CHAPTER V

DISCUSSIONS AND CONCLUSION

5.1 Discussions

Proteomics is an emerging field in medical science focused on the library of proteins specific to a given biosystem, the proteome, and understanding relationships therein. This field comprises technologies that can be applied to serum and tissue in order to extract important biological information to aid clinicians and scientists in understanding the dynamic biology of their interest, such as a patient with cancer (Posadas et al., 2005). The application of this field has assisted in the discovery of new biomarkers and may lead to new diagnostic tests and improvements in therapeutics. By this approach, we hope to investigate the new serum biomarkers for CCA diagnosis because of current biomarkers show low sensitivity, specificity, and predictive value, particularly when applied to population screening programs. However, the milestone of this study is the unequal concentration of proteins that present in serum. High abundant proteins, such as serum albumin and immunoglobulin, are accounted for more than 90% of total serum protein by weight, thus decrease a chance to detect the other proteins, particularly low abundant proteins. We decided to remove albumin and immunoglobulin from sera before proteomic analysis, intense to concentrate the low abundance proteins.

5.1.1 Increased the detection of low abundance protein has improved the 2D pattern analysis

Sample preparation is one of the most times consuming, error prone aspect of analytical chemistry, but it is increasingly being an important area in proteomic study (Luque-Garcia and Neubert, 2007). As mentioned previously, it is difficult to analyses serum proteins, especially for low abundance proteins, unless high abundance proteins should be removed prior to the analysis. In this study, we decided to remove the two most abundance proteins from serum, albumin and

immunoglobulin, before 2DE analysis by using commercial columns that available in the market. The results were confirmed by both SDS-PAGE and 2-DE, which concluded that those columns increase the sensitivity of 2-DE for detection of low abundant proteins. However, there are handfuls of antibodies columns that have better sensitivity and specificity, but the priced are also higher.

Pooling sample is one of things that should be concerned because it can affect the quality of the result. In 2008, Sadiq and Agranoff demonstrated that pooling serum can lead to the loss of potential biomarker in proteomic profiling. They used surface enhanced laser desorption/ionization time of flight (SELDI-ToF) to analyses the peak intensity of both individual and pooled serum of 20 patients and 20 controls. The result showed that pooled cases had 50% loss of peak clusters that can be detected in individual samples. Also, they suggested that low abundant proteins, even when represented in a majority of individual samples, may still be lost during pooling (Sadiq and Agranoff, 2008).

5.1.2 MS/MS result and candidate proteins

This study showed the benefit of proteomic profiling in identifying potential biomarker candidates in serum using small discovery sets of selected samples of similar clinical feature, with both pre- and post-operative sera. However, we obtained a few candidate proteins when compared between pre- and post-operative sera but more candidates were obtained from pre-operative sera against healthy sera. Totally 23 candidate proteins were identified among all analysis. The functions, post translational modifications (PTM), cellular component, GI number, theoretical and observed molecular mass (Mr)/Isoelectric point (pI), percentage of sequence coverage, number of matched peptide, and protein score of each candidate protein were showed in Table 5-1.

Of these proteins, afamin was moreexpressed in CCA pre-operative sera than healthy sera in all of IPAP, WD, and all type analysis. The different appearance of afamin was 2 isoforms with Mr/pI of ~ 80kDa/5.4, with ratio (-1.71) – (-8.38) (for demonstrated picture see Figure 5-1).

Table 5-1 Information of all candidate proteins from MS/MS results.

