CHAPTER III
RESEARCH METHODOLOGY

3.1 Apparatus and chemicals

3.1.1 Apparatus

HiTrap Blue HP column, HiTrap Protein G HP column, Vivaspin 3 kDa MWCO, Immobiline polyacrylamide gradient (IPG) strip holders (18 cm), IPGphor Isoelectric Focusing Unit, Ettan DALTsix electrophoresis unit, and Image Scanner were obtained from Amersham Phamacia Biotech (Uppsala, Sweden).

3.1.2 Chemicals

All chemicals are analytical grade and obtained from companies listed below.

Chemicals	Companies
(1) Albumin depletion:	
- Potassium phosphate	Fluka Chemical Company
- Potassium chloride	Fluka Chemical Company
(2) Immunoglobulin depletion:	
- Sodium phosphate	Fluka Chemical Company
- Glycine	Sigma
- Hydrochloric acid (HCl)	AnalaR®
(3) Protein determination:	
- Bovine serum albumin (BSA)	Boehringer Mannheim
- Sodium carbonate anhydrous	Fluka Chemical Company
- Sodium hydroxide	Merck
- Copper sulfate pentahydrate	Famitalia Carlo Erba
- Sodium potassium tartrate	M&B Laboratory Chemicals

Chemicals	Companies
- Phenol (Folin-Ceocalteau)	Merck
(4) Isoelectric focusing:	
- Urea	Amersham Phamacia Biotech
- Thiourea	Amersham Phamacia Biotech
- Dithiothreitol (DTT)	Amersham Phamacia Biotech
- IPG Cover Fluid	Amersham Phamacia Biotech
- IPG buffer (pH 4-7), linearity (L)	Amersham Phamacia Biotech
- IPG Strip (pH 4-7), L, 18 cm-length	Amersham Phamacia Biotech
- CHAPS	Sigma
- Bromophenol blue	Sigma
- Tris-(hydroxymethyl) aminometane (Tris)	Sigma
- Iodoacetamide	Amersham Phamacia Biotech
- Glycerol	AnalaR®
- Lauryl sulphate or sodium dodecyl sulfate (SDS)	Sigma
(5) SDS-PAGE:	
- Acrylamide	Amersham Phamacia Biotech
- N, N' –methylene bis-acrylamide (Bis)	Amersham Phamacia Biotech
- Agarose	USB™
- N, N, N', N' – tetramethylethylenediamine (TEMED)	Sigma
- Ammonium persulphate (APS)	Sigma
- Glycine	Sigma
(6) Colloidal coomassie staining:	
- Ammonium sulfate	Fluka Chemical Company
- Phosphoric acid	Farmitalia Carlo Erba
- Coomassie Brilliant Blue G-250	Sigma
- Absolute methanol	AnalaR®
(7) Silver staining:	
- Sodium acetate	Fluka Chemical Company

Chemicals	Companies
- Sodium thiosulfate pentahydrate	Fluka Chemical Company
- Silver nitrate	M&B Laboratory Chemicals
- Sodium carbonate	Fluka Chemical Company
- EDTA-disodium salt	Ajax Finechem
- Glutaraldehyde	Fluka Chemical Company
- Absolute ethanol	AnalaR®
- Acetic acid	QRëC™

3.2 Subjects

There were 2 groups of subject in this study, cholangiocarcinoma (CCA) and healthy person group. Six patients with histological proven CCA who had undergone surgical resection at Srinagarind Hospital, Khon Kaen University were selected for CCA group. Age, tumor location, histology, tumor size, and pathological data were obtained from hospital records of these patients. Ten blood donors who go for annual health check up at Srinagarind hospital, with no sign and symptom for any diseases, normal blood smear, normal liver function and normal kidney function, were included in the healthy person group.

3.3 Serum Sample collections

Blood sample were collected by standard protocol for collection and storage of serum from Specialized Cooperative Centers Program in Reproductive and Infertility Research (SCCPRR) protocol. Venous blood samples were collected into BD tube was allowed to clot by standing tubes vertically at room temperature (25°C) for 10 minutes. The clotted blood tubes were then placed on ice for no longer than 2 hours before centrifuging at 3000 x g for 15 minutes at 4 °C and the supernatant was transfer to a new centrifuge tube. To further remove microplatelets, additional centrifugation was applied at 15000 x g for 15 minutes at 4 °C. Within 30 minutes of centrifugation, aliquots of 1-mL serum in the new 1.5 mL tubes were kept at -70 °C until use.

3.4 Serum albumin depletion

All sera were depleted albumin with HiTrap Blue affinity column (GE Healthcare, Sweden) and the process was performed according to the instruction protocol of the product.

The serum sample was diluted with 50 mM Potassium phosphate buffer (binding buffer) and then applied to the column using a peristaltic pump with a flow rate of 0.5 mL/minute. Elution was performed using 50 mM Potassium phosphate buffer containing 1.5 M KCl (Appendix A).

Unbound and bound fractions were collected during this process and both fractions were then concentrated and buffer was changed to 20 mM sodium phosphate, pH 7, by using spin membrane, Vivaspin (GE Healthcare, Sweden) with 3 kDa MWCO. Only the retentates were used for further immunoglobulin depletion.

3.5 Serum immunoglobulin depletion

From the serum albumin depletion experiment, the albumin depleted serum was diluted with 20 mM sodium phosphate, pH 7 and applied to the Hitrap Protein G HP column (GE Healthcare, Sweden) by using peristaltic pump with a flow rate of 0.5 mL/minute. Elution was done with 0.1 M Glycine-HCl, pH 2.7 (Appendix A).

Unbound and bound fractions were collected during this process, then applied to spin membrane 3 kDa MWCO and retentates were collected for future analysis.

3.6 Protein determination

Serum proteins were estimated according to the method of Lowry et al. (1951). Diluted serum (100 µl) was mixed with 1 mL of solution D (Appendix A), and left at room temperature for 30 minutes. A 1N of follin phenol reagent (100 µl) was added, mixed and left at room temperature for 30 minutes. The absorbance at 650

nm was recorded for triplicate in each sample. Various amount of BSA (50-600 μg) treated in the same manner as samples were used as standard.

3.7 Two-dimensional electrophoresis

Protein pattern of each sample was obtained from triplicate experiments in CCA group and duplicate experiment in healthy group; two were performed at the same time as pre- and post-operative samples, and one on the different day.

3.7.1 First dimension: isoelectric focusing (IEF)

Serum albumin and immunoglobulin depleted sample of 500 μg protein was added to rehydration solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM DTT, and 2% IPG buffer, (Appendix A) to a final volume of 340 μl. The mixture was then applied into a IPG strip holder. An IPG strip was placed (gel side-down) into the holder and covered with 1.36 mL of IPG Cover Fluid. Then, the holder was closed with lid. The IPG strip was subjected to IEF using IPGphor at 20 °C. Isoelectric focusing steps were performed with increasing voltages and time as shown in Table 3-1.

Table 3-1 Isoelectric focusing guideline; 50 μA per IPG strip

Step	Voltage	Hour	Volt-hours
1) Step and hold	30	6	180
2) Step and hold	60	6	360
3) Step and hold	500	1	500
4) Gradient	1000	6	6000
5) Gradient	8000	1:41	13500
6) Step and hold	8000	2:39	21200

3.7.2 Equilibration of IPG strip for SDS-PAGE

Just before use, 100 ug of DTT was added into 10 mL of equilibration solution containing 6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) Bromophenol blue (Appendix A). The IPG strip was immerged into the equilibration solution (15 mL per strip) for 15 minutes with gentle shaking. After first equilibration, the IPG strip was then immerged into the new equilibration solution but added iodoacetamide (250 mg/10 mL) instead of DTT and gentle shaking for 15 minutes. The IPG strip was briefly washed with the SDS electrophoresis buffer, containing 25 mM Tris pH 8.3, 192 mM glycine, and 0.1% (w/v) SDS, (Appendix A). Agarose solution (40-50 ^{o}C) (Appendix A) was slowly (to avoid introducing bubbles) applied into the place between the plates of second dimension gel then gently placed the IPG strip with the plastic backing against one of the glass plates. The IPG strip was gently pushed down untill the entire lower edge of the IPG strip was in contact with the top surface of the slab gel. Ensure that no air bubble was trapped between the IPG strip and the gel surface of the second dimension or between the gel backing and the glass plate. The agarose was left at least 5 minutes to solidify.

3.7.3 Second dimension: sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The vertical SDS-PAGE was carried out as described by Leammli, 1970 (Appendix A). A homogeneous 12 percent separating gel was prepared using Ettan DALTsix system (25.5 x 20.5 x 0.1 cm). The upper chamber was added with 4x SDS electrophoresis buffer and used 1x SDS electrophoresis buffer for lower chamber. Electrophoresis was performed with constant watt as described in Table 3-2 and the temperature was maintained at 15 ^{o}C.

Table 3-2 Electrophoresis condition for second dimension vertical gel

Step	Voltage	Current(mA)	Watt (w/gel)	Duration(Hrs:min)
1	600	400	1	17:30

3.8 Colloidal coomassie staining

Colloidal coomassie staining was performed by the protocol of Neuhoff et al. (1988) (Appendix A). All steps were carried out with gentle shaking and done at room temperature (25 °C). Each step required 300 mL of solution per gel. The gel was fixed in fixative solution (40% ethanol and 10% acetic acid) for more than 3 hours and then briefly washed twice with distilled water for 30 seconds. The gel was stained overnight in colloidal coomassie blue G-250 working solution (8% ammonium sulfate, 0.8% phosphoric acid, 0.08% coomassie blue G-250, and 20% methanol). Finally, the gel was washed in distilled water twice for 1 minute each.

3.9 Silver staining

Silver staining protocol of Heukeshoven and Dernick (1985) was performed with minor modification (Appendix A). All steps were carried out with gentle shaking and done at room temperature (25 °C). Two gels were performed together with required 500 mL of solution. The gel was fixed in fixative reagent (40% ethanol and 10% acetic acid) for more than 2 hours then briefly washed with distilled water for 1 minute then sensitized with sensitizing solution (30% ethanol, 6.8% Sodium acetate,0.2% Sodium thiosulfate and 0.125% glutaraldehyde) for 30 minutes. The gels were washed 3-times with distilled water for 5 minutes and then soaked in silver solution (0.25% Silver nitrate, 0.015% formaldehyde) for 20 minutes, and washed twice with distilled water for 1 minute. The gels were then developed by developing solution (2.5% sodium carbonate, 0.0074% formaldehyde) for 5 minutes and stopped the reaction with stopping solution (1.5% Na_2EDTA) for 10 minutes. Finally, the gels were washed 3-times with distilled water for 5 minutes.

3.10 Image record and data processing

Serum proteins pattern of each gel was recorded using ImageScanner (Amersham Biosciences). Each image was scanned and saved in ImageMaster File Format (*.mel) using LabScan Version 5 (Amersham Biosciences)

3.11 Data analysis by 2-DE program

Serum proteins pattern was analyzed using ImageMaster 2D platinum 7.0 (GE Healthcare). Some parameters were set to ensure that the image obtained was corresponded to the gel pattern; these are spot detection and spot matching.

3.11.1 Spot detection

Spot detection is the first option needed to be conducted. Spot detection is the analysis to decide which areas of the image represent spots of the gel, by setting spot detection parameters.

Spot detection parameters:

(1) Smooth: This parameter is a setting to detect all real spots and split as many overlapping spots as possible without being concerned about noise spots. In this study, the smooth was set at 6 in both coomassie and silver stained gel images.

(2) Saliency: This parameter is a measure based on the spot curvature. It indicates how far a spot stands out with respect to its environment. Real spots generally have high saliency values whereas artifacts and background noise have small saliencies. Although the saliency is an efficient quantity for filtering spots, it is also highly dependent on the images. In this study, the saliency was set at 135 and 100 for coomassie and silver stained gel images respectively.

(3) Min Area: After setting an appropriate saliency to filter out all noise spots, there may still be noise in the gel that cannot be eliminated without suppressing real spots. Get rid of these artifacts by using the min area parameter. It eliminates spot that have an area smaller than the specified threshold. After several

experiences, this parameter was set at 5 in both coomassie and silver stained gel images.

3.11.2 Spot matching

"Automatic matching" is the option to analyze the spots from series of gels. In general, three spots distributed over the gel in both axes are defined as a "landmarks" for spot matching. All landmarks in each gel have to be present and matched. An "Edit matches" option was performed after the matching in case of incorrectly and mismatched to make sure that the existing matches are correct.

Analysis representative patterns:

Three patterns obtained from each CCA subject and 2 patterns from each healthy subject were analyzed for spot matching. The spot, which matched more than half of the represent gels, were counted and recorded as a reference spot in the representative pattern. In the present study, 22 representative patterns from 3 cholangiocarcinoma papillary type patients (pre-&post-operative serum), 3 cholangiocarcinoma well differentiated type patients (pre-&post-operative serum) and 10 healthy persons were constructed and analyzed for spot matching (Figure 3-1).

3.12 Data analysis and statistics

The sum of all the sample values divided by the sample size were given as mean of central tendency and the square root of the average squared difference of each sample value to the center location is calculated as mean squared deviation (M.S.D.) of dispersion. The central tendency and dispersion are used for descriptive statistic analysis. The difference of expression between 2 groups was calculated using one-way ANOVA test available in ImageMaster 2D Platinum 7.0 program. It is based on the same assumptions as the t-test. Only spot that had significantly different expression ($P < 0.01$) will be picked as a candidate protein spot to furture identified by mass spectrometry.

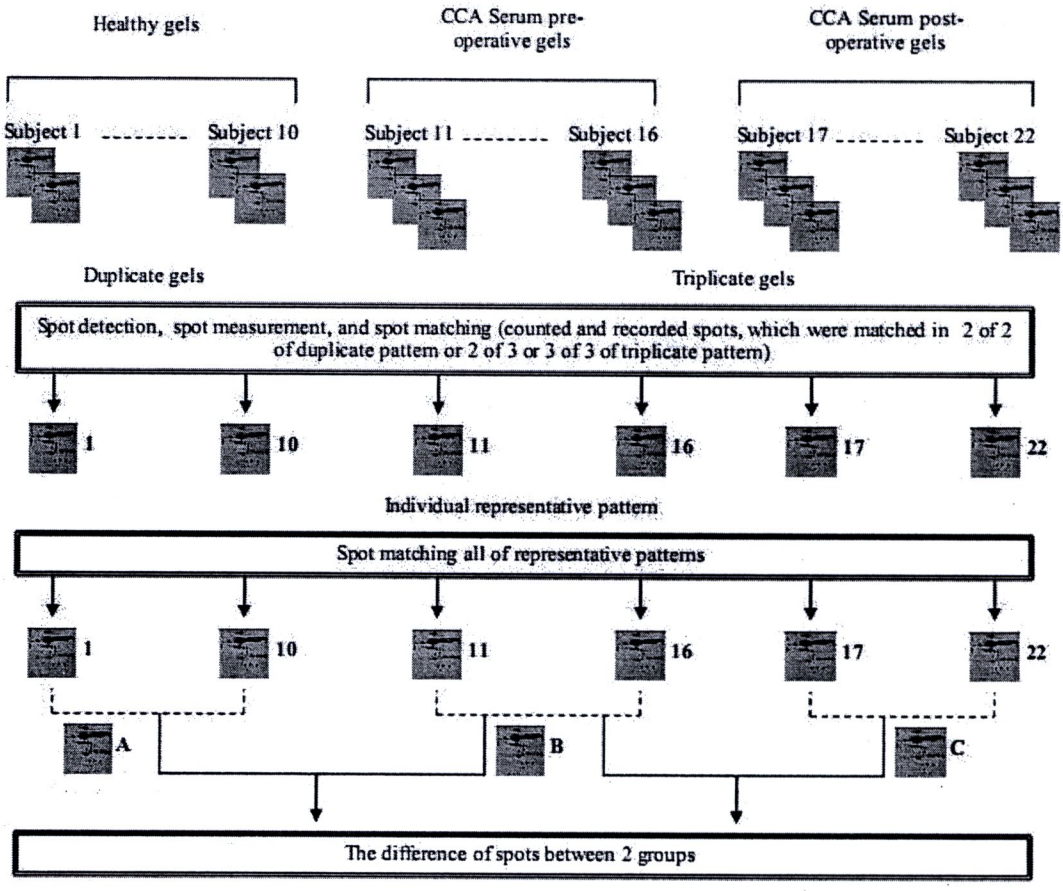

Figure 3-1 Protocol for data analysis

3.13 Gel picked for mass spectrometry (MS) analysis

Gels for spot picked-up were done separately from analytical gels and all gels were stained by colloidal Coomassie stain. Each candidate spot was manually punched by 1 mL pipette tip and kept in 1.5 mL microfuge tube having 20 µl sterile distilled water. All gel plugs were kept at 4 °C until in-gel digestion step.

