CHAPTER VI
SUBSTRACTIVE PROTEOMIC APPROACH TO THE CHOLANGIOCARICINOMA INVASIVE FRONT

6.1 Introduction

The heterogeneity of tumor tissue has been largely illustrated not only in terms of space with miscellany in morphology and biology among different areas of the same tumor, but also in terms of time as a continuous tumor evolution. These evidences are nurturing functional proteomics and genomics as a more realistic perspective in cancer research than a steady-state analysis where molecular profiling of bulk tumor specimens are correlated with carcinogenesis. Direct