Original article **P66**

Identification of protein targets for development of diagnostics and chemotherapeutics for cholanigiocarcinoma

Pramote Sriwanitchrak[1*], Wanna Chacharoenkul[1], Petcharin Srivatanakul[2], Kesara Na-Bangchang[1], Hans Rudi Gram[1], Vithoon Viyanant[1]

[1] Graduate Program in Biomedical Sciences, Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand
[2] National Cancer Institute of Thailand
* Presenting author

Abstract

Cholangiocarcinoma (CA) is a rare but devastating neoplasm that accounts for about 3% of all gastrointestinal cancers and 15% of all primary liver cancers worldwide. The lack of early detection and limited therapeutic options are major problems in controlling CA.
This study was aimed to identify differentially expressed proteins in plasma obtained from CA patients (n=30) and healthy subjects (n=30). Protein patterns and spots were identified in pooled and each individual sample from each group using two-dimensional gel electrophoresis (2-DE) and mass spectrometry. There were a number of protein spots which were either up- or down-regulated in pooled and individual plasma samples from CA patients. Five spots with up-regulation of proteins were focused on for further analysis, one of which was identified as α_1-antitrypsin. The up-regulation of α_1-antitrypsin was observed in all CA patients (10/10), and the spot density was significantly higher in these patients than the control group (75.49 *vs* 19.98). Apart from α_1-antitrypsin, it is noteworthy that among the down-regulated protein spots, one spot identified as transferrin, had markedly low expression in CA patients. Study to investigate the association between the up- and down-regulation of α_1-antitrypsin and transferrin, and the levels of both proteins in plasma/serum of patients is underway.

Keywords: Cholangiocarcinoma, proteomics, two-dimensional gel electrophoresis

Introduction

Cholangiocarcinoma (CA) is an adenocarcinoma arising from cholangiocyte, the epithelial cell lining the bile duct apparatus. Several epidemiologic studies have demonstrated an increase in the incidence of CA in Southeast Asia (1). Infection with liver fluke *Opisthorchis viverini* (OV) has been identified as one significant factor associated with the risk of development of CA (2). OV is endemic in Southeast Asia, particularly in the northeastern part of Thailand, where the daily habit of eating raw and salt-fermented freshwater fish repeatedly exposed this local population to both OV and nitrosamine-contaminated food. The prevalence of OV infection is up to 70.8%, and the incidence of CA in this region is up to 317.6 *per* 100,000 person-years (3). The lack of early detection and limited therapeutic options are major problems ib controlling the disease. Even those with operable tumor, the recurrence rate was extremely high, with a 5-year survival rate of less than 40% (4). At present, there is no effective tool or specific biomarkers that can predict the early stage and status of CA. Established serum tumor markers include carbohydrate antigen 19.9 (CA19.9) and carcinoembryonic antigen (CEA); however, these markers are not always helpful, with sensitivities of approximately 70% and 50%, respectively. A specific protein marker for either early detection or monitoring of the tumor would significantly improve the prognosis and therapeutic management of such patients. In addition, information of the pattern of proteins that were up- or down-regulated in CA patients would be exploited for

development of chemotherapeutics that act to inhibit the growth of this cancer. The objective of this study was to identify differentially expressed proteins in plasma obtained from CA patients and healthy subjects by using two-dimensional gel electrophoresis (2-DE) and mass spectrometry.

Methods

Patient Samples: A total of 60 plasma samples collected from patients with CA (n=30) and healthy subjects (n=30) were included in the analysis. Sample collection was performed at The National Cancer Institute of Thailand. The study protocol was approved by the ethics committee of the ministry of public health of Thailand. Written informed consents were obtained from all subjects before sample collection. The diagnosis of CA was based on abdominal ultrasound and serological. We firstly investigated protein patterns from the pooled plasma samples to provide an overall picture of the protein profiles of samples from both groups. Equal volume of plasma from each subject was pooled for each group (case and control). The pooled plasma samples were depleted for removal of high abundant protein by using a Proteome Lab IgY-12 Spin Column from Beckman Coulter. The depleted pooled plasma sample was then applied to a Microcon YM-3 (YM-3, MWCO 3 KD, Millipore MA) and centrifuged at 5,000 rpm ($4^{O}C$). The supernatant was then filtered by using 2-D Clean-Up**.** Total protein content of depleted plasma samples was determined using the DC Protein assay (Bio-Rad) with bovine serum albumin (BSA) as a standard protein. In the second step, proteomic profile of each individual plasma sample in both groups (10 each) was examined. The procedures followed that described above for the pooled plasma samples.