Mass spectrometry experiment was done at the Proteomic Research Unit, Genome Institute, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand under supervision of Dr.Sittiruk Roytrakul. Gel plugs were sepsrately transferred to new 96 well plates #1 (1 gel plug per 1 well) and destained by adding 200 µl 50 mM NH_4HCO_3 / 50% methanol with shaking at room

temperature for 3 times or until the blue color disappeared, then washed with 200 µl sterile distilled water for 2 times. Water was discarded and 200 µl of 100% acetronitrile was added to dehydrate the gel for 5 minutes with shaking. The solution was removed and the gel was dried for 5-10 minutes then 30 µl 10 mM DTT / 10 mM NH_4HCO_3 was added and incubated at 56 °C for 1 hour to break protein disulfide bond before the solution was discarded. Adding 30 µl of 100 mM IAA / 10 mM NH_4HCO_3 into gel plugs and incubated in dark for 1 hour to prevent the re-formation of disulfide bond then removed the solution. The gel plugs were dehydrated again with 200 µl of 100% acetronitrile twice with shaking for 5 minutes each then removed the solution. Air drying the gel plug at room temperature again for 5 minutes before adding 10 ul of 10 ng Trypsin in 50% acetronitrile / 10 mM NH_4HCO_3 and incubated at 37 °C for 3 hours. Transferring the liquid phase from each well into the new 96 well plates #2, the 30 µl/well 50% acetronitrile / 0.1% formic acid was added into the old plate that has gel plugs before mixed well and incubated at room temperature for 20 minutes. The liquid was separately pooled into plate #2. Repeating this step for 3 times before air drying the solution in plate #2 at 37 °C overnight or until it completely dried. Samples were resuspended with 0.1% formic acid before centrifuged at 10,000 rpm for 5 minutes then transferred to insert tube to subject into LC-MS/MS (Appendix I).

The format of the MS/MS spectra is a micromass (PKL) file that supports multiple MS/MS datasets in a single file. The first line of a PKL dataset contains the observed m/z, intensity, and charge state of the precursor peptide as a triplet of space separated values. Subsequent lines contain space separated pairs of fragment ion m/z and intensity values.

3.14 MS/MS Ion search

The micromass files from LC-MS/MS experiment were further analyzed to identify the protein in each sample. MASCOT program is one of the search engine program that use mass spectrometric data to identify proteins by compared with primary sequence databases of protein. The micromass files were submitted into

MASCOT program to obtain the possible protein that match with our MS/MS data by using MS/MS Ion Search function (Figure 3-2). There are several parameters that should be concerned in order to obtain the good result from databases;

(1) *Name and Email*: filled in the valid name and email to the box, incase when disconnection occurred, they will send the search result to the mail box.
(2) *Search title*: defined the name in each search.
(3) *Enzyme*: in our experiment, we used trypsin to digest protein
(4) *Missed cleavage*: this value depended on the quality of experiment and personal exception, in this case, we vary this value from 0-2 (more value more bias result)
(5) *Taxonomy*: it depended on your source of sample, in this case, we used Homo sapiens (Human)
(6) *Fixed Modifications*: there are many types of modification that provided by the program, in our experiment, we used IAA to prevent the reformation of disulfide bond that referred to carbamidomethyl (C) reaction.
(7) *Variable Modification*: we used oxidation (M) is this filled.
(8) *Peptide charge*: we use 1+, 2+ and 3+ in this filled.
(9) *Data format*: it depended on the raw data file format, we used micromass (.PKL) in this filled.
(10) *Instrument*: we used ESI-QUAD-TOF.
(11) *Other parameters:* we used the default value from the program.
(12) *Data file:* browse the raw file format then click Start Search button.

The search report page was appeared as for example in Figure 3-3. In "Peptide Summary Report", choose "Protein Summary" instead of "Peptide Summary" then click "Format As" button. The result page will report the possible proteins that the data hits to the databases. The number of hit will be ranked by the score of each protein. The protein that has the highest score may be the protein that present in the gel. However, there are several values, which should be considered which one was the protein that represent in the gel, that

are calculated pI and molecular mass of the protein from Mascot report page of each protein should be related with the pI and molecular mass of the protein that present in the gel. Besides, the protein score, pI and molecular mass should be considered together to get the best result of candidate protein.

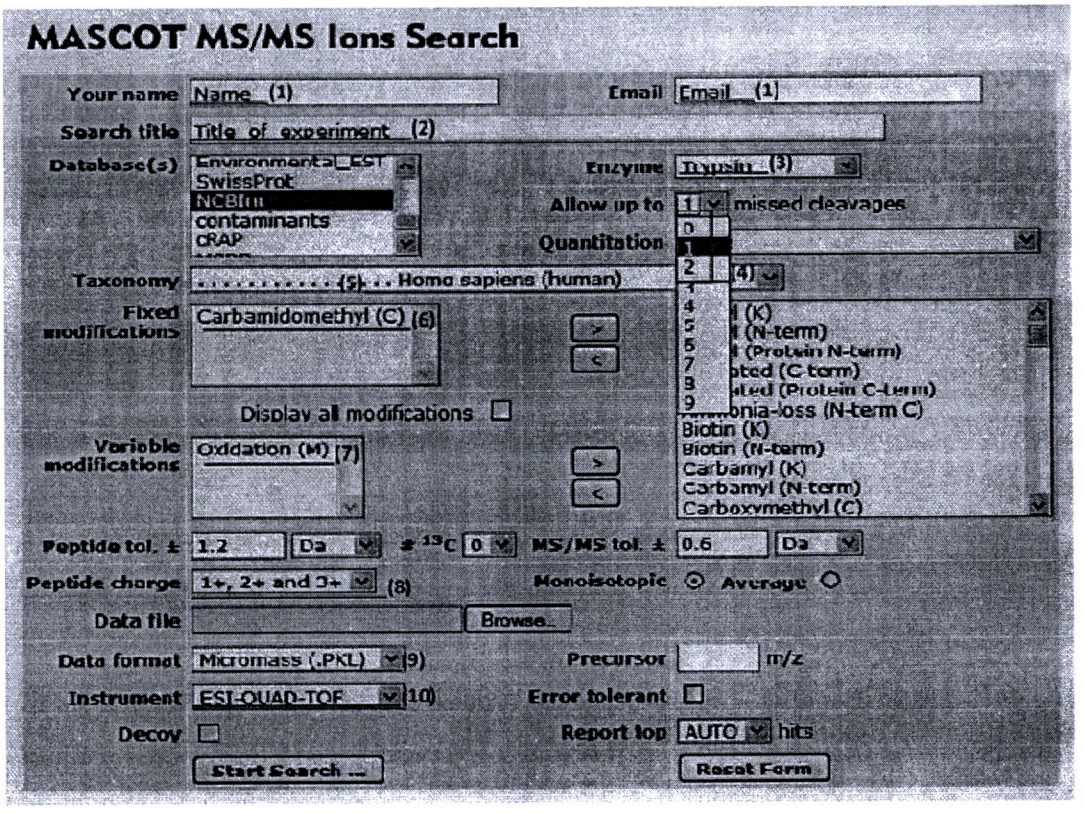

Figure 3-2 MASCOT search page and setting parameters.

3.15 Candidate Proteins selection for further validation

From the data that obtained from MASCOT analysis, we could collect some of protein species that had potential to be marker protein, but only a few of it will be selected to further validation study. There are some criteria that have placed to select the candidate protein;

(1) associated with CCA

(2) had commercial antibody available

(3) literature review, that had some previous study of the interested proteins

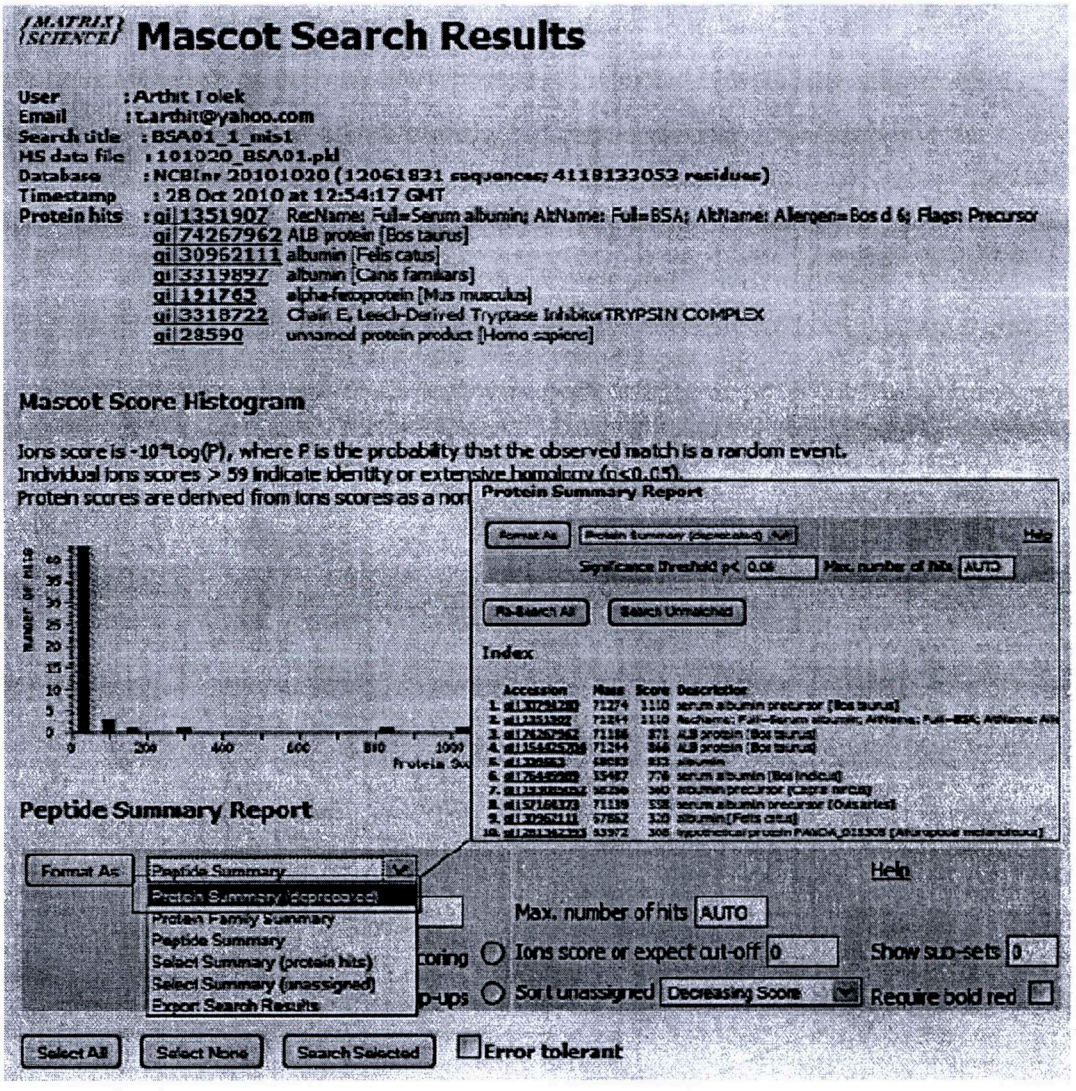

Figure 3-3 MASCOT search result page. The default of the result page is peptide summary but it can change to protein summary by formatting the platform.

(4) should be low abundance protein in serum

(5) gave consistency result (always increase/decrease in one group)

(6) the localization of spots of interested protein in gel should be in the closet area.

CHAPTER IV

RESULTS

4.1 Characterization of cholangiocarcinoma samples

All CCA patients were histological proven cases and distributed over 2 common groups of histological differentiation. There were 3 cases of invasive papillary carcinoma (IPAP) and 3 cases of well-differentiated (WD) (Table 4-1). Subjects in this study covered the common CCA found in northeast of Thailand and hence was a good representative of CCA subjects.

Ten healthy sera are used in this experiment to compare with those disease subjects (Table 4-2). Since serum protein can be altered by several factors, comparison of 2-DE serum protein patterns of disease and non-disease persons will be valid when the two subjects possess only one variable that is disease and non-disease.

4.2 Sample Preparation

4.2.1 Serum albumin and immunoglobulin depletion

There are 2 methods for depletion albumin and immunoglobulin from the serum; firstly using HiTrap Blue affinity column to deplete albumin and then the flow-through fraction was applied to HiTrap Protein G HP column to deplete immunoglobulin. All 22 individual serum samples were subjected to both HiTrap columns and 4 fractions of each were collected. All fractions were determined protein concentration before subjected to SDS-PAGE and 2-DE (Table 4-3a-d). The final fraction from both depletetions gave a much better pattern than the crude serum. According to SDS-PAGE experiment to check the quality of the serum fractions before subjected to 2-DE, albumin and immunoglobulin were almost completely remove from the serum (Figure 4-1) and low abundance proteins were concentrated in the final fraction.

Table 4-1 CCA subjects information. Three cases for CCA papillary type (Y76, Y83, Y100) and 3 cases for well differentiated type (W97, X31, X42)

Code	Sex	Age (year)	CCA histopathological differentiation	Serum Post-OR (month)	Hepatectomy	Invasion
Y76	Female	61	Invasive papillary carcinoma (IPAP)	7	Right hepatectomy	Distance
Y83	Female	51	Invasive papillary carcinoma (IPAP)	7	Right hepatectomy	Distance
Y100	Male	57	Invasive papillary carcinoma (IPAP)	3	Left hepatectomy	Local
W97	Female	55	Tubular adenocarcinoma, well differentiated (WD)	7	Left hepatectomy	Local
X31	Male	57	Tubular adenocarcinoma, well differentiated (WD)	3	Right hepatectomy	Distance
X42	Female	56	Tubular adenocarcinoma, well differentiated (WD)	8	Left hepatectomy	Local

Table 4-2 Blood test for 10 healthy sera

Code	Sex	Age	Sugar	BUN	Creatinine	Cholesterol	ALT	AST	ALP	Hb	Hct	WBC	PMN	Lymphocyte	Monocyte	Eosinophile	Basophile	Platlet smear	Platlet count
HE01	F	52	85	8.2	0.5	236	19	24	67	12.8	39.9	5.9	48.3	40.2	5.9	4.9	0.7	adq	284
HE02	F	50	80	12.3	0.8	246	14	17	83	14.4	43.4	9.3	60.4	31.0	5.4	2.7	0.5	adq	317
HE03	M	48	85	8.1	0.9	132	24	23	49	13.1	41.4	6.7	51.0	31.7	12.9	3.8	0.6	adq	187
HE04	M	54	97	14.3	0.8	210	22	19	40	15.0	44.6	7.0	59.5	28.2	8.5	3.0	0.8	adq	279
HE05	F	61	91	13.7	0.8	277	21	20	92	14.2	42.3	6.9	53.9	35.7	6.0	3.8	0.6	adq	268
HE06	M	49	106	10.0	0.9	244	6	18	55	14.0	42.0	6.4	62.8	28.8	4.1	4.0	4.0	adq	327
HE07	F	52	93	13.3	0.6	196	11	18	68	12.7	38.0	8.3	64.6	27.9	6.0	1.1	0.4	adq	244
HE08	M	56	89	10.2	1.0	190	37	40	84	14.5	44.3	6.1	73.0	17.0	4.0	4.0	0.0	adq	185
HE09	M	48	93	10.9	1.2	193	27	36	59	15.5	46.8	8.3	60.4	28.0	7.2	3.8	0.6	adq	296
HE10	F	47	89	12.3	0.5	184	11	15	66	13.1	39.8	5.1	59.5	33.4	5.1	1.3	0.7	adq	254

Table 4-3a Protein determination of serum fractions from CCA pre-operative sera included crude serum, (B1) Bound fraction from HiTrap Blue affinity column, (UB1) Unbound fraction from HiTrap Blue affinity column, (B2) Bound fraction from HiTrap Protein G HP column, and (UB2) Unbound fraction (final) from HiTrap Protein G HP column.