Protein	Function(s)	Post Translational Modification(s)	Cellular component	GI no.	Theoretical Mr/pI	Observed Mr/pI	Sequence coverage	Matched peptide	Protein score
CD5 antigen-like	regulation of the immune system, inhibitor of apoptosis	Disulfide bond	Secreted	gi|5174411	39603/ 5.28	43kDa/5.6	53%	16	727
Apolipoprotein M	lipid transport	Disulfide bond Glycoprotein	HDL, Secreted	gi|55961583	13327/7.66	20kDa/5.7	24%	4	122
Ceruloplasmin	iron transport	Disulfide bond, Glycoprotein, Phosphoprotein	Secreted	gi|119599289	123779/5.46	130kDa/5.4	11%	14	359
Haptoglobin	cellular iron ion homeostasis, defense response, proteolysis	Disulfide bond, Glycoprotein	Secreted	gi|47124562, gi|119579599	41518/7.46	40kDa/5.5	45%	18	467
Antithrombin-III	regulates the blood coagulation	Disulfide bond, Glycoprotein, Phosphoprotein	extracellular space, plasma membrane	gi|52695711	49454/5.95	50kDa/5.4	24%	13	217

Table 5-1 Information of all candidate proteins from MS/MS results (Cont.).

Protein	Function(s)	Post Translational Modification(s)	Cellular component	GI no.	Theoretical Mr/pI	Observed Mr/pI	Sequence coverage	Matched peptide	Protein score
Alpha-1-antitrypsin	Irreversibly inhibits trypsin, chymotrypsin and plasminogen activator	Glycoprotein	Extracellular matrix, Secreted	gi|13787109, gi|226192646, gi|253723069, gi|196049621, gi|11514321, gi|83754916, gi|7245932, gi|28948408, gi|157831596, gi|15080499	42817/5.40	53kDa/5.4	35%	20	549
Alpha-2-macroglobulin	inhibit all four classes of proteinases	Disulfide bond Glycoprotein Isopeptide bond Thioester bond	Secreted	gi|177872, gi|224053	116697/5.71	110kDa/6.0	7%	7	179
Interleukin-1 beta	Inflammatory response	-	Secreted	gi|119594012	22774/5.61	18kDa/5.2	11%	6	67

Table 5-1 Information of all candidate proteins from MS/MS results (Cont.).

Protein	Function(s)	Post Translational Modification(s)	Cellular component	GI no.	Theoretical Mr/pI	Observed Mr/pI	Sequence coverage	Matched peptide	Protein score
Apolipoprotein A-I	Cholesterol metabolism, Lipid metabolism, Lipid transport, Steroid metabolism	Glycation, Glycoprotein, Lipoprotein, Palmitate, Phosphoprotein,	Amyloid, HDL, Secreted,	gi|4557321, gi|90108664	29410/5.42	25kDa/5.3	46%	20	552
Serum amyloid A protein	Acute phase	Methylation	Amyloid, HDL, Secreted,	gi|40316910	13581/6.28	10kDa/6.1	54%	7	298
Retinol-binding protein 4	Sensory transduction, Transport, Vision,	Disulfide bond	Secreted	gi|296672	13290/7.71	20kDa/5.5	23%	6	180
Alpha-2-HS-glycoprotein	Mineral balance	Disulfide bond, Glycoprotein, Phosphoprotein	Secreted	gi|119598593	39970/5.43	70kDa/5.1	16%	6	170

Table 5-1 Information of all candidate proteins from MS/MS results (Cont.).

Protein	Function(s)	Post Translational Modification(s)	Cellular component	GI no.	Theoretical Mr/pI	Observed Mr/pI	Sequence coverage	Matched peptide	Protein score
Transthyretin	Transport	Gamma-carboxyglutamic acid, Glycoprotein	Amyloid, Cytoplasm, Secreted	gi|14719497	12671/5.26	14kDa/5.7	22%	5	162
Vitamin D-binding protein	Transport	Disulfide bond, Glycoprotein	Secreted	gi|455970	54513/5.34	40kDa/5.7	20%	15	343
Serotransferrin	Ion transport, Iron transport, Transport	Disulfide bond, Glycoprotein, Methylation, Phosphoprotein	Secreted	gi|115394517, gi|110590599, gi|49258810, gi|119599572	60461/6.91	46kDa/6.9	20%	15	385
Alpha-1B-glycoprotein	Interacts with CRISP3	Disulfide bond Glycoprotein	Secreted	gi|69990	52479/5.65	70kDa/5.4	31%	18	474
Apolipoprotein A-IV	Lipid transport, Transport	Phosphoprotein	Chylomicron, HDL, Secreted	gi|178779	43358/5.22	44kDa/5.5	59%	37	942