Two-Dimensional Gel Electrophoresis (2-DE): For 2-DE, each 100 µg of depleted plasma was exchanged with sample buffer (8 M urea, 2 M thiourea, 2%CHAPS, 65 mM DTT, 0.5% ampholyte). After dilution to 125 µl with rehydration buffer (8 M urea, 4% CHAPS, 65 mM DTT, 0.001% bromphenol blue, 1% Bio-Lyte ampholytes), the samples were loaded onto IPG strips (7 cm, covering the pH ranges 3-10 NL; Bio-Rad) for isoelectric focusing using the PROTEAN IEF cell. After rehydration overnight at room temperature, the voltage/time profile applied as as follows: 250 V for 15 min, 4,000 V for 1 hour, and then 4,000 V to total of 15,000 V-hr. After equilibration with a solution containing 6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2%(w/v) DTT, IPG strips were equilibrated again with the same solution containing 2.5% (w/v) iodoacetamide instead of DTT. IPG strips were placed on top of 12% SDS- PAGE for second dimension separation will follow by 200 V for 45 min with Mini-PROTEAN 3 Electrophoresis.

Protein identification by MALDI-TOF-MS: The protein spots from either the pooled plasma or individual plasma sample from both groups were used for identification of the proteins by peptide mass fingerprinting by MALDI-TOF-MS. Analysis of selected protein spots was performed using PDQuest^{TM} software version 7 (BioRad, USA). Peptides identified was investigated with the MASCOT search engines (http://www. matrixscience.com).

Results

Figure 1 shows protein patterns separated by 2-DE, of pooled plasma samples of healthy subjects (control group) and CA patients. There were a number of protein spots which were either up- or down-regulated in pooled plasma samples from CA patients. Table 1 shows examples of six protein spots which were identified by MALDI-TOF. Figure 2 presents the analysis of separated spots from pooled plasma samples of CA patients using PDQuest^{TM} software. Among these, five spots (ID 1375, 1427, 1512, 1324, 1368) with up-regulation of proteins were focused on for further analysis (Table 2). The first spot was identified as α_1-antitrypsin, whereas for the other four spots, identification was not possible

due to poor resolution from the nearby spots. The ratio of protein density in pooled plasma collected from patients to that from healthy subjects were 4.39, 2.51, 2.11, 4.18, and 6.09, respectively. Table 2 shows the mean density of protein spots in each individual plasma samples of both groups. The number in parenthesis indicate the proportion of spots with up-regulation in each group. The up-regulation of the protein spot number 1375 was observed in all samples obtained from patients with CA, and in addition, the spot density was significantly higher ($p < 0.00001$; Mann-Whitney U test) in the patient group than the control group (75.49 *vs* 19.98).

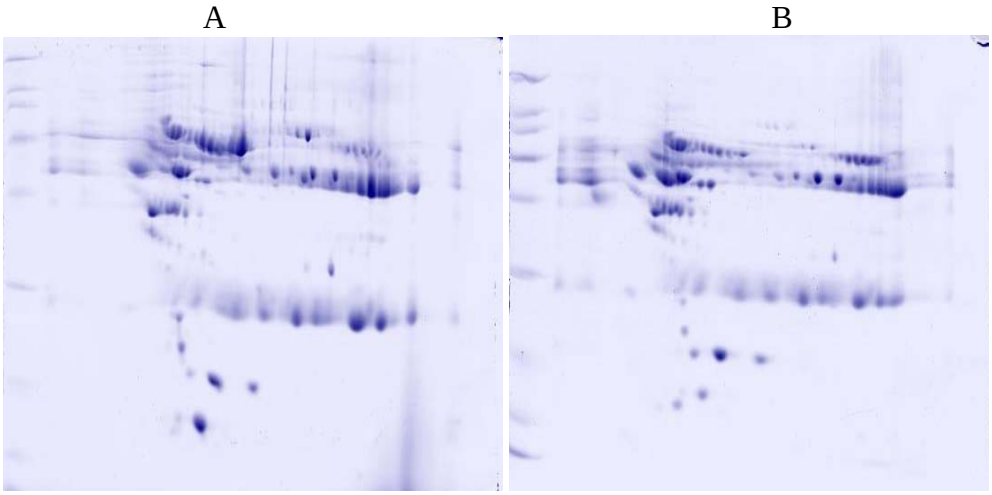

Figure 1 Protein patterns separated from pooled plasma samples of (A) healthy subjects (control group) and (B) CA patients by 2DE