Sample	A650	µg/ul	Vol. (µl)	Amount protein (mg)	%
Pre-operative sera					
Y76pre-crude	0.224	90.32	570	51.48	100
Y76pre-B1	0.442	89.12	202	18.00	35
Y76pre-UB1	0.366	73.78	276	20.36	40
Y76pre-B2	0.200	13.44	267	3.59	7
Y76pre-UB2	0.328	66.12	174	11.50	22
Y83pre-crude	0.163	65.72	530	34.83	100
Y83pre-B1	0.270	54.44	320	17.42	50
Y83pre-UB1	0.201	40.52	227	9.20	26
Y83pre-B2	0.161	10.82	168	1.82	5
Y83pre-UB2	0.133	26.82	163	4.37	13
Y100pre-crude	0.127	79.40	500	39.70	100
Y100pre-B1	0.274	110.36	185	20.42	51
Y100pre-UB1	0.361	36.36	238	8.65	22
Y100pre-B2	0.068	6.85	183	1.25	3
Y100pre-UB2	0.163	32.84	129	4.24	11
W97pre-crude	0.192	69.48	540	37.52	100
W97pre-B1	0.312	56.31	358	20.16	54
W97pre-UB1	0.242	43.62	201	8.77	23
W97pre-B2	0.095	5.76	145	0.84	2
W97pre-UB2	0.112	20.30	239	4.85	13
X31pre-crude	0.086	34.96	480	16.78	100
X31pre-B1	0.182	37.00	253	9.36	56
X31pre-UB1	0.098	19.99	218	4.36	26
X31pre-B2	0.037	2.48	189	0.47	3
X31pre-UB2	0.105	10.67	214	2.28	14
X42pre-crude	0.176	73.48	450	33.06	100
X42pre-B1	0.413	86.37	209	18.05	55
X42pre-UB1	0.200	41.76	228	9.52	29
X42pre-B2	0.316	16.52	166	2.74	8
X42pre-UB2	0.263	27.54	146	4.02	12

Table 4-3b Protein determination of serum fractions from CCA post-operative sera

Sample	A650	µg/ul	Vol. (µl)	Amount protein (mg)	%
Post-operative sera					
Y76post-crude	0.252	102.37	540	55.28	100
Y76post-B1	0.557	113.20	206	23.32	42
Y76post-UB1	0.339	68.92	242	16.68	30
Y76post-B2	0.284	19.23	154	2.96	5
Y76post-UB2	0.196	39.74	152	6.04	11
Y83post-crude	0.245	99.39	540	53.67	100
Y83post-B1	0.383	77.79	261	20.30	38
Y83post-UB1	0.316	64.25	341	21.91	41
Y83post-B2	0.393	26.63	151	4.02	7
Y83post-UB2	0.217	44.14	183	8.08	15
Y100post-crude	0.215	76.80	510	39.17	100
Y100post-B1	0.276	56.92	478	27.21	69
Y100post-UB1	0.339	34.96	325	11.36	29
Y100post-B2	0.183	12.56	208	2.61	7
Y100post-UB2	0.129	26.60	182	4.84	12
W97post-crude	0.262	95.09	540	51.35	100
W97post-B1	0.455	82.47	285	23.50	46
W97post-UB1	0.315	57.09	275	15.70	31
W97post-B2	0.327	19.74	128	2.53	5
W97post-UB2	0.234	42.41	168	7.13	14
X31post-crude	0.211	85.78	480	41.18	100
X31post-B1	0.280	56.99	305	17.38	42
X31post-UB1	0.374	76.03	199	15.13	37
X31post-B2	0.458	31.01	137	4.25	10
X31post-UB2	0.403	40.93	148	6.06	15
X42post-crude	0.216	90.48	450	40.72	100
X42post-B1	0.514	107.42	205	22.02	54
X42post-UB1	0.294	61.55	170	10.46	26
X42post-B2	0.398	20.83	144	3.00	7
X42post-UB2	0.286	29.91	128	3.83	9

Table 4-3c Protein determination of serum fractions from healthy sera (HE01-05)

Sample	A650	µg/ul	Vol. (µl)	Amount protein (mg)	%
Healthy sera					
HE01-crude	0.233	89.73	450	40.38	100
HE01-B1	0.642	123.68	165	20.41	51
HE01-UB1	0.312	60.14	171	10.28	25
HE01-B2	0.015	0.74	175	0.13	0.3
HE01-UB2	0.301	28.95	150	4.34	11
HE02-crude	0.256	98.58	450	44.36	100
HE02-B1	0.575	110.78	193	21.38	48
HE02-UB1	0.336	64.70	161	10.42	23
HE02-B2	0.061	2.95	141	0.42	1
HE02-UB2	0.328	31.58	137	4.33	10
HE03-crude	0.242	91.53	450	41.19	100
HE03-B1	0.608	114.99	195	22.42	54
HE03-UB1	0.318	60.14	162	9.74	24
HE03-B2	0.106	5.03	159	0.80	2
HE03-UB2	0.268	25.34	155	3.93	10
HE04-crude	0.219	82.96	410	34.01	100
HE04-B1	0.655	123.81	154	19.07	56
HE04-UB1	0.340	64.30	134	8.62	25
HE04-B2	0.020	0.96	163	0.16	0.5
HE04-UB2	0.347	32.78	117	3.84	11
HE05-crude	0.206	83.91	440	36.92	100
HE05-B1	0.535	108.85	184	20.03	54
HE05-UB1	0.260	52.94	165	8.73	24
HE05-B2	0.026	1.34	174	0.23	1
HE05-UB2	tion f27.86	27.86	136.5	3.80	10

Table 4-3d Protein determination of serum fractions from healthy sera (HE06-10)

Sample	A650	µg/ul	Vol. (µl)	Amount protein (mg)	%
Healthy sera					
HE06-crude	0.209	85.13	450	38.31	100
HE06-B1	0.577	117.26	169	19.82	52
HE06-UB1	0.242	49.21	192	9.45	25
HE06-B2	0.374	19.01	158	3.00	8
HE06-UB2	0.253	25.72	139	3.58	9
HE07-crude	0.225	92.98	450	41.84	100
HE07-B1	0.549	113.43	199	22.57	54
HE07-UB1	0.267	55.10	203	11.18	27
HE07-B2	0.400	20.68	174	3.60	9
HE07-UB2	0.308	31.85	134.5	4.28	10
HE08-crude	0.229	94.49	450	42.52	100
HE08-B1	0.481	99.31	240	23.84	56
HE08-UB1	0.317	65.57	176	11.54	27
HE08-B2	0.418	21.61	165	3.57	8
HE08-UB2	0.349	36.02	140	5.04	12
HE09-crude	0.201	87.58	450	39.41	100
HE09-B1	0.572	124.99	187	23.37	59
HE09-UB1	0.320	69.89	167	11.67	30
HE09-B2	0.428	23.39	171	4.00	10
HE09-UB2	0.273	29.85	157	4.69	12
HE10-crude	0.202	88.09	430	37.88	100
HE10-B1	0.514	112.33	203	22.80	60
HE10-UB1	0.305	66.54	159	10.58	28
HE10-B2	0.341	18.64	167	3.11	8
HE10-UB2	0.287	31.30	136.5	4.27	11

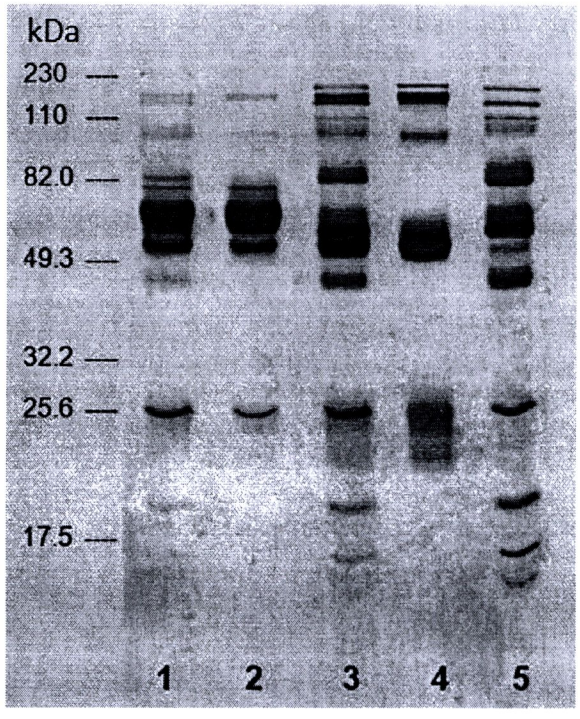

Figure 4-1 Comparative silver-stained SDS-PAGE gel (12.5%); (1) Crude serum, (2) Bound fraction from HiTrap Blue affinity column, (3) Unbound fraction from HiTrap Blue affinity column, (4) Bound fraction from HiTrap Protein G HP column, and (5) Unbound fraction (final) from HiTrap Protein G HP column.

4.2.2 Sample preparation and sample loading

The crucial factors for overall performance of the 2-DE technique are sample preparation and solubilization. In order to avoid artifact, the protein complexes and aggregates should be completely disrupted. Principally, the preparation solution contains urea, thiourea, a non-ionic detergent such as CHAPS, and a reducing agent such as DTT. In our study, serum sample were added into rehydration solution that contained chemicals similar to the solution recommended by GE Healthcare (Appendix D).

The pH gradient and the length of the IPG strip gel are depended on the complexity of the sample, as well as on the amount of protein loading. In our

study, 18 cm long and pH gradient 4-7 were used to perform 2-DE. Various amount of proteins (300, 500, 1000 μg) were tried to get the best pattern and 500 μg of serum protein was found to be appropriate for this study.

4.3 Optimization of 2-DE procedure

4.3.1 First dimension: IEF

The IPG strips gels were rehydrated with rehydration buffer that already including serum sample in strip holder individually. It is convenient for sample containing very high molecular weight, very alkaline, and hydrophobic protein (Görg et al., 2009). In this study, we also improved entry of high molecular weight proteins into the IPG strip gel by using active rehydration method, by applying low voltages (30, 60 V) during rehydration step. The focusing steps were continued to reach the final approximately 41740 Vh (Table 3-1).

4.3.2 Second dimension: SDS-PAGE

After the IEF step and the equilibration of the IPG strips, separation of proteins in second dimension (SDS-PAGE) was performed on the vertical electrophoresis with 12% homogeneous polyacrylamide gel by using DALTsix electrophoresis unit (Table 3-2). However, to get a good 2-DE pattern, factors influencing the gel pattern (gel thickness, gel size, etc) and the limitation of instrument in a particular laboratory have to be optimized and considered.

4.3.3 Coomassie staining

Coomassie Brilliant Blue (CBB) is the most commonly used total protein gel stain. Coomassie stains are generally treated as endpoint stains, allowing significant time flexibility for fixation and staining steps. The effectiveness of Coomassie staining methods is colloidal formulations because the stain does not effectively enter the gel matrix while binding specifically to protein spots, that allowing low background staining with reliable quantitation and increased sensitivity relative to the standard CBB-R 250 staining method (Steinberg, 2009). In the present

study, gels were stained according to the method of Neuhoff et al. The stained gels gave a good reproducibility and clearing background.

4.3.4 Silver staining

Silver staining is the alternative colorimetric method, for increased detection sensitivity over Coomassie staining. The basis of protein detection is reduction of protein-bound silver ion to metallic silver.

Mass spectrometry of proteins following silver staining may be problematic due to protein modification resulting by using glutaraldehyde in fixation and sensitization steps. Besides, there are protocols that compatible with mass spectrometry by omitting glutaraldehyde, which reduce relative sensitivity. However, in this study, we used the conventional silver stain protocol of Heukeshoven and Dernick to obtain the best sensitivity and reproducibility. The stained gels gave a good reproducibility, clearing background, and increase-visualizing spot of proteins.

4.4 Reproducibility of 2-DE patterns

To obtain a standard 2-DE pattern of an individual subject, the artifacts generated from the 2-DE technique have to be minimized. Triplicate 2-DE pattern of each subject were performed under the same condition but on different days. Two setes of serum pre-operative and post-operative in the same case were run together on the same day and the remaining was run on the other day. Patterns of a set of triplicate gels from CCA patients and duplicate for normal subjects are shown in Figure 4-2 to 4-17 (Appendix B).

The triplicate gels had good reproducible patterns. Almost every detected spot were matched among the triplicate gels. These results implied that the 2-DE and staining procedures set up in this study are validated and appropriate for further analysis.

4.5 Spot detection

Detected spots of serum proteins from 10 healthy persons and 6 CCA patients for pre- and post-operative sera patterns are shown in table 4-3a and 4-3b. The minimum-maximum, mean, and standard deviation of mean of the detected spots of the healthy subjects were 118-214, 168, and 29 respectively in Coomassie stained gel and 415-789, 618, and 105 respectively in silver stained gel (Table 4-4), whereas those of CCA subjects for pre-operative serum were 104-202, 152, and 26 respectively in Coomassie stained gel and 417-788, 548, and 118 respectively in silver stained gel. For post-operative serum, those were 104-248, 158, and 30 respectively in Coomassie stained gel and 432-948, 585, and 139 respectively in silver stained gel (Table 4-5).

Two-DE study of human plasma from SWISS-2DPAGE database showed 1966 of total detected spots, with 629 identified spots and 69 identified proteins (http://au.expasy.org/swiss-2dpage/). However, the range of pI from database is 3.50 – 10.00, that is broader than our experiment. The other factor affecting the impact in spot present from difference experiment is sample preparation. To get better, we removed some of abundant protein from serum sample that may make our represent spots lower than the database.