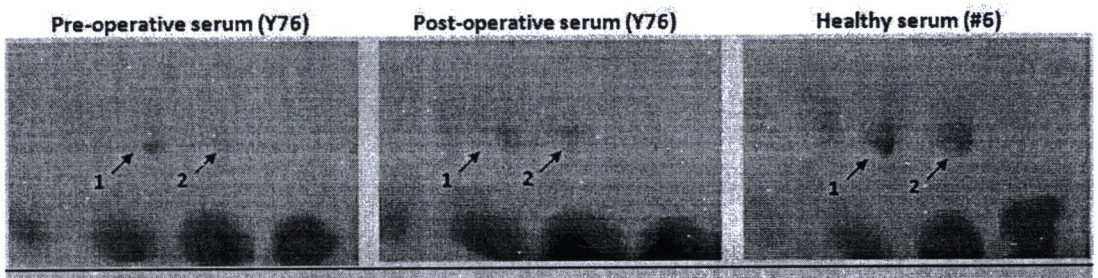
Table 5-1 Information of all candidate proteins from MS/MS results (Cont.).

Protein	Function(s)	Post Translational Modification(s)	Cellular component	GI no.	Theoretical Mr/pI	Observed Mr/pI	Sequence coverage	Matched peptide	Protein score
Glutathione peroxidase 3	Protects cells and enzymes from oxidative damage	The N-terminus is blocked	Secreted	gi|404108	16760/8.93	20kDa/6.0	57%	12	378
Zinc-alpha-2-glycoprotein	antigen processing and presentation, cell adhesion, immune response, lipid catabolic process, negative regulation of cell proliferation	Disulfide bond, Glycoprotein, Pyrrolidone carboxylic acid	Secreted	gi|58176763, gi|4699583	32104/5.70	41kDa/5.0	36%	13	438
Afamin	Transport	Disulfide bond, Glycoprotein	Secreted	gi|4501987	70963/5.64	80kDa/5.4	17%	14	356

Table 5-1 Information of all candidate proteins from MS/MS results (Cont.).

Protein	Function(s)	Post Translational Modification(s)	Cellular component	Database GI no.	Theoretical Mr/pI	Observed Mr/pI	Sequence coverage	Matched peptide	Protein score
CAG-ISL 7	rRNA processing, ribosomal large subunit biogenesis, translational elongation	Acetylation, Phosphoprotein	cytosolic large ribosomal subunit	gi|3126984	23389/10.94	45kDa/5.6	37%	14	73
Serine/arginine-rich splicing factor 6	mRNA processing, mRNA splicing	Acetylation, Phosphoprotein	Nucleus	gi|62087532	32074/10.95	35kDa/4.8	23%	11	101
PP2A B subunit isoform B56-delta	nervous system development, signal transduction	Phosphoprotein	Cytoplasm, Nucleus	gi|62089308	59526/7.01	60kDa/6.6	14%	12	69

Afamin is the newest member of the albumin family that included albumin, α-fetoprotein, and vitamin D-binding protein. It has a predicted mass of ~ 65kDa and apparent molecular mass of 87kDa due to N-glycosylation. Afamin presents in plasma/serum, cerebrospinal fluid, and follicular fluid with at least three different forms in the 5.05 to 5.25 isoelectric point ranges. In 2007, Jackson had studied the expression of afamin in ovarian cancer patients by proteomic-based approaches. They found the significant decrease of total afamin concentration in patients with ovarian cancer compared with healthy controls (P = 0.002) and patients with benign disease (P = 0.046) (Jackson et al., 2007). Back to 2000, Wu had studied afamin in human hepatoma. They found that, afamin was significantly reduced in hepatocellular carcinoma (HCC) tumor tissue as compared with a paired peritumor tissue from 16 patients in both mRNA and protein levels. Afamin was also shown to be significantly down-expressed in HCC cell lines (Wu et al., 2000).

