>Category</i>	<i>Protein name</i> <i>Coagulation and complement factors</i>	<i>Plasma</i>	<i>Serum</i>
IPI00883273	31 kDa protein (54% to ZP1_human, Zona pellucida sperm-binding protein 1)	4.75	4.00
IPI00575264	ZP3 Zona pellucida sperm-binding protein 3	9.75	NA
IPI00577729	LOC431522 similar to This CDS feature is included to show the translation of the corresponding V_region. Presently translation qualifiers on V_region features are illegal (63% to Q0ZCI1_HUMAN, Immunglobulin heavy chain variable region)	NA	18.00
IPI00581347	LOC423629 hypothetical protein	NA	1.50
IPI00577274	TGFBI RGD-CAP (78% to BGH3_HUMAN, Transforming growth factor-beta-induced protein ig-h3)	2.00	9.00
IPI00584625	PROS1 Uncharacterized protein (64% to PROS_human, Vitamin K-dependent protein S)	NA	4.33
IPI00596201	OLFML3 Olfactomedin-like protein 3	1.75	0.33
IPI00810708*	GIF similar to pancreatic intrinsic factor (51% to IF_human, isoform 2 of gastric intrinsic factor)	2.00 NA	NA NA
IPI00593765*	Putative uncharacterized protein (31% to IF_human, gastric intrinsic factor)	71.00 NA	26.33 NA
IPI00599828*	LOC769110 similar to KIAA1430 (34% to K1420_human, isoform 2 of UPF0501 protein KIAA1430)	NA NA	0.67 NA
IPI00581992*	Uncharacterized protein (49% to VWDE_human, von Willebrand factor D and EGF domain-containing protein)	NA NA	1.33 NA
IPI00599180*	Uncharacterized protein (31% to LRC19_human, leucine-rich repeat-containing protein 19)	3.67 NA	NA NA

Proteins are grouped into functional groups. For uncharacterized or hypothetical proteins, the human counterparts obtained by pBLAST search are noted in the parentheses with percentage of identical sequences between chicken and human protein included. Average PSM (spectral counts) to each protein from either plasma or serum samples across the whole experiment are listed. Protein IDs marked with * were identified from the preliminary test comparing ACN method with CPLL. The average PSM for ACN or CPLL treated sample are all listed, separated by a | notation. NA refers to no qualified spectra counted.

method to the CPLL method to maximize the proteome coverage. Alternatively, the flow-through fractions from the CPLL can be analyzed to potentially recover the lost proteins.

The animal model we used, although it has its own limitations, provides a good alternative to rodents and other animals. Chickens are considered a valid model to study ovarian cancer because they have high rates of developing spontaneous ovarian tumors (Wilson, 1953; Fredrickson, 1987). Other authors have searched for potential cancer biomarkers

in chicken plasma as an alternative to human samples (Huang et al., 2006; Barua et al., 2009; Hawkrigge et al., 2010). Among the 264 proteins identified in this study, several proteins are related to ovarian cancer pathogenesis or are potential diagnostic biomarkers. For instance, beta-2 microglobulin, transthyretin, and apolipoprotein A1 were included in the FDA-cleared OVA1 multivariate assay for ovarian cancer diagnosis (Fung, 2010). In a large-scale proteomic study based on human serum samples, apolipoprotein A1,

TABLE 3. DIFFERENTIALLY EXPRESSED BIOMARKER CANDIDATES IDENTIFIED FROM BLOOD SAMPLE OF CHICKEN OVARIAN CANCER MODEL

<i>Protein</i>	<i>Protein name</i>	<i>Human homologue</i>	<i>Ratio*</i>	<i>quasi.p</i>
IPI00577279	ITIH2 Inter-alpha inhibitor heavy chain 2	Inter-alpha-trypsin inhibitor heavy chain H2	19.50	0.043
IPI00680258	SERPINF2 similar to alpha-2-plasmin inhibitor	Alpha-2-antiplasmin (SERPINF2)	5.15	0.001
IPI00573007	F2 Thrombin	Prothrombin	4.00	0.0008
IPI00598749	GPLD1 Uncharacterized protein	Phosphatidylinositol-glycan-specific phospholipase D	0.40	0.005
IPI00602550	C1S Uncharacterized protein	Complement C1s subcomponent	0.30	0.003
IPI00819590	APOB Uncharacterized protein (Fragment)	Apolipoprotein B-100	0.26	0.019
IPI00684448	LOC417848 Uncharacterized protein	Cathepsin E	0.23	0.01
IPI00820086	TPRXL Uncharacterized protein	Dentin sialophosphoprotein	0.23	0.011
IPI00584356	C1R hypothetical protein	Complement C1r subcomponent	0.12	0.028
IPI00575264	ZP3 Zona pellucida sperm-binding protein 3	Zona pellucida sperm-binding protein 3	0.07	0.006

*Diseased/Healthy.

transferrin, and inter-alpha-trypsin inhibitor heavy chain H4 have demonstrated the potential to be used in early detection of ovarian cancer (Zhang et al., 2004). Another human serum-based proteomic study identified potential ovarian cancer biomarker candidates such as inter-alpha-trypsin inhibitor heavy chain H1, insulin-like growth factor-binding protein 6, insulin-like growth factor-binding protein 3, angiopoietin-like protein 3 (ANGPTL3), and phosphatidylcholine-sterol acyltransferase (Lin et al., 2009). Moreover, clusterin has been identified from chicken serum and plasma samples. Elevated expression of clusterin was found in primary ovarian tumor tissues (Hassan et al., 2011, Yang et al., 2012) and paclitaxel-resistant ovarian cancer cells (Hassan et al., 2011) and was correlated with impaired prognostic outcome (Wei et al., 2009, Yang et al., 2009).