Table 4-4 Number of detected spots in each triplicate gel for healthy cases with the percentage of coefficient of variation (%CV)

Code	Sex	Age (year)	Sample	Number of detected spot on 2-DE							
				Coomassie stain				Silver stain			
				#1	#2	Mean ± SD	%CV	#1	#2	Mean ± SD	%CV
HE01	Female	52	Blood donor	182	197	189 ± 10	5.29	789	753	771 ± 25	3.24
HE02	Female	50	Blood donor	118	132	125 ± 9	7.2	505	750	627 ± 173	27.59
HE03	Male	48	Blood donor	151	162	156 ± 7	4.49	415	574	494 ± 112	22.67
HE04	Male	54	Blood donor	129	149	139 ± 14	10.07	574	594	584 ± 14	2.40
HE05	Female	61	Blood donor	181	150	165 ± 21	12.73	489	566	527 ± 54	10.25
HE06	Male	49	Blood donor	188	208	198 ± 14	7.07	606	600	603 ± 4	0.66
HE07	Female	52	Blood donor	199	193	196 ± 4	2.04	674	781	727 ± 75	10.32
HE08	Male	56	Blood donor	180	175	177 ± 3	1.69	705	630	667 ± 53	7.95
HE09	Male	48	Blood donor	214	193	203 ± 14	6.90	614	710	662 ± 67	10.12
HE10	Female	47	Blood donor	147	123	135 ± 16	11.85	494	544	519 ± 35	6.74
Mean ± SD		51 ± 4				168 ± 29				618 ± 105	

Table 4-5 Number of detected spots in each triplicate gel for CCA cases with the percentage of coefficient of variation (%CV)

Code	Sex	Age (year)	CCA type	Serum	Number of detected spots on 2-DE									
					Coomassie stain					Silver stain				
					#1	#2	#3	Mean ± SD	%CV	#1	#2	#3	Mean ± SD	%CV
Y76	Female	61	IPAP	Pre-OR	155	132	130	139±14	10.07	561	449	501	503±56	11.13
				Post-OR	132	143	138	137±5	3.65	633	497	591	573±69	12.04
Y83	Female	51	IPAP	Pre-OR	166	128	104	132±31	23.48	493	478	554	508±40	7.87
				Post-OR	156	141	104	133±26	19.55	564	558	602	574±23	4.01
Y100	Male	57	IPAP	Pre-OR	156	175	173	168±10	5.95	552	444	442	479±62	12.94
				Post-OR	165	165	139	156±15	9.62	520	481	432	477±44	9.22
W97	Female	55	WD	Pre-OR	151	126	118	131±17	12.98	625	417	488	510±105	20.59
				Post-OR	167	146	159	157±10	6.37	587	464	522	524±61	11.64
X31	Male	57	WD	Pre-OR	149	137	159	148±11	7.43	468	505	554	509±43	8.45
				Post-OR	156	159	161	158±2	1.27	479	486	545	503±36	7.16
X42	Female	56	WD	Pre-OR	202	191	184	192±9	4.69	785	767	788	780±11	1.41
				Post-OR	248	194	187	209±33	15.79	818	948	816	860±75	8.72
Mean ± SD		56±3		Pre-OR	152±26					548±118				
				Post-OR	158±30					585±139				

4.6 Differential expression and mass spectrometry analysis

In this experiment, we divided serum 2D pattern analysis into 6 sub groups: (1) CCA IPAP type pre-operative sera compared to post-operative sera; (2) CCA IPAP type pre-operative sera compared to healthy sera; (3) CCA WD type pre-operative sera compared to post-operative sera; (4) CCA WD type pre-operative sera compared to healthy sera; (5) CCA all type (6 cases) pre-operative sera compared to post-operative sera; and (6) CCA all type pre-operative sera compared to healthy sera. These groups are detailed in the following section 4.6.1 – 4.6.6

4.6.1 CCA IPAP type pre-operative sera compared to post-operative sera

Nine gels of pre-operative sera from 3 CCA IPAP type patients are compared with 9 gels of post-operative sera from the same cases. There were 3 protein spots showing statistic significantly different between 2 groups using Coomassie stained gels (code AC) but when compared the silver stained gels (code AS), 6 spots were differentially expressed ($P<0.01$) (Figure 4-18a,b).

All spots were picked-up and identified by LC-MS/MS. After submitted the mass spectrometry data to Mascot program, the results showed that, for Coomassie stained gels, antithrombin and retinol binding protein (RBP) were higher expressed in pre-operative group while apolipoprotein A-IV was higher expressed in post-operative group with all ratio more than 2 (Table 4-6a). For silver stained gels, the results showed only antithrombin that was higher expressed in pre-operative group but apolipoprotein A-IV, arginine/serine-rich splicing factor 6, and one of unnamed protein were higher expressed in post-operative group. Besides, there was one protein spot that found express only in post-operative group which was identified as apolipoprotein A-I isoforms (Table 4-6b). However, there was one spot, code AS01, which has no significant hits.

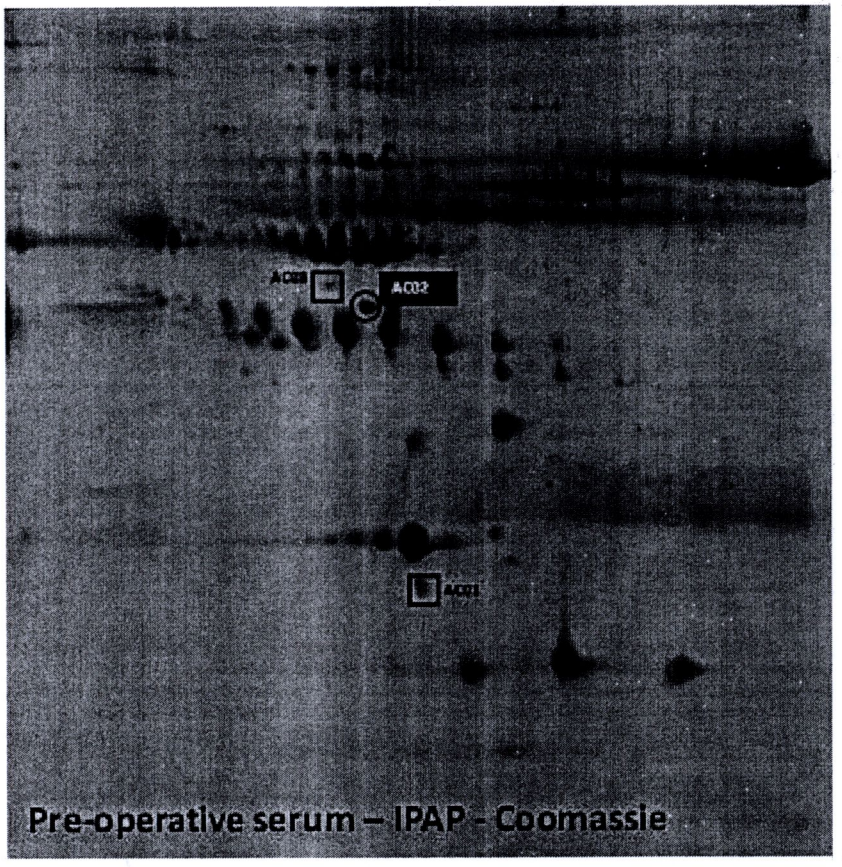

Figure 4-18a Differential expression between CCA IPAP type pre- operative sera (left represent gel) and post-operative sera (right represent gel) in Coomassie stained gels. Two spots were higher expressed in pre-operative group (box) and 1 spot was higher expressed in post-operative group (circle).

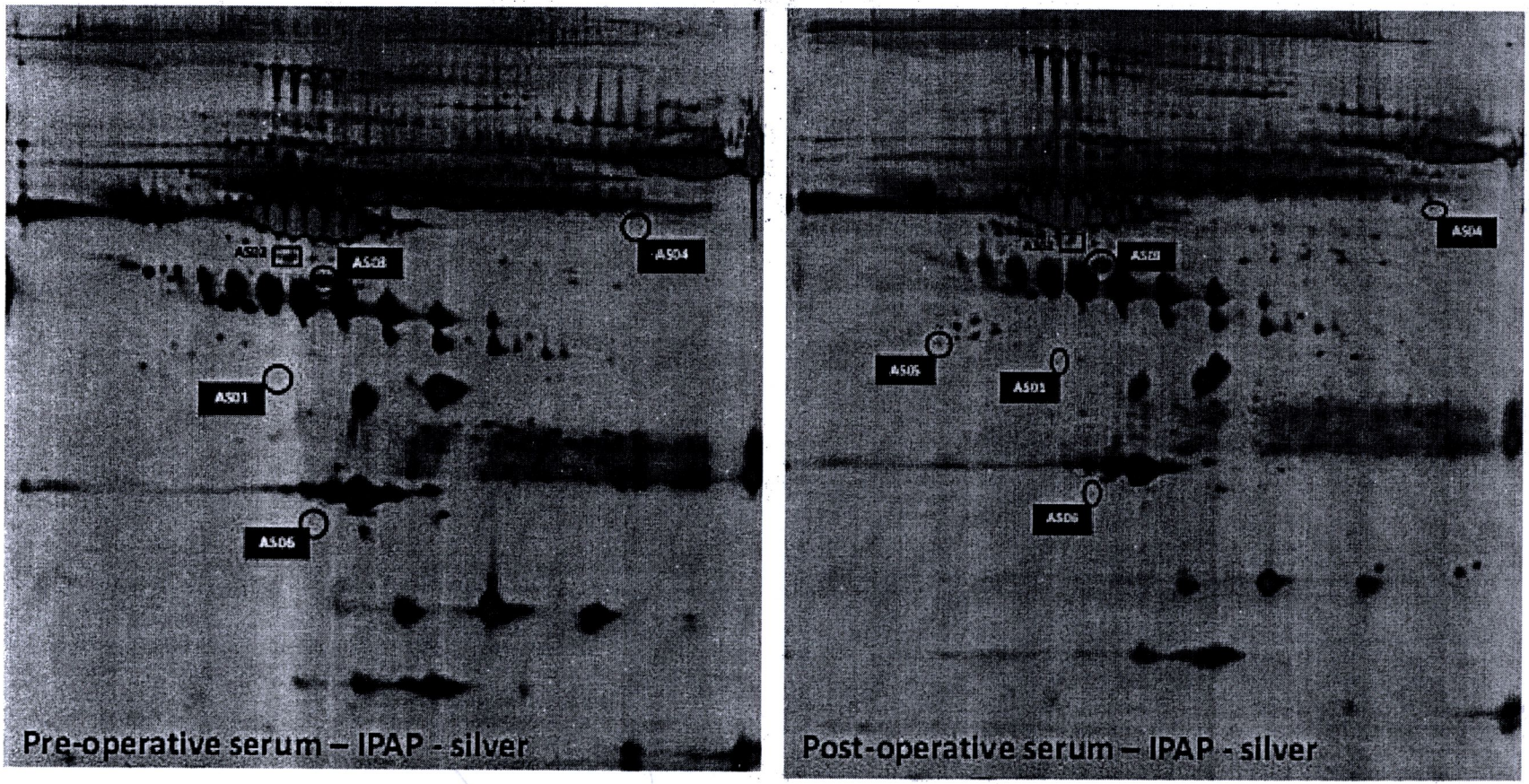

Figure 4-18b Differential expression between CCA IPAP type pre- and post-operative sera in silver stained gels. One spots was higher expressed in pre-operative group (box), 5 spot were higher expressed in post-operative group (circle).

Table 4-6a Identified protein spots in Figure 4-18a (Coomassie stained gel) with both ratio and ID of each spot.

Proteins found in both pre- and post-op serum		Expressed in pre-op only (ID)	Expressed in post-op only (ID)
High expressed in pre-op (ratio, ID)	**High expressed in post-op (ratio, ID)**		
1. Antithrombin (6.82, AC03) 2. RBP (2.36, AC01)	1. apolipoprotein A-IV precursor (2.40, AC02)	-	-

Table 4-6b Identified protein spots in Figure 4-18b (silver stained gel) with both ratio and ID of each spot.

Proteins found in both pre- and post-op serum		Expressed in pre-op only (ID)	Expressed in post-op only (ID)
High expressed in pre-op (ratio, ID)	**High expressed in post-op (ratio, ID)**		
1. Antithrombin (2.26, AS02)	1. apolipoprotein A-IV precursor (1.64, AS03) 2. unnamed protein product (5.41, AS04) 3. arginine/serine-rich splicing factor 6 variant (16.23, AS05)	-	1. apolipoprotein A-I preproprotein (AS06)

4.6.2 CCA IPAP type pre-operative sera compared to healthy sera

For this group, 9 gels of pre-operative sera from 3 CCA IPAP type patients are compared with 20 gels of healthy sera. There were 27 protein spots that were statistic significantly different between 2 groups for Coomassie stained gels (code BC), which 13 spots were higher expressed in pre-operative group, 10 spots were lower expressed in pre-operative group, and 4 spots were only found in healthy group. For silver stained gels (code BS), up to 38 spots were found differentially expressed, which 9 spots were higher expressed in pre-operative group with 2 spots that only expressed in pre-operative group, 20 spots were lower expressed in pre-operative group, and 7 spots that only expressed in healthy group ($P<0.01$) (Figure 4-19a, b).

Only the different spots from the previous group analysis were additionally picked-up and identified by LC-MS/MS, then submitted the mass spectra to Mascot program. As previously report, antithrombin was higher expressed in pre-operative group but apolipoprotein A-IV was lower expressed in pre-operative group than healthy group in both Coomassie and silver stained gel. Moreover, for Coomassie stained gel, 6 isoforms of haptpglobin (HP), alpha-1-antitrypsin, 2 isoforms of alpha-1-B-glycoprotein, ceruloplasmin, and 1 isoform of albumin were higher expressed in pre-operative group, but 2 isoforms of HP, 2 isoforms of afamin, alpha-1-antitrypsin, Zn-alpha-2-glycoprotein, CD5 antigen-like, and apo-serum transferrin were lower expressed in pre-operative group than healthy group. Besides, there were protein spots found only in healthy group which were identified as 2 isoforms of alpha-1-antitrypsin, Zn-alpha-2-glycoprotein, and serum transferrin with one spot that was not analyzed (BC01) and one that had no significant hits (BC20) (Table 4-7a). When compared the silver stained gel, there were 4 isoforms of HP, alpha-1-antitrypsin, CAG-isl 7, and transthyretin that were higher expressed in pre-operative group with 1 isoform of serpin protease that had expressed only in pre-operative group. However, 3 isoforms of HP, alpha-1-antitrypsin, regulatory subunit B56, afamin, apo-serum transferrin, 2 isoforms of Zn-alpha-2-glycoprotein, 3 isoforms of serum transferrin, alpha-2-macroglobulin, interleukin 1-beta, 2 isoforms of vitamin D-binding protein, and 2 unnamed proteins were lower expressed in pre-

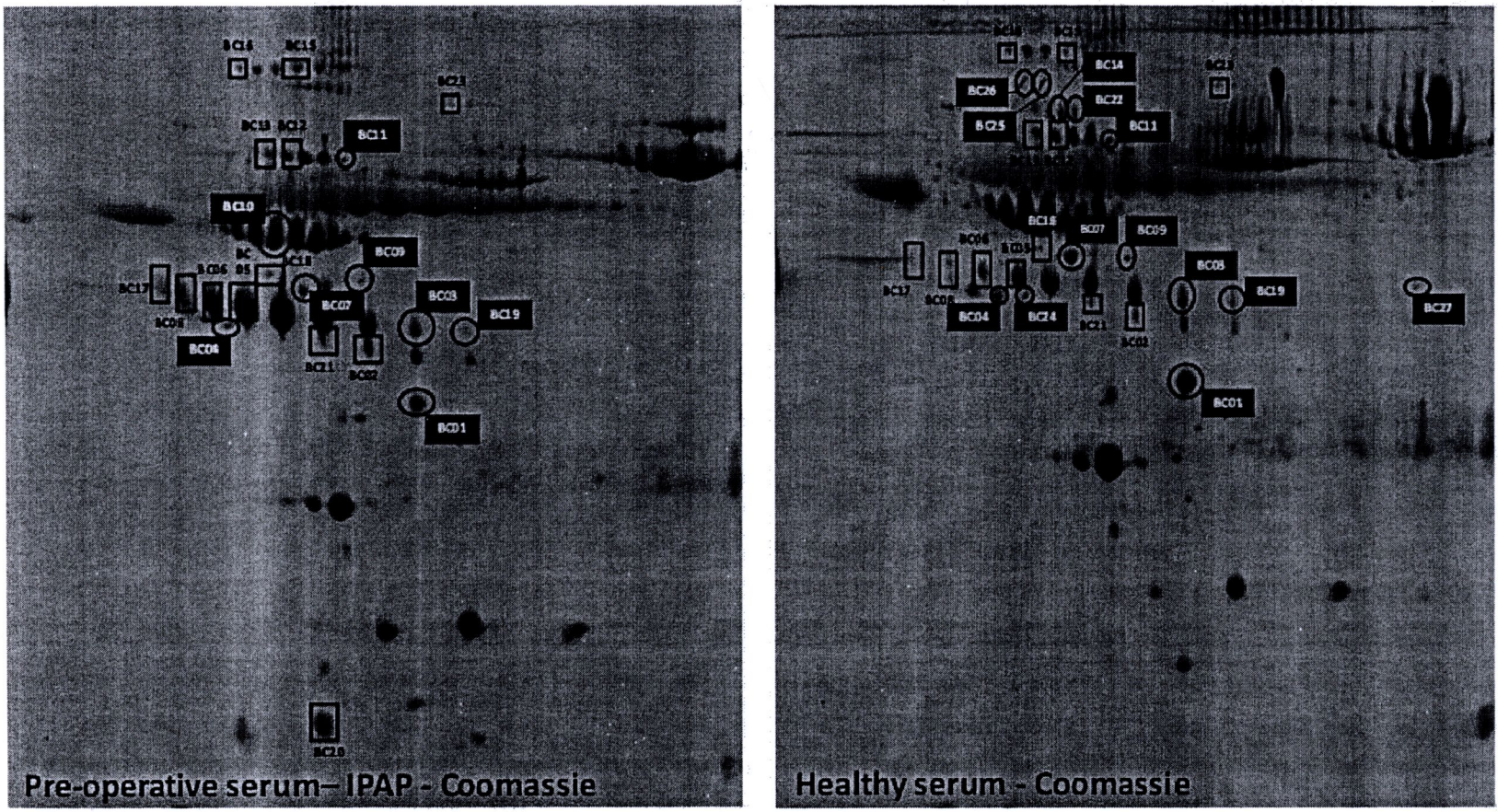

Figure 4-19a Differential expression between CCA IPAP type pre- operative sera (left represent gel) and healthy sera (right represent gel) in Coomassie stained gels. Thirteen spots were higher expressed in pre-operative group (box) and 14 spots were lower expressed in pre-operative group (circle).

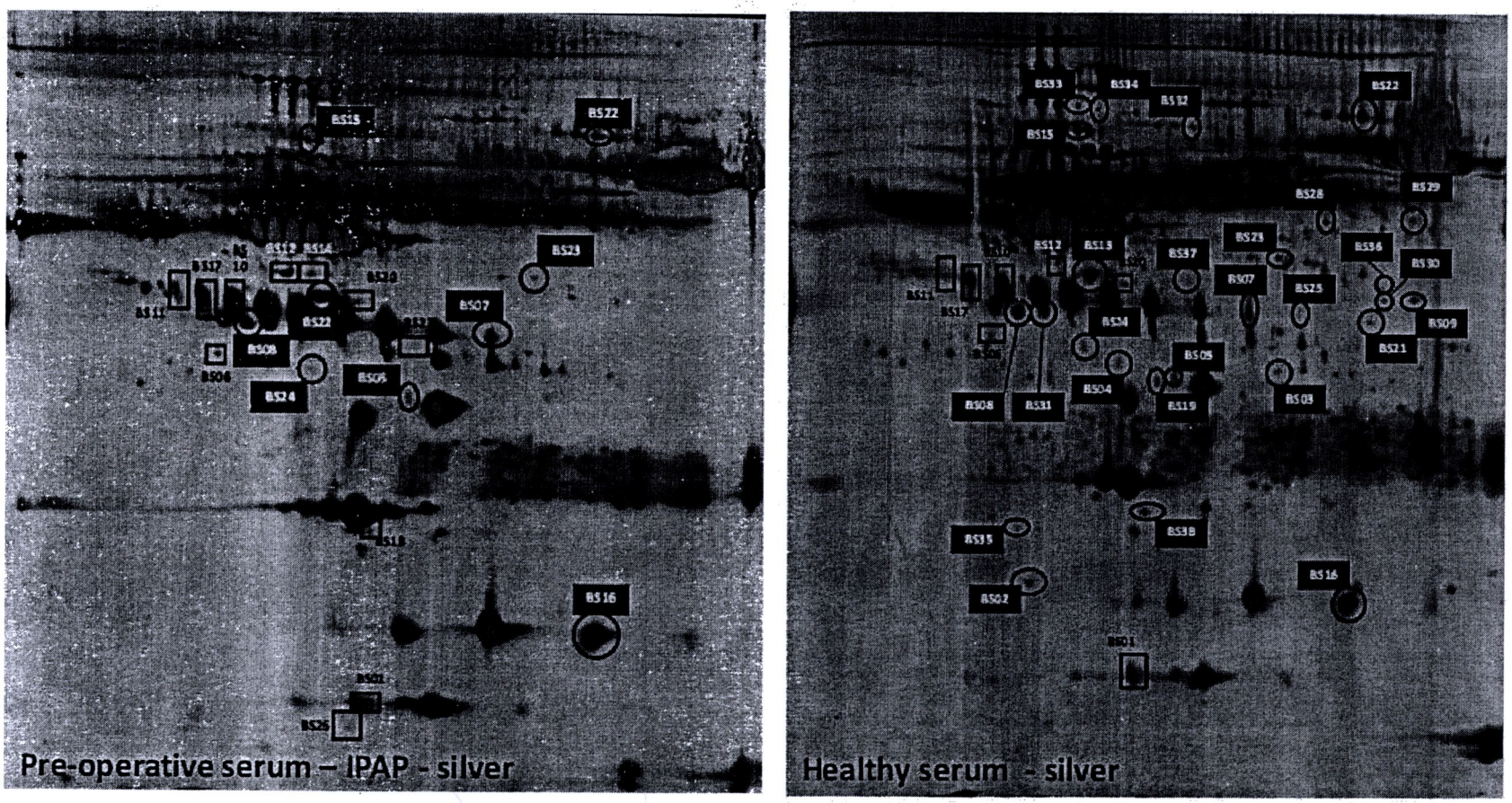

Figure 4-19b Differential expression between CCA IPAP type pre- operative sera (left represent gel) and healthy sera (right represent gel) in silver stained gels. Eleven spots were higher expressed in pre-operative group (box) and 27 spots were lower expressed in pre-operative group (circle).

Table 4-7a Identified protein spots in Figure 4-19a (Coomassie stained gel) with both ratio and ID of each spot.

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
1. HP protein (1.75, BC02)	1. HP, isoform CRA_b (2.30, BC03)	-	1. Chain B, Human Zinc-Alpha-2-Glycoprotein (BC24)
2. HP protein (1.87, BC05)	2. HP protein (4.30, BC19)		2. Uncleaved Alpha-1-Antitrypsin (BC25)
3. HP protein (2.67, BC06)	3. afamin precursor (4.24, BC14)		3. Uncleaved Alpha-1-Antitrypsin (BC26)
4. HP protein (3.18, BC08)	4. afamin precursor (5.95, BC22)		4. Chain A, Human Serum Transferrin (BC27)
5. HP protein (11.22, BC17)	5. Chain A, Alpha1-Antitrypsin (1.47, BC10)		
6. HP protein (6.33, BC21)	6. Chain A, Zn-Alpha-2-Glycoprotein (1.56, BC04)		
7. albumin, isoform CRA_h (4.32, BC23)	7. apolipoprotein A-IV precursor (1.77, BC07)		
8. Antithrombin (3.78, BC18)	8. CD5 antigen-like precursor (1.92, BC09)		
9. alpha-1-B-glycoprotein (1.29, BC12)	9. Chain A, Apo-Human Serum Transferrin (1.21, BC11)		
10. alpha-1-B-glycoprotein (1.44, BC13)			
11. ceruloplasmin (ferroxidase), isoform CRA_b (2.84, BC15)			
12. Chain A, Interactions Causing The Kinetic Trap In Serpin Protein Folding (2.18, BC16)			

Table 4-7b Identified protein spots in Figure 4-19b (Silver stained gel) with both ratio and ID of each spot.

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	**High expressed in healthy (ratio, ID)**		
1. HP protein (1.79, BS10)	1. HP protein (3.55, BS07)	1. Chain A, Crystal Structure Of A Serpin:protease Complex (BS14)	1. alpha 2 macroglobulin (BS32)
2. HP protein (4.03, BS11)	2. HP protein (32.19, BS25)		2. apolipoprotein M (BS38)
3. HP protein (2.72, BS17)	3. HP isoform 1 preproprotein (1.55, BS16)		3. albumin, isoform CRA_j (BS37)
4. HP protein (5.94, BS27)	4. Chain A, Alpha1-Antitrypsin (4.67, BS03)		4. Chain A, Human Serum Transferrin (BS36)
5. alpha-1-antitrypsin (3.15, BS06)	5. delta isoform of regulatory subunit B56, protein phosphatase 2A isoform 1 variant (3.71, BS28)		5. Chain A, Cleaved Alpha-1-Antitrypsin Polymer (BS34)
6. Antithrombin (1.92, BS12)	6. afamin precursor (1.88, BS15)		6. Chain A, Cleaved Antitrypsin With P10 Pro, And P9-P6 Asp (BS33)
7. CAG-isl 7 (1.57, BS20)	7. apolipoprotein A-IV precursor (1.46, BS13)		
8. Chain A, Transthyretin Thr119met Protein Stabilisation (1.53, BS01)	8. Apo-Human Serum Transferrin (Glycosylated) (2.19, BS23)		
	9. Chain A, Zn-Alpha-2-Glycoprotein (1.69, BS08)		
	10. Chain B, Human Zinc-Alpha-2-Glycoprotein (32.09, BS31)		
	11. Chain A, Human Serum Transferrin (3.06, BS09)		
	12. Chain A, Human Serum Transferrin (10.93, BS30)		

Table 4-7b Identified protein spots in Figure 4-19b (Silver stained gel) with both ratio and ID of each spot (Cont.).

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
-	13. macroglobulin alpha2 (1.88, BS22)	-	-
	14. transferrin (3.05, BS21)		
	15. interleukin 1, beta, isoform CRA_b (7.16, BS02)		
	16. vitamin D-binding protein/group specific component (2.27, BS24)		
	17. serum vitamin D-binding protein precursor (4.01, BS04)		
	18. unnamed protein product (5.07, BS05)		
	19. unnamed protein product (4.19, BS19)		

operative group than healthy group with 2 isoforms of antitrypsin, serum transferrin, albumin isoforms, alpha-2-maacroglobulin, and apolipoprotein M that only found in healthy group. Besides, there was 1 spot protein that was not identified (BS29) and 3 protein spots that had no significant hits (Table 4-7b).

4.6.3 CCA WD type pre-operative sera compared to post-operative sera

Similar to the analysis of the first group, 9 gels of pre-operative sera were used to compare with 9 gels of post-operative sera from 3 CCA WD type patients. The results showed only 2 protein spots were statistical significantly different between 2 group for Coomassie stained gel (Code CC) and 3 spots for silver stained gel (Code CS) ($P<0.01$) (Figure 4-20a,b).

The different spots were identified like the previous analysis. The results showed RBP was higher expressed in pre-operative group in both Coomassie and silver stained gels. Moreover, for Coomassie stained gel, a serpin protease was higher expression in post-operative group (Table 4-8a) but unlikely to IPAP group, for silver stained gel, apolipoprotein A-I was higher expressed in pre-operative group instead of post-operative group (Table 4-8b). Unfortunately, spot code CS02 has no significant hits. There are less candidate proteins obtained from CCA WD type than IPAP type when compare between pre- and post-operative sera.

4.6.4 CCA WD type pre-operative sera compared to healthy sera

Nine gels of pre-operative sera from 3 CCA WD type patients were compared to 20 gels from 10 healthy volunteers. The results showed 39 protein spots were significantly different between 2 group for Coomassie stained gel (Code DC) and up to 69 spots for silver stained gel (Code DS) ($P<0.01$). The represented gels are showed in Figure 4-21a and 4-21b, respectively.

Only different spots from the previous groups' analysis were picked to identify. The results showed that, for Coomassie stained gel, 8 isoforms of HP, 7 isoforms of alpha-1-antitrypsin, 4 isoforms of alpha-1-B-glycoprotein, antithrombin, RBP, Zn-alpha-2-glycoprotein, lipid-free apolipoprotein A-I, and 3 unnamed proteins had higher expression in pre-operative group with 1 isoform of alpha-1-antitrypsin,

that only express in pre-operative group. Despite, 1 isoform of HP, 2 isoforms of afamin, apolipoprotein A-IV, 1 isoform of lipid-free apolipoprotein A-I, and Apo-serum transferrin were lower expressed in pre-operative group with 1 isoform of Zn-alpha-2-glycoprotein, serum transferrin isoforms, macroglobulin isoforms, and 1 isoform of apo-serum transferrin were expressed only in healthy group (Table 4-9a). However, there were 2 protein spots that had no significant hits. For silver stained gel, 10 isoforms of HP, 9 isoforms of alpha-1-antitrypsin, 2 isoforms of albumin, 2 isoforms of serpin protease, antithrombin, lipid-free apolipoprotein A-I, proapo-A-I protein, vitamin D-binding protein, and 5 unnamed proteins had higher expression in pre-operative group with 3 isoforms of alpha-1-antitrypsin, HP isoform, apolipoprotein M, serum amyloid A protein, vitamin D-binding protein, serpin protease, and 1 isoform of unnamed protein, that had only expressed in pre-operative group. Whereas, 2 isoform of HP, 2 isoforms of serum transferrin, albumin isoform, transthyretin, Zn-alpha-2-glycoprotein, 2 isoform of macroglobulin, apolipoprotein A-IV, apolipoprotein M, plasma glutathione peroxidase, and 3 isoform of unnamed proteins had lower expression in pre-operative group with 2 isoforms of serum transferrin, Zn-alpha-2-glycoprotein isoform, alpha-2-HS-glycoprotein isoform, and regulatory subunit B56 isoform, which only expressed in healthy group (Table 4-9b). Although, there were 6 protein spots that had no significant hits with 1 spot that identified as keratin (DS42).