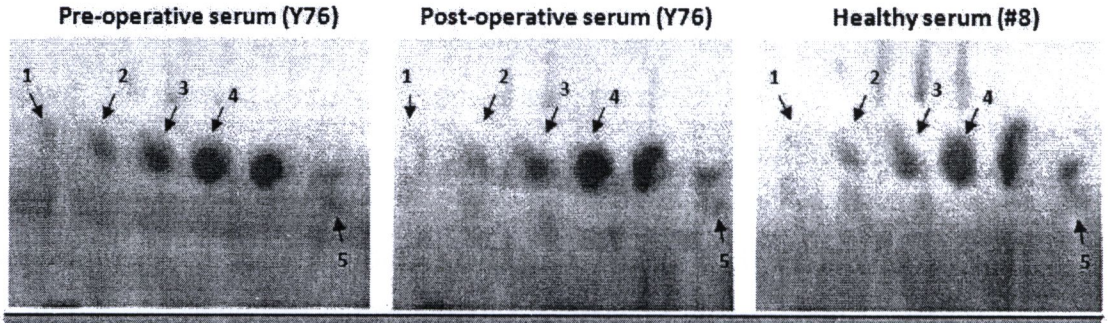


Spot No.	$Volume_{av}$		Ratio	Frequency (CCA/HE)	
	CCA	Healthy		CCA	Healthy
1	0.030623	0.118604	-3.87	6/6	10/10
2	0.013543	0.105989	-7.83	6/6	10/10

Figure 5-1 The expression level of AFM in all groups.

Alpha-1-B-glycoprotein, a protein with unknown function that present in human plasma/serum (Ishioka et al., 1986), belongs to the immunoglobulin supergene family because it exhibited sequence similarity to the other members of the family such as the receptor for transepithelial transport of IgA and IgM and the secretory component of human IgA. We found the expression level of this protein was increase in CCA patients when compared with healthy subjects, with ratio 1.16 – 2.73 fold (for demonstrated picture see Figure 5-2). This protein has molecular mass of ~ 63kDa and consists of the single polypeptide that has N-linked glycosylation to

four glucosamine oligosaccharides. Alpha-1-B-glycoprotein presents in normal adult serum at an average concentration of 22 mg/dl. However, alpha-1-B-glycoprotein is one of human plasma glycoproteins with unidentified physiological function, they have been highly purified and have been characterized by physicochemical methods.



Spot No.	Volume$_{av}$		Ratio	Frequency (CCA/HE)	
	CCA	Healthy		CCA	Healthy
1	0.067331	0.024696	2.73	5/6	10/10
2	0.182120	0.119187	1.53	6/6	10/10
3	0.444081	0.307423	1.44	6/6	10/10
4	0.911687	0.784254	1.16	6/6	10/10
5	0.112281	0.053298	2.11	6/6	10/10

Figure 5-2 The expression level of A1BG in all groups.

In 2004, Udby had studied the association of alpha-1-B-glycoprotein with human cysteine-rich secretory protein 3 (CRISP-3), a plasma protein (28 kDa) that believed to play role in innate immunity (Udby et al., 2004). They found that CRISP-3 is a specific and high-affinity ligand of alpha-1-B-glycoprotein with a dissociation constant in the nanomolar range as evidenced by surface plasmon resonance. Also, they suggested that alpha-1-B-glycoprotein–CRISP-3 complex displays a function in protecting the circulation from a potential harmful effect of free CRISP-3. Moreover, in 2008, Tian had investigated new biomarkers for pancreatic ductal adenocarcinoma (PDAC) patient by proteomic base method (Tian et al., 2008). They found matrix metalloproteinase-9, oncogene DJI, and alpha-1-B-glycoprotein are up-regulated in patient when compared with cancer-free control. They also confirmed the result by western blot and immunohistochemical analysis, alpha-1-B-

glycoprotein was found to 0.86 fold higher express in pancreatic cancer tissues higher than normal tissues.

However, there are other proteins such as antithrombin, which consistently found high level of expression in CCA group when compared to healthy subjects. Moreover, apolipoprotein A-IV, glutathione peroxidase 3, macroglobulin alpha 2, and transferrin also have decreased the level of expression in CCA group like each other (Table 5-2).

5.2 Conclusions

Six CCA subjects were selected for analysis of their serum proteins in both pre- and post-operation. The CCA subjects were divided into 2 groups; 3 CCA IPAP type and 3 CCA WD type. In each group, 2D patterns of pre-operative sera were compared with post-operative sera. Also, patterns from both groups were also compared. Moreover, sera from 10 healthy were compared with those of 6 CCA subjects. Only statistical significantly different spots were picked up to identify by LC-MS/MS. From this study, several observations and analysis results can be concluded as follow:

(1) Albumin and immunoglobulin were almost completely removed from crude sera. The final fraction of treated serum gave the better pattern of protein than crude serum. The low abundant proteins were concentrated and gave a better chance to identify the new protein markers in serum for CCA patients.

(2) Two-DE analysis of serum proteins showed some specific characteristic of CCA patterns.

(2.1) When compared between pre- and post-operative sera, RBP was significantly higher expressed in pre-operative group for all 3 groups analysis (IPAP, WD, and all type) with ratio +1.74 - +2.36 (P<0.01). Antithrombin was higher expressed in pre-operative group for only IPAP type with ratio +2.26 - +6.82 (P<0.01). Moreover, apolipoprotein A-IV was higher expressed in post-operative group for both IPAP and all type analysis with ratio around +1.64 - +2.40 (P<0.01).

Table 5-2 The frequency of expression for all candidate proteins that have consistently expressed.

Protein name	No. of spot(s)	Expression in CCA group (frequency)	Expression in healthy group (frequency)	Frequency of expression change (%)	
				CCA	Healthy
Antithrombin	1	Up (6/6)	Normal (10/10)	100% (6/6)	20% (2/10)
Alpla-1-B-glycoprotein	5	Up (5-6/6)	Normal (10/10)	50-100% (3-6/6)	0-40% (0-4/10)
Afamin	2	Down (6/6)	Normal (10/10)	100% (6/6)	0% (0/10)
Macroglobulin alpha 2	1	Down (6/6)	Normal (8/10)	67% (4/6)	20% (2/10)
Apolipoprotein A-IV	1	Down (6/6)	Normal (10/10)	83% (5/6)	10% (1/10)
Glutathione peroxidase 3	1	Down (6/6)	Normal (10/10)	67% (4/6)	20% (2/10)
Transferrin	4	Down (6/6)	Normal (7-10/10)	83-100% (5-6/6)	10-30% (1-3/10)
		Expression in CCA pre-operative group	**Expression in CCA post-operative group**	**Frequency of expression change (%)**	
				CCA pre-operation	**CCA post-operation**
Apolipoprotein A-IV	1	Down (6/6)	High (6/6)	83% (5/6)	33% (2/6)

(2.2) When compared between pre-operative and healthy sera. HP protein, alpha-1-antitrypsin and Zn-alpha-2-glycoprotein were differently expressed in many isoforms, and had most vary expression in CCA sera because they had both increase and decrease expression all together. The interesting proteins were antithrombin and alpha-1-B-glycoprotein, that showed higher expression level in pre-operative group for all 3 groups analysis with ratio (+1.92) – (+6.68) and (+1.16) – (+2.73), respectively. Afamin and apolipoprotein A-IV were constantly found to have lower expression in pre-operative group for all 3 groups analysis with ratio (-1.71) – (-8.38) and (-1.46) – (-2.00), respectively.

(3) Two candidate proteins, afamin and alpha-1-B-glycoprotein, were further validated with more patient's sera to explore the clinical values of this study.

5.3 Future works

(1) To further verify afamin and alpha-1-B-glycoprotein using immunoblotting.

(2) To test for diagnostic values of the candidate proteins by increasing number of CCA cases and healthy subjects into cohort study.