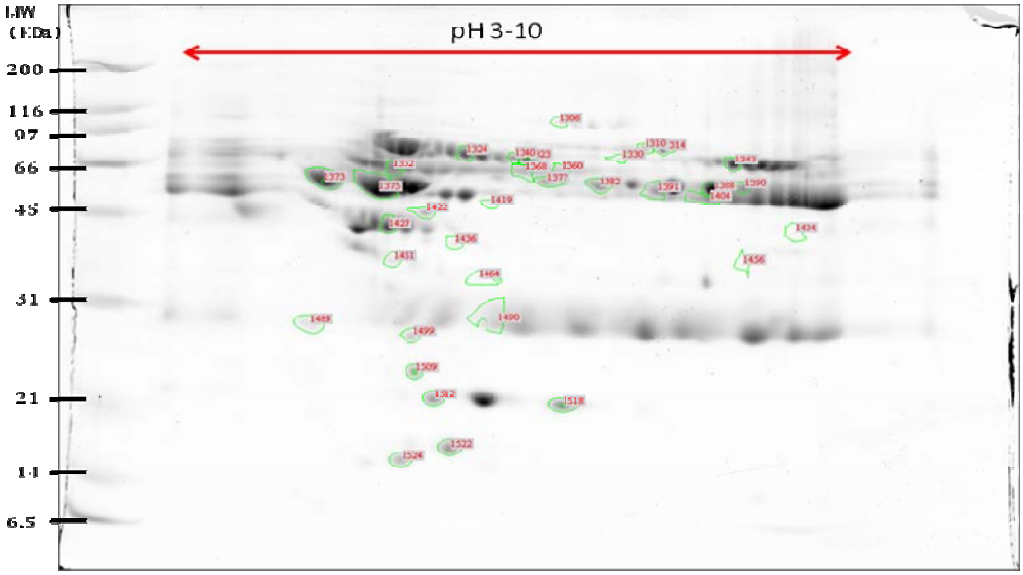

Figure 2 Analysis of separated spots by PDQuest^{TM} software.

Discussion

Our preliminary results demonstrate significantly different protein patterns of plasma from CA patients with a number of protein spots which were either up- or down-regulated. Spot ID 1375 seems prominent in its up-regulation in all plasma samples (10/10), with significantly higher protein density (75.49 *vs* 19.98). This spot was identified by MALDI-TOF as α_1-antitrypsin (A1AT). α_1-antitrypsin is a glycoprotein and generally known as

serum trypsin inhibitor or α_1-proteinase inhibitor (A1PI). It is a serine protease inhibitor (serpin) which inhibits a wide variety of proteases (5). α_1-antitrypsin protects tissues from enzymes of inflammatory cells, especially elastase, and has a reference range in blood of 1.5 - 3.5 gram/liter (in US the reference range is generally expressed as mg/dL or micromoles), but the concentration can rise many folds upon acute inflammation (6). Marked increase in α_1-antitrypsin was also previously reported in CA (7).

Table 1 Examples of spots identified by MALDI-TOF

Spot ID	Protein	Accession No NCBI	Number of matched peptides	MALDITOF-MS sequence coverage (%)	Protein score	MW (kDa)	Pi	Expression
1375	α_1-antitrypsin	151302818	13/27	34	82	46978	53.5	+4.4
1314	Transferrin	553788	12/39	33	90	55207	6.0	-3.2
1321	Hemopexin	11321561	11/41	29	79	52385	6.55	-3.3
1340	Chain A, crystal structure of human serum albumin	3212456	18/53	31	117	68425	5.67	-4.5
1330	RAB39 member RAS oncogene family	39930371	8/40	52	68	25390	7.57	-3.4
1352	Kimimogen 1 isoform 2	4504893	12/51	28	81	48936	6.29	-5.0

Table 2 Mean density of protein spots in each individual plasma samples of both groups.