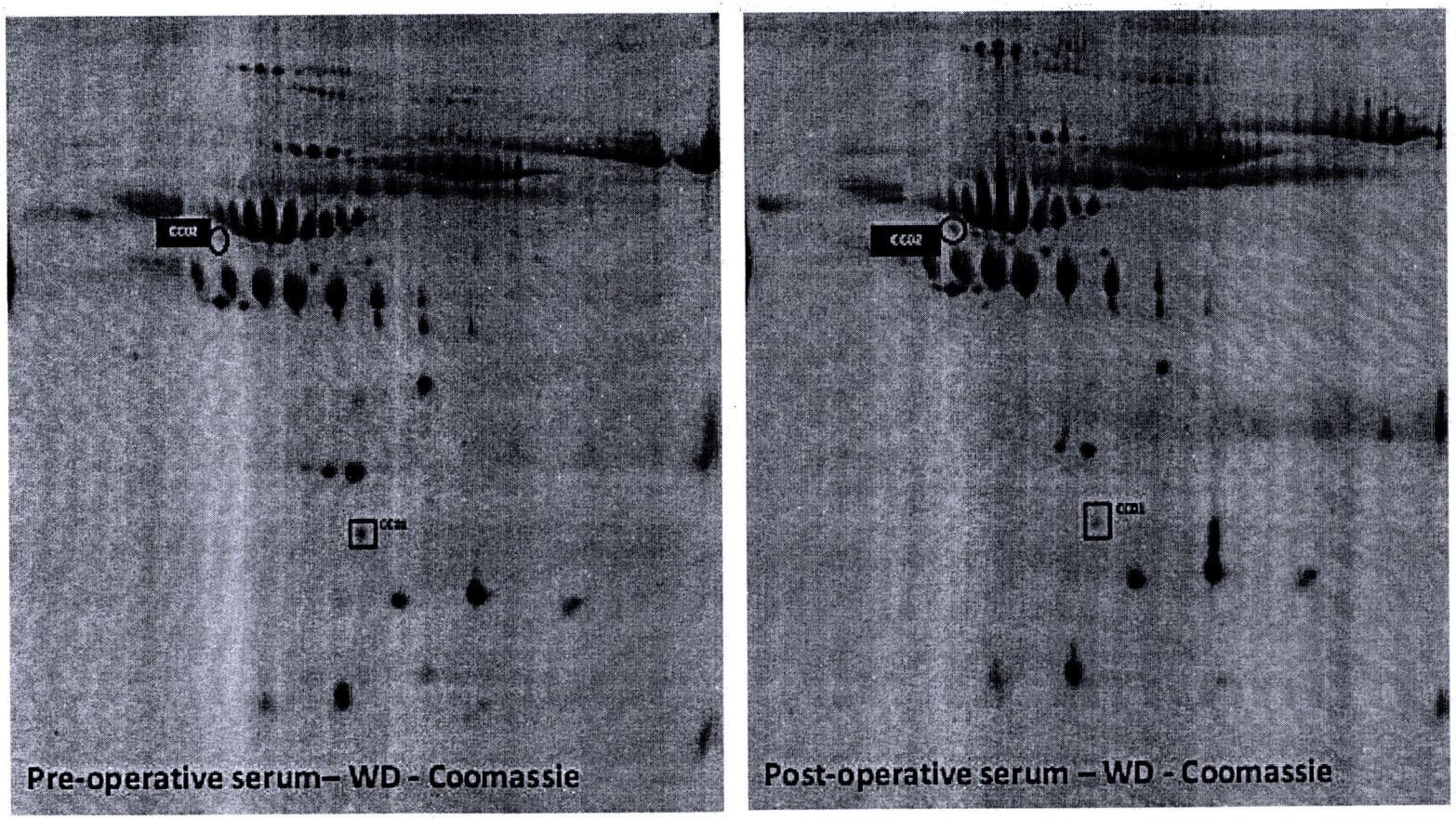

Figure 4-20a Differential expression between CCA WD type pre- operative sera (left represent gel) and post-operative sera (right represent gel) in Coomassie stained gels. One spots was higher expressed in pre-operative group (box) and 1 spot was higher expressed in post-operative group (circle).

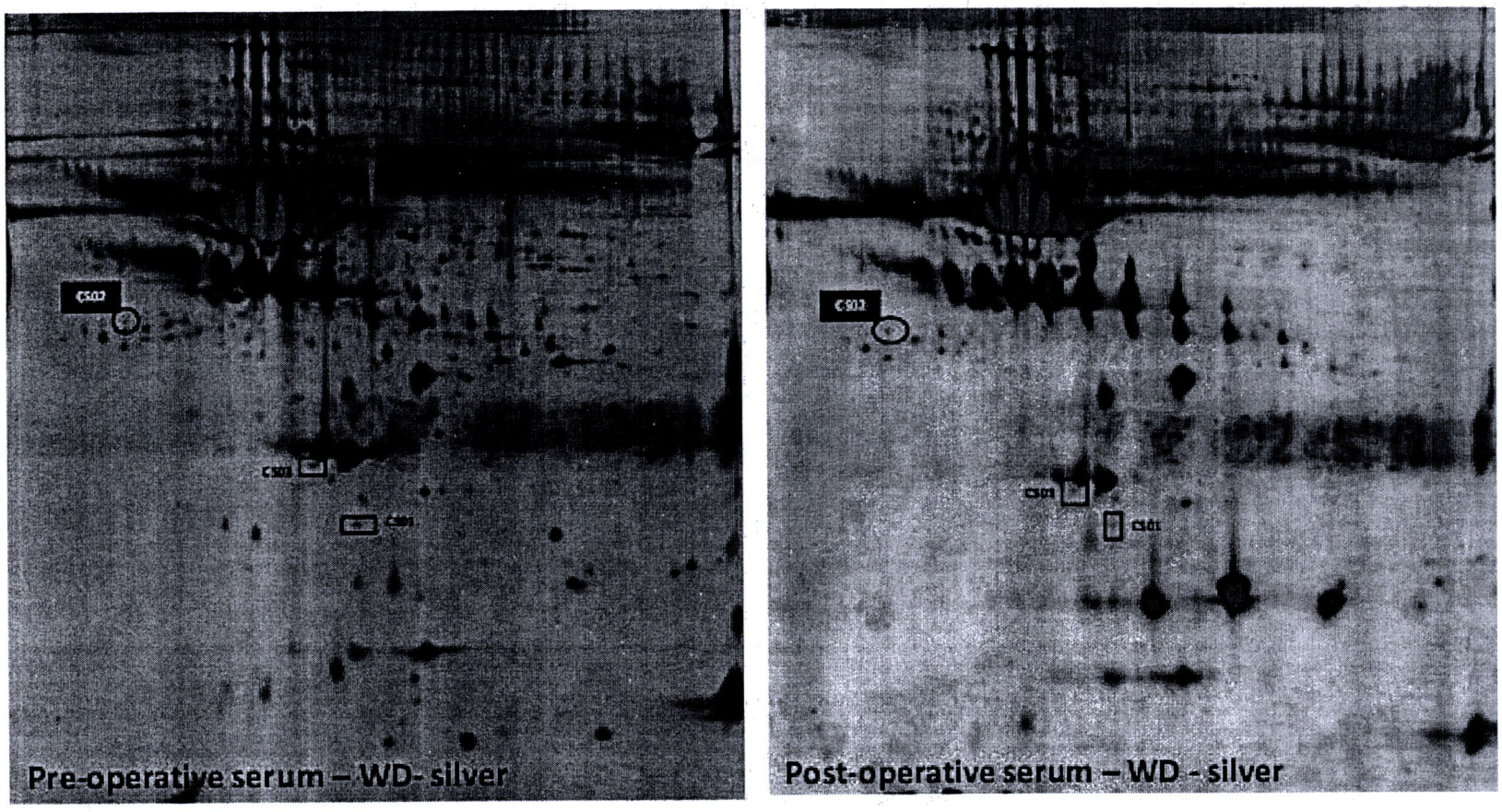

Figure 4-20b Differential expression between CCA WD type pre- operative sera (left represent gel) and post-operative sera (right represent gel) in silver stained gels. Two spots was higher expressed in pre-operative group (box) and 1 spot was higher expressed in post-operative group (circle).

Table 4-8a Identified protein spots in Figure 4-20a (Coomassie stained gel) with both ratio and ID of each spot.

Proteins found in both pre- and post-op serum		Only Expressed in pre-op (ID)	Only Expressed in post-op (ID)
High expressed in pre-op (ratio, ID)	High expressed in post-op (ratio, ID)		
1. RBP (2.05, CC01)	1. Chain A, Crystal Structure Of A Serpin:protease Complex (1.82, CC02)	-	-

Table 4-8b Identified protein spots in Figure 4-20b (silver stained gel) with both ratio and ID of each spot.

Proteins found in both pre- and post-op serum		Only Expressed in pre-op (ID)	Only Expressed in post-op (ID)
High expressed in pre-op (ratio, ID)	High expressed in post-op (ratio, ID)		
1. RBP (1.75, CS01)	-	-	-
2. apolipoprotein A-I preproprotein (2.87, CS03)			

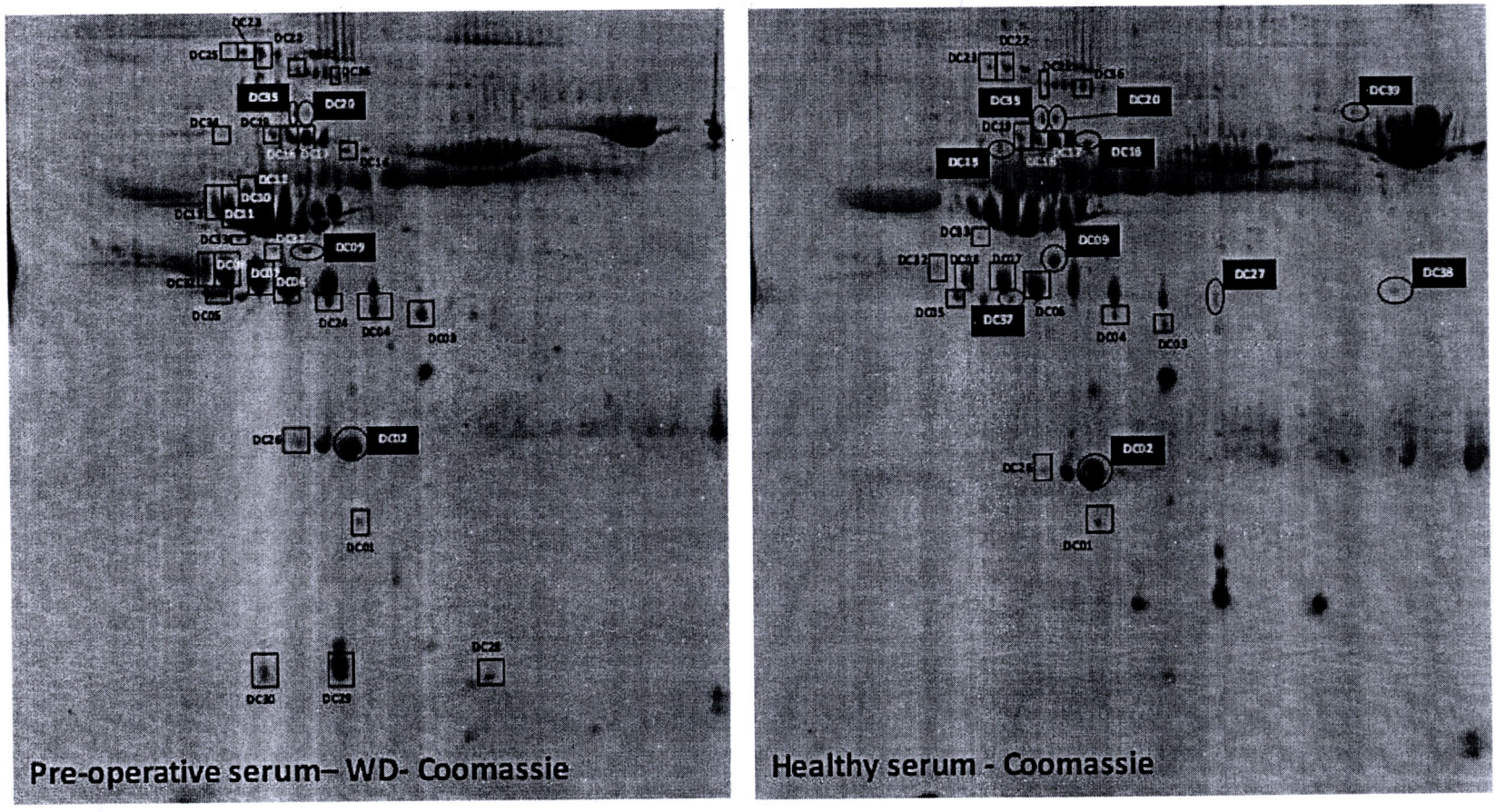

Figure 4-21a Differential expression between CCA WD type pre- operative sera (left represent gel) and healthy sera (right represent gel) in Coomassie stained gels. Twenty-nine spots were higher expressed in pre-operative group (box) and 10 spots were lower expressed in pre-operative group (circle).

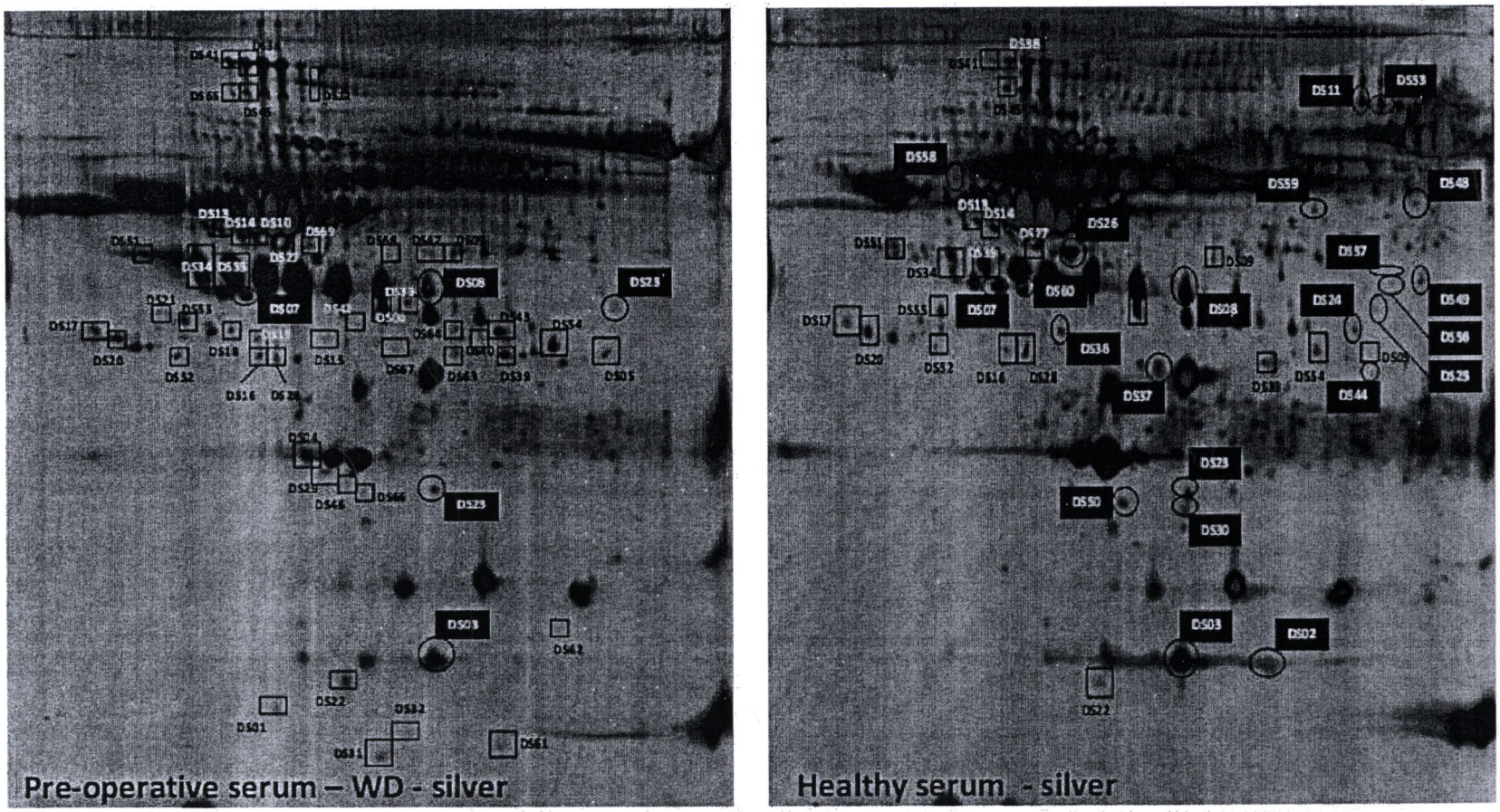

Figure 4-21b Differential expression between CCA WD type pre- operative sera (left represent gel) and healthy sera (right represent gel) in silver stained gels. Forty-seven spots were higher expressed in pre-operative group (box) and 22 spots were lower expressed in pre-operative group (circle).

Table 4-9a Identified protein spots in Figure 4-21a (Coomassie stained gel) with both ratio and ID of each spot.

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op	Only Expressed in healthy
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)	(ID)	(ID)
1. HP protein (1.50, DC03)	1. HP protein (3.37, DC27)	1. Chain A, Alpha1-Antitrypsin (DC25)	1. Apo-Human Serum Transferrin (Glycosylated) (DC15)
2. HP protein (2.22, DC04)	2. apolipoprotein A-IV precursor (2.22, DC09)		2. Human Zinc-Alpha-2-Glycoprotein (DC37)
3. HP protein (1.70, DC06)	3. afamin precursor (8.38, DC20)		3. Human Serum Transferrin (DC38)
4. HP protein (2.42, DC07)	4. afamin precursor (3.49, DC35)		4. macroglobulin alpha2 (DC39)
5. HP protein (3.84, DC08)	5. Lipid-Free Human Apolipoprotein A-I (1.33, DC02)		
6. HP protein (5.26, DC32)	6. Apo-Human Serum Transferrin (Glycosylated) (1.48, DC16)		
7. HP protein (7.06, DC24)			
8. HP protein (56.75, DC30)			
9. RBP (1.59, DC01)			
10. alpha-1-B-glycoprotein (2.09, DC14)			
11. alpha-1-B-glycoprotein (1.32, DC17)			
12. alpha-1-B-glycoprotein (1.63, DC18)			
13. alpha-1-B-glycoprotein (1.65, DC19)			
14. Zinc-Alpha-2-Glycoprotein (3.04, DC05)			
15. Lipid-Free Human Apolipoprotein A-I (2.56, DC26)			

Table 4-9a Identified protein spots in Figure 4-21a (Coomassie stained gel) with both ratio and ID of each spot (Cont.).

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
16. Antithrombin (6.68, DC31)	-	-	-
17. Chain A, Alpha1-Antitrypsin (3.75, DC11)			
18. Chain A, Alpha1-Antitrypsin (1.36, DC12)			
19. Chain A, Alpha1-Antitrypsin (16.53, DC13)			
20. Chain A, Alpha1-Antitrypsin (2.68, DC23)			
21. Thr114phe Alpha1- Antitrypsin (2.03, DC10)			
22. Thr114phe Alpha1- Antitrypsin (1.80, DC22)			
23. Cleaved Antitrypsin With P10 Pro, And P9-P6 Asp (3.29, DC33)			
24. unnamed protein product (1.91, DC36)			
25. unnamed protein product (4.22, DC21)			
26. unnamed protein product (8.66, DC34)			

Table 4-9b Identified protein spots in Figure 4-21b (silver stained gel) with both ratio and ID of each spot.

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
1. HP protein (1.95, DS01)	1. HP protein (1.80, DS24)	1. apolipoprotein M (DS66)	1. alpha-2-HS-glycoprotein, isoform CRA_a (DS58)
2. HP protein (1.59, DS06)	2. albumin, isoform CRA_a (8.74, DS44)	2. Chain A, Alpha1-Antitrypsin (DS63)	2. Chain A, Human Serum Transferrin (DS56)
3. HP protein (3.60, DS34)	3. Transthyretin Thr119met Protein Stabilisation (1.53, DS03)	3. Uncleaved Alpha-1- Antitrypsin (DS64)	3. Chain A, Human Serum Transferrin (DS57)
4. HP protein (1.84, DS35)	4. haptoglobin, isoform CRA_b (1.85, DS08)	4. Uncleaved Alpha-1- Antitrypsin (DS65)	4. Chain B, Human Zinc-Alpha-2-Glycoprotein (DS60)
5. HP protein (6.25, DS18)	5. Chain A, Zn-Alpha-2-Glycoprotein (1.40, DS07)	5. HP protein (DS68)	5. delta isoform of regulatory subunit B56, protein phosphatase 2A isoform 1 variant (DS59)
6. HP protein (9.71, DS52)	6. macroglobulin alpha2 (2.56, DS11)	6. serum amyloid A protein preproprotein (DS61)	
7. HP protein (6.43, DS33)	7. macroglobulin alpha2 (2.31, DS53)	7. vitamin D-binding protein/group specific component (DS67)	
8. HP protein (6.00, DS40)	8. transferrin (2.04, DS25)	8. Chain A, Crystal Structure Of A Serpin:protease Complex (DS69)	
9. HP protein (2.87, DS42)	9. apolipoprotein A-IV precursor (1.97, DS26)	9. unnamed protein product (DS62)	
10. HP protein (2.13, DS47)	10. apolipoprotein M (2.73, DS50)		
11. Antithrombin (2.39, DS27)	11. plasma glutathione peroxidase (1.86, DS23)		
12. ALB protein (1.20, DS31)	12. Chain A, Human Serum Transferrin (7.03, DS49)		
13. Lipid-Free Human Apolipoprotein A-I (2.08, DS04)			
14. Uncleaved Alpha-1- Antitrypsin (7.12, DS05)			

Table 4-9b Identified protein spots in Figure 4-21b (silver stained gel) with both ratio and ID of each spot (Cont.).

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
15 Chain A, Alpha1-Antitrypsin (3.99, DS39)	13. Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (3.67, DS54)	-	-
16. Chain A, Alpha1-Antitrypsin (4.60, DS41)	14. unnamed protein product (8.76, DS30)		
17. Chain A, Cleaved Alpha-1-Antitrypsin Polymer (3.57, DS55)	15. unnamed protein product (3.71, DS37)		
18. Chain A, Cleaved Alpha-1-Antitrypsin Polymer (3.35, DS21)	16. unnamed protein product (6.01, DS48)		
19. proapo-A-I protein (4.82, DS29)			
20. Uncleaved Alpha-1- Antitrypsin(3.28, DS45)			
21. Uncleaved Alpha-1- Antitrypsin (7.11, DS43)			
22. vitamin D-binding protein/group specific component (4.61, DS15)			
23. Chain A, Serpin:protease Complex (2.95, DS10)			
24. Chain A, Serpin:protease Complex (1.91, DS13)			

Table 4-9b Identified protein spots in Figure 4-21b (silver stained gel) with both ratio and ID of each spot (Cont.).

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
25. Chain A, Human Serum Albumin Complexed With Myristate And Azapropazone (3.68, DS09)	-	-	-
26. Chain A, Cleaved Antitrypsin With P10 Pro, And P9-P6 Asp (1.55, DS14)			
27. Chain A, Interactions Causing The Kinetic Trap In Serpin Protein Folding (2.08, DS38)			
28. unnamed protein product (1.59, DS12)			
29. unnamed protein product (3.17, DS20)			
30. unnamed protein product (3.01, DS51)			
31. unnamed protein product (4.12, DS16)			
32. unnamed protein product (9.46, DS17)			

4.6.5 CCA all type pre-operative sera compared to post-operative sera

Nine gels from CCA IPAP type and 9 gels from CCA WD type of pre-operative sera from CCA patients were compared against 18 gels of post-operative sera. The results showed that, for both Coomassie (code EC) and silver stained gel (code ES), only 2 spots were either significantly different between 2 groups ($P<0.01$). The represented gels are shown in Figure 4-22a and 4-22b, respectively.

The results from LC-MS/MS and Mascot program showed that, apolipoprotein M was higher expressed in pre-operative group but apolipoprotein A-IV was higher expressed in post-operative group in Coomassie stained gel (Table 4-10a). For silver stained gel, RBP protein and 1 isoform of unnamed protein were higher expressed in pre-operative group (Table 4-10b).

4.6.6 CCA all type pre-operative sera compared to healthy sera

Eighteen gels of pre-operative sera from 6 CCA patients were compared to 20 gels from 10 healthy people. The 37 spots were significantly different between 2 groups ($P<0.01$) for Coomassie stained gel (code FC) (Figure 4-23a). For silver stained gels (code FS), there were up to 47 spots protein that significantly different between 2 groups ($P<0.01$) (Figure 4-23b).

After the analysis by LC-MS/MS and Mascot program, 8 isoforms of HP, 5 isoforms of alpha-1-B-glycoprotein, 4 isoform of alpha-1-antitrypsin, serpin protease, antithrombin, Zn-alpha-2-glycoprotein isoform, and lipid-free apolipoprotein A-I isoform were higher expressed in pre-operative group with 1 isoform of antitrypsin that only expressed in pre-operative group. Three isoforms of HP, 2 isoforms of afamin, apolipoprotein A-IV, plasma glutathione peroxidase, alpha-2-macroglobulin isoform, and apo-serum transferrin were lower expressed in pre-operative group with 2 isoforms of apo-serum transferrin, Zn-alpha-2-glycoprotein isoform, and serum transferrin isoform were only expressed in healthy group (Table 4-11a). However, 1 spot has not been done (code FC02) and 1 spot has no significant hit. For silver stained gel, 8 isoforms of HP, 4 isoforms of alpha-1-antitrypsin, antithrombin, apolipoprotein A-I, and 1 isoform of unnamed protein were higher

expressed in pre-operative group, instead of 5 isoforms of HP, 2 isoforms of albumin, 2 isoforms of afamin, 4 isoforms of serum transferrin, apolipoprotein M, apolipoprotein A-IV, 2 isoforms of apo-serum transferrin, 2 isoforms of Zn-alpha-2-glycoprotein, plasma glutathione peroxidase, interleukin 1-beta, regulatory subunit B56 isoform, and 3 isoforms of unnamed proteins, that were lower expressed in pre-operative group with alpha-2-HS-glycoprotein isoform that only expressed in healthy group (Table 4-11b). However, there were 6 protein spots that had no significant hits.

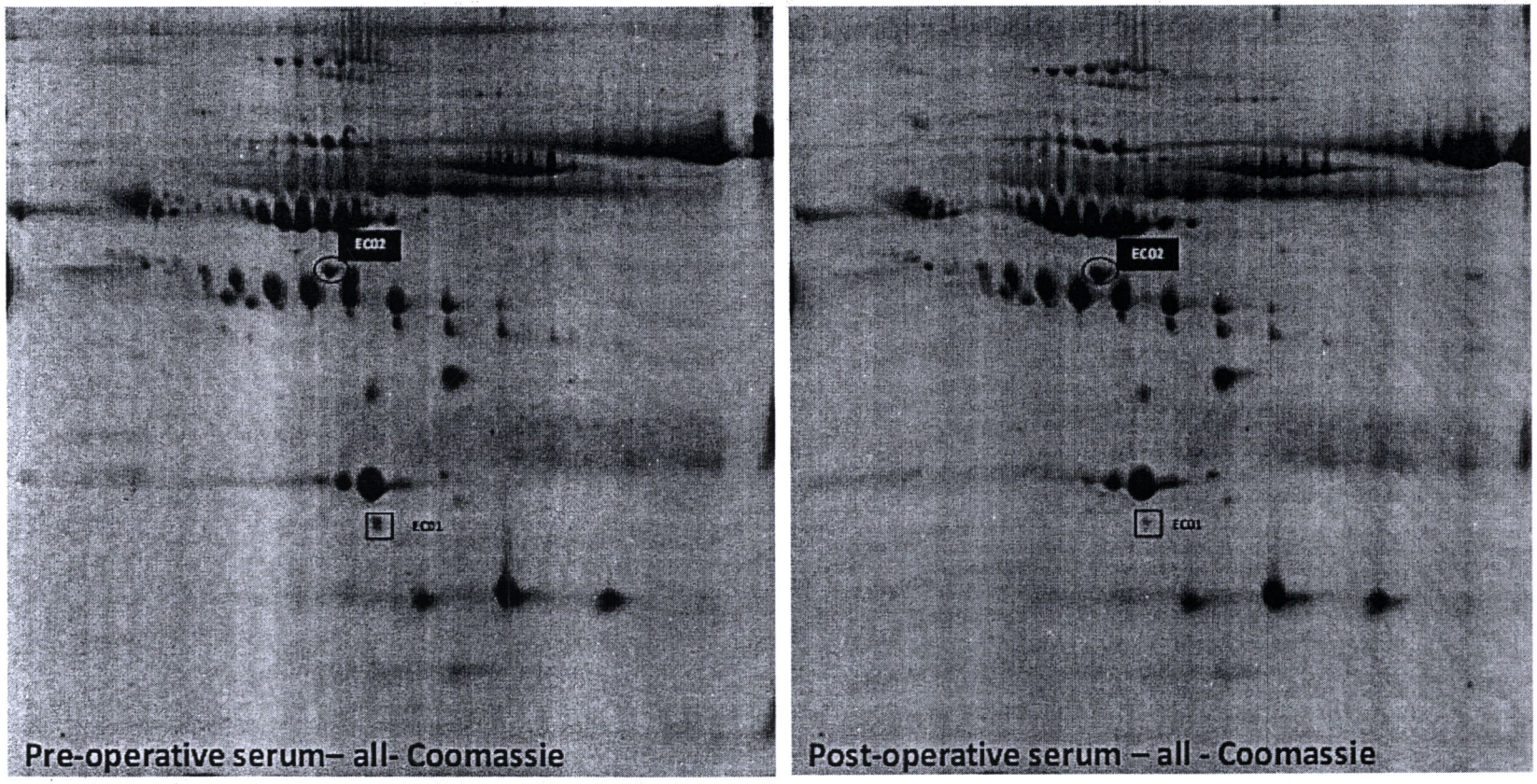

Figure 4-22a Differential expression between CCA all type pre- operative sera (left represent gel) and post-operative sera (right represent gel) in Coomassie stained gels. One spot was higher expressed in pre-operative group (box) and 1 spot was higher expressed in post-operative group (circle).

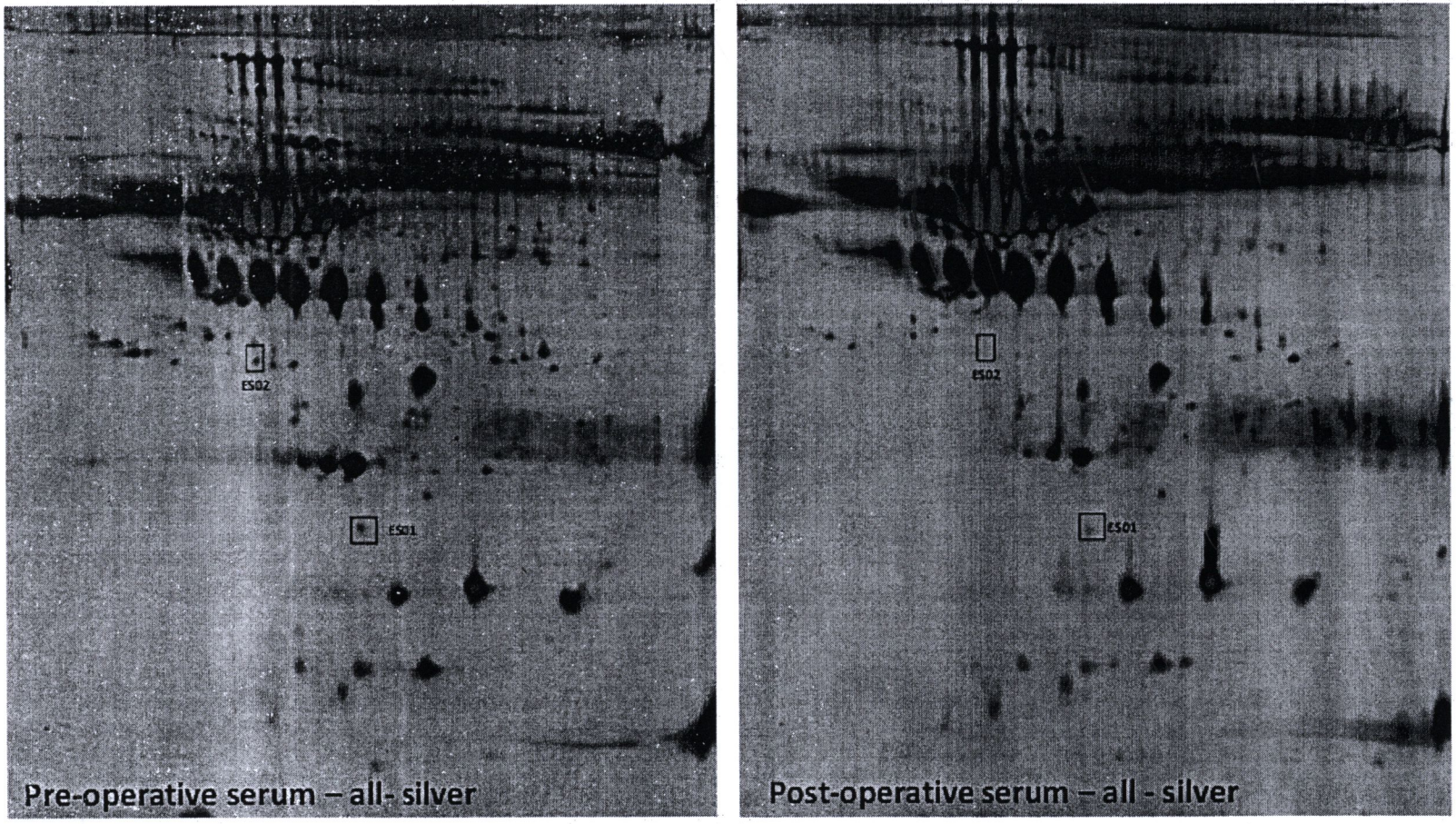

Figure 4-22b Differential expression between CCA all type pre- operative sera (left represent gel) and post-operative sera (right represent gel) in silver stained gels. Only 2 spots were higher expressed in pre-operative group (box).

Table 4-10a Identified protein spots in Figure 4-22a (Coomassie stained gel) with both ratio and ID of each spot.

Proteins found in both pre- and post-op serum		Only Expressed in pre-op (ID)	Only Expressed in post-op (ID)
High expressed in pre-op (ratio, ID)	High expressed in post-op (ratio, ID)		
1. apolipoprotein M (2.18, EC01)	1. apolipoprotein A-IV precursor (2.07, EC02)	-	-

Table 4-10b Identified protein spots in Figure 4-22b (silver stained gel) with both ratio and ID of each spot.

Proteins found in both pre- and post-op serum		Only Expressed in pre-op (ID)	Only Expressed in post-op (ID)
High expressed in pre-op (ratio, ID)	High expressed in post-op (ratio, ID)		
1. RBP (1.74, ES01)	-	-	-
2. unnamed protein product (4.38, ES02)			

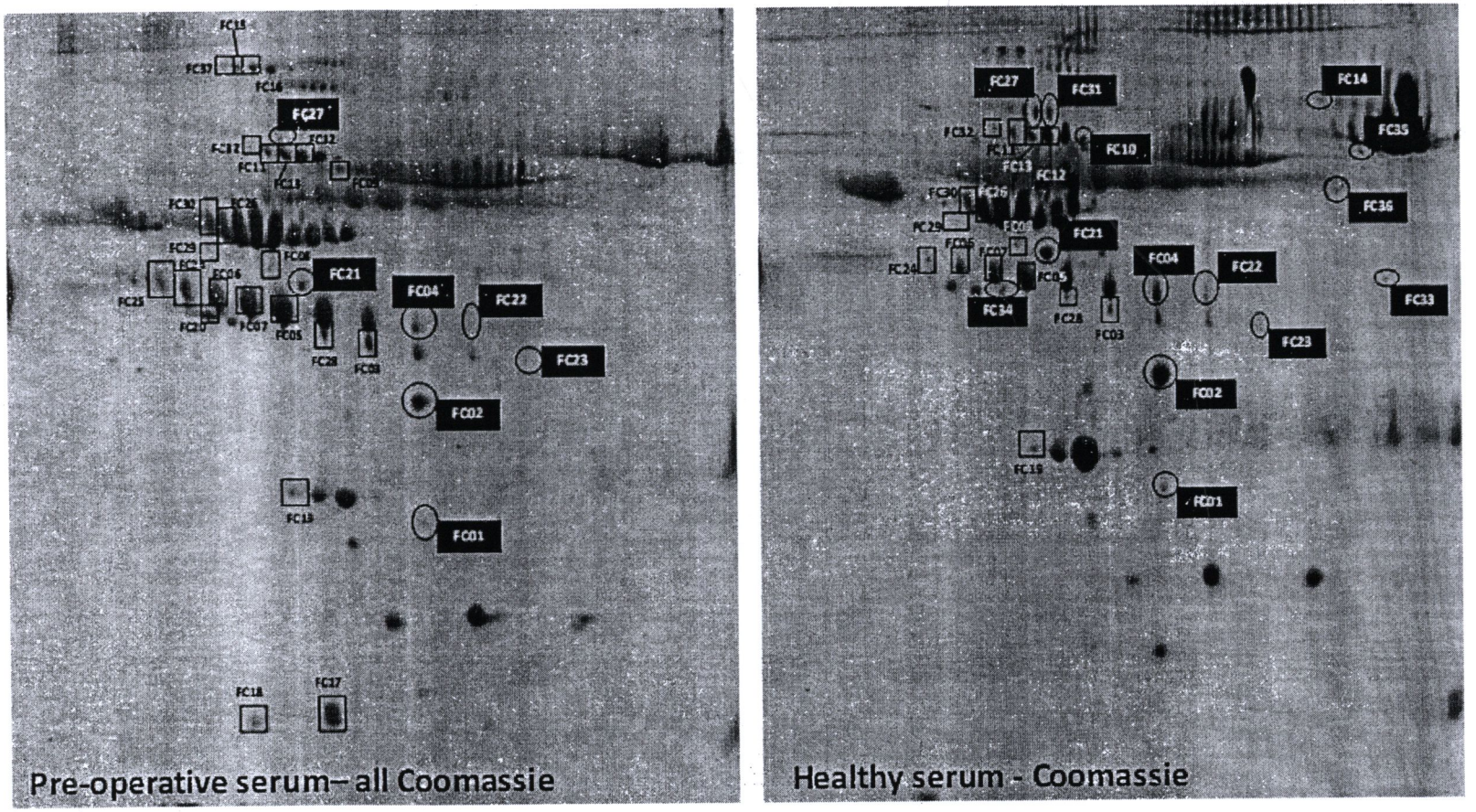

Figure 4-23a Differential expression between CCA all type pre- operative sera (left represent gel) and healthy sera (right represent gel) in Coomassie stained gels. Twenty-three spots were higher expressed in pre-operative group (box) and 14 spots were lower expressed in pre-operative group (circle).

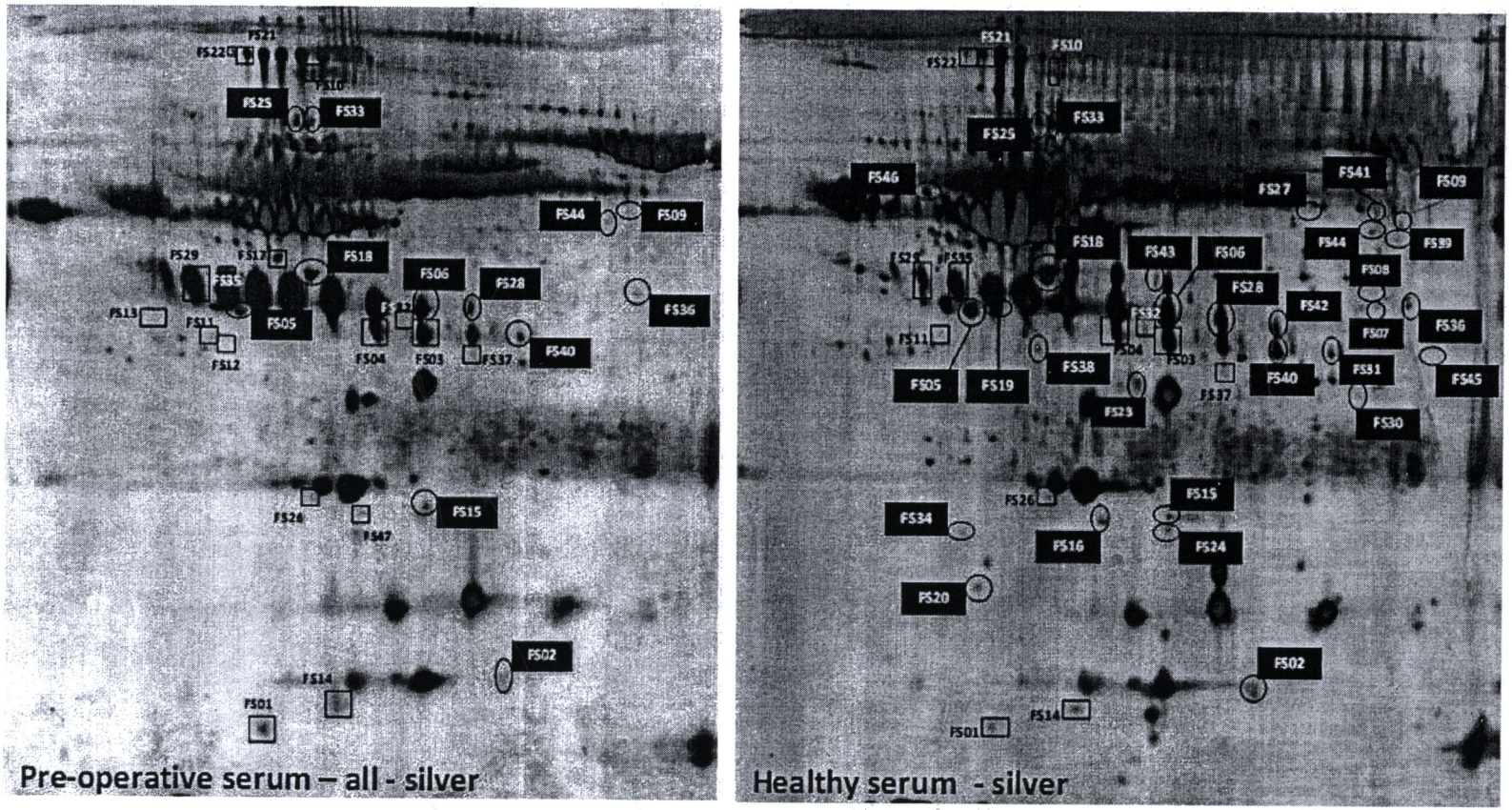

Figure 4-23b Differential expression between CCA all type pre- operative sera (left represent gel) and healthy sera (right represent gel) in silver stained gels. Seventeen spots were higher expressed in pre-operative group (box) and 30 spots were lower expressed in pre-operative group (circle).

Table 4-11a Identified protein spots in Figure 4-23a (Coomassie stained gel) with both ratio and ID of each spot.

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
1. HP protein (1.97, FC03)	1. HP protein (3.81, FC22)	1. Chain A, Alpha1-Antitrypsin (FC37)	1. Apo-Human Serum Transferrin (Glycosylated) (FC35)
2. HP protein (1.54, FC05)	2. HP protein (3.76, FC23)		2. Apo-Human Serum Transferrin (Glycosylated) (FC36)
3. HP protein (3.23, FC06)	3. haptoglobin, isoform CRA_b (2.22, FC04)		3. Human Serum Transferrin (FC33)
4. HP protein (2.13, FC07)	4. afamin precursor (3.87, FC27)		4. Human Zinc-Alpha-2-Glycoprotein (FC34)
5. HP protein (4.19, FC24)	5. afamin precursor (7.83, FC31)		
6. HP protein (6.63, FC28)	6. plasma glutathione peroxidase (1.45, FC01)		
7. HP protein (16.14, FC25)	7. macroglobulin alpha2 (5.95, FC14)		
8. HP protein (46.94, FC18)	8.apolipoprotein A-IV precursor (2.00, FC21)		
9. Antithrombin (5.15, FC08)	9. Apo-Human Serum Transferrin (Glycosylated) (1.35, FC10)		
10. alpha-1-B-glycoprotein (2.11, FC09)			
11. alpha-1-B-glycoprotein (1.53, FC11)			
12. alpha-1-B-glycoprotein (1.16, FC12)			
13. alpha-1-B-glycoprotein (1.44, FC13)			
14. alpha-1-B-glycoprotein (2.73, FC32)			

Table 4-11a Identified protein spots in Figure 4-23a (Coomassie stained gel) with both ratio and ID of each spot (Cont.).

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
15. Zinc-Alpha-2-Glycoprotein (2.07, FC20)	-	-	-
16. Lipid-Free Human Apolipoprotein A-I (1.84, FC19)			
17. Chain A, Alpha1-Antitrypsin (2.40, FC15)			
18. Chain A, Alpha1-Antitrypsin (2.62, FC26)			
19. Chain A, Alpha1-Antitrypsin (12.75, FC30)			
20. Thr114phe Alpha1- Antitrypsin (1.62, FC16)			
21. Chain A, Crystal Structure Of A Serpin:protease Complex (4.14, FC29)			

Table 4-11b Identified protein spots in Figure 4-23b (silver stained gel) with both ratio and ID of each spot.

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	High expressed in healthy (ratio, ID)		
1. HP protein (1.72, FS01)	1. HP, isoform CRA_b (1.65, FS06)	-	1. alpha-2-HS-glycoprotein, isoform CRA_a (FS46)
2. HP protein (1.23, FS03)	2. HP protein (3.43, FS28)		
3. HP protein (1.45, FS04)	3. HP protein (4.95, FS31)		
4. HP protein (3.21, FS29)	4. HP protein (3.05, FS40)		
5. HP protein (1.77, FS35)	5. HP protein (3.77, FS42)		
6. HP protein (8.09, FS12)	6. albumin, isoform CRA_a (3.49, FS30)		
7. HP protein (6.17, FS32)	7. albumin, isoform CRA_j (8.09, FS43)		
8. HP protein (4.61, FS37	8. apolipoprotein M (8.43, FS16		
9. apolipoprotein A-I preproprotein (3.40, FS26)	9. apolipoprotein A-IV precursor (1.69, FS18)		
10. Antithrombin (2.03, FS17)	10. afamin precursor (1.71, FS25)		
11. Chain A, Alpha1-Antitrypsin (1.76, FS21)	11. afamin precursor (2.66, FS33)		
	12. Apo-Human Serum Transferrin (Glycosylated) (6.97, FS39)		
	13. Apo-Human Serum Transferrin (Glycosylated) (5.69, FS41)		

Table 4-11b Identified protein spots in Figure 4-23b (silver stained gel) with both ratio and ID of each spot (Cont.).

Proteins found in both pre-op and healthy serum		Only Expressed in pre-op (ID)	Only Expressed in healthy (ID)
High expressed in pre-op (ratio, ID)	**High expressed in healthy (ratio, ID)**		
12. Chain A, Alpha1-Antitrypsin (3.85, FS22)	14. Chain A, Human Serum Transferrin (22.82, FS07)	-	-
13. Chain A, Cleaved Alpha-1-Antitrypsin Polymer (21.39, FS13)	15. Chain A, Human Serum Transferrin (4.76, FS08)		
14. alpha-1-antitrypsin NULL(BRESCIA) variant (2.84, FS11)	16. Chain A, Human Serum Transferrin (4.05, FS36)		
15. unnamed protein product (1.63, FS10)	17. transferrin, isoform CRA_c (10.07, FS45)		
	18. Chain A, Zn-Alpha-2-Glycoprotein (1.54, FS05)		
	19. Chain B, Human Zinc-Alpha-2-Glycoprotein (61.24, FS19)		
	20. plasma glutathione peroxidase (1.54, FS15)		
	21. interleukin 1, beta, isoform CRA_b (4.77, FS20)		
	22. delta isoform of regulatory subunit B56, protein phosphatase 2A isoform 1 variant (7.62, FS27)		
	23. unnamed protein product (4.20, FS09)		
	24. unnamed protein product (4.98, FS23)		
	25. unnamed protein product (4.34, FS24)		