Apart from those high- and medium-abundance proteins, several low-abundance potential biomarkers were also found in our list. For example, interleukin-6 receptor subunit beta and glycoprotein 130 are key participants in IL-6 signaling pathways that lead to ovarian cancer progression (Wang et al., 2005, 2012b), and they have been investigated as potential ovarian cancer biomarkers (Kovacs, 2001). Another example is creatine kinase B, which has been found to be upregulated in ovarian cancer cells *in vitro* and *in vivo*, and its enzyme activity is significantly elevated in sera from ovarian cancer patients (Huddleston et al., 2005). We also identified potential low-abundance biomarkers such as alpha-1 acid glycoprotein (Imre et al., 2008; Budai et al., 2009) (average PSM of 4.5), cysteine endopeptidases calpain (average PSM of 0.5) (Salehin et al., 2012), SPARC (Mok et al., 1996, Said et al., 2008; Chen et al., 2012) (average PSM of 1.5), tetranectin (average PSM of 8.17) (Hogdall et al., 1993, Hogdall et al., 1994; Deng et al., 2000; Begum et al., 2009, 2010), and periostin (Zhu et al., 2010; Morra and Moch, 2011) (average PSM of 4.5) in chicken serum, and peroxiredoxin-1 (average PSM of 2) (Hoskins et al., 2011) in chicken plasma in this study. Therefore, our dataset provides many potential ovarian cancer biomarkers that await validation using MRM-based targeted proteomics because no antibodies to these proteins are available.

Our semiquantitative spectral counting results also suggest additional biomarkers may exist. We found diseased chicken had higher ITIH2 inter-alpha inhibitor heavy chain 2 and F2 thrombin levels in blood compared with healthy chicken. In a previous report, another heavy chain of inter-alpha inhibitor heavy chain, ITIH4, was found to be a potential biomarkers for early detection of ovarian cancer (Zhang et al., 2004). The human homolog of F2 thrombin (prothrombin fragment F2) was also significantly elevated in 18 patients with ovarian cancer (Gadducci et al., 1996). These pilot data support our hypothesis that chicken blood is a valuable surrogate reservoir for developing human ovarian cancer biomarkers.

Conclusion

We developed an integrated shotgun proteomic workflow based on CPLL enrichment and 1DGE-LC-MS/MS to increase the identification rate of low-abundance blood proteins from a chicken ovarian cancer model. Compared with the ACN precipitation method, the CPLL method added 83.3%–96.2% and 70.7%–148.3% more protein IDs to our proteomic survey of chicken plasma and serum samples, respectively.

Based on the established CPLL-assisted proteomic workflow, a total of 264 non-redundant proteins with >2 peptide hits and 176 “single-hit wonder” proteins were identified. To our knowledge, this is the largest dataset of blood proteins reported to date in chicken. Functional analysis suggested that most identified proteins were coagulation and complement factors, blood transport and binding proteins, immune- and defense-related proteins, proteases, protease inhibitors, cellular enzymes, or cell structure and adhesion proteins. In addition, we found that many proteins within this list have been reported previously as diagnostic biomarkers for ovarian cancers. Finally, semiquantitative analysis discovered 10 proteins with significantly different expression levels in the cancerous chicken group compared to the healthy group. We conclude that the CPLL-assisted proteomic workflow described in this study has strengthened our ability to explore the blood proteome from this unique animal model when antibody-based immunodepletion methods are not available. Using this workflow, this study provided a long list of blood proteins for developing effective biomarker assay platforms in chicken models. The identified list of chicken blood proteins is an excellent resource for the research community not only for biomarker discovery for ovarian cancers but also for studying chicken diseases, such as the emerging chicken influenza epidemics. To the best of our knowledge, we thus report here the most comprehensive survey of the chicken blood proteome to date.

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Author Disclosure Statement

The authors declare that there are no conflicting financial interests.

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Address correspondence to:
Professor Biaoyang Lin, PhD
*State Key Laboratory for Diagnosis and Treatment
 of Infectious Diseases
 The First Affiliated Hospital
 School of Medicine
 Zhejiang University
 79 Qingchun Road
 Hangzhou, China 310003*

E-mail: bylin@u.washington.edu

Abbreviations Used

ABC = ammonium bicarbonate
 ACN = acetonitrile
 CPLL = combinatorial peptide ligands library
 FA = formic acid
 FDR = false discovery rate
 IAA = iodoacetamide
 LC-MS/MS = liquid chromatography-tandem mass spectrometry
 RP = reversed-phase
 SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 TCEP = Tris [2-carboxyethyl] phosphine
 TPP = trans-Proteomic Pipeline