Spot ID	Density of Protein Spot (Number of sample found/Total sample)	
	Control (n=10)	Cancer (n=10)
1375 (α_1–antitrypsin)	19.98 (8/10)	75.49 (10/10)
1427	16.93 (6/10)	30.0 (6/10)
1512	2.2 (5/10)	8.2 (5/10)
1524	1.6 (5/10)	8.2 (5/10)
1368	1.46 (2/10)	7.59 (2/10)

Apart from α_1-antitrypsin, it is noteworthy that among the down-regulated protein spots, spot ID 1314 identified as transferrin, the iron binding protein, which had markedly low expression in plasma of CA patients. High rate of iron intake has been observed in most cancer cells (8). In addition, the cells also express high cell surface concentration of transferrin receptors (9) which allow binding of iron to the iron transporter holotransferrin. Iron then enters into the cells *via* a receptor-mediated endocytosis process (10). Therefore, the iron storage of tumor cells is generally greater in tumor cells than in normal cells (8). Holo-transferrin and other iron sources have clearly been shown to increase the potency of artemisinin (antimalarial drug with anticancer activity) in killing cancer cells (11). In our recent study however, a controversial result was observed. Pretreatment with holo-transferrin did not alter the cytotoxicity of artemisinin and its derivatives against the CA cell line-- CL6 (12). The possible explanation is that expression of transferrin receptor in CA may be lower than normal cell. In a previous study, the level of transferrin was shown to be significantly lower in intrahepatic CA cell lines when compared with normal liver tissues which is specific for this protein (12). Study to investigate the association between the up- and down-

regulation of α_1-antitrypsin and transferrin, and the levels of both proteins in plasma/serum of patients is underway. Further study to investigate the expression of transferrin receptor in CA cells is required to support this supposition.

Conclusion

Our preliminary results demonstrate significantly different protein patterns of plasma from CA patients with a number of protein spots which were either up- or down-regulated. The roles of the two proteins identified as α_1-antitrypsin (up-regulated) and transferrin (down-regulated) in CA are under investigation.

Acknowledgement

The study was supported by The Commission on Higher Education, Ministry of Education of Thailand.

References

1. Jinawath N, Chamgramol Y, Furukawa Y, Obama K, Tsunoda T, Sripa B, Pairojkul C, Nakamura Y. Comparison of gene expression profiles between Opisthorchis viverrini and non-Opisthorchis viverrini associated human intrahepatic cholangiocarcinoma. Hepatol 2006; 44: 1025-38.
2. Srisomsap C, Sawangareetrakul P, Subhasitanont P, Panichakul T, Keeratichamroen S, Lirdprapamongkol K, Chokchaichamnankit D, Sirisinha S, Svasti J. Proteomic analysis of cholangiocarcinoma cell line. Proteomics 2004; 4: 1135-44.
3. Khuhaprema T, Srivatanakul P . Liver and Bile Duct. In: Khuhaprema T, Srivatanakul P, H S, Wiangnon S, Sumitsawan Y, Attasara P (eds). Cancer in Thailand, 2007, Vol. IV, 1998–2000. Bangkok Medical Publisher, Bangkok pp. 36-8.
4. Thongprasert S. The role of chemotherapy in cholangiocarcinoma. Ann Oncol 2005; 16 Suppl 2: ii93-6
5. Gettins PG. Serpin structure, mechanism, and function. *Chem Rev* 2002; 102: 4751–804.
6. Kushner, Mackiewicz A. *The acute phase response: an overview*. CRC Press, 1993, pp. 3–19
7. Changbumrung S, Migasena P, Supawan V, Juttijudata P, Buavatana T. Serum protease inhibitors in opisthorchiasis, hepatoma, cholangiocarcinoma, and other liver diseases. Southeast Asian J Trop Med Pub Hlth 1988; 19: 299-305.
8. Shterman N, Kupfer B, Moroz C. Comparison of transferrin receptors, iron content and isoferritin profile in normal and malignant human breast cell lines. Pathobiol 1991; 59: 19-25
9. Raaf HN, Jacobsen DW, Savon S, Green R. Serum transferrin receptor level is not altered in invasive adenocarcinoma of the breast. Am J Clin Pathol 1993; 99: 232-7
10. May WS, Jr., Cuatrecasas P. Transferrin receptor: its biological significance. J Membr Biol 1985; 88: 205-15
11. Singh NP, Lai H. Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. Life Sci 2001; 70: 49-56
12. Chaijaroenkul W, Viyanant V, Mahavorasirikul W, Na-Bangchang K. Cytotoxic activities of artemisinin derivatives against cholangiocarcinoma cell line (CL6) (manuscript submitted)
13. Wang AG, Yoon SY, Oh JH, Jeon YJ, Kim M, Kim JM, Byun SS, Yang JO, Kim JH, Kim DG, Yeom YI, Yoo HS, Kim YS, Kim NS. Identification of intrahepatic cholangiocarcinoma related genes by comparison with normal liver tissues using expressed sequence tags. Biochem Biophys Res Commun 2006; 345: 1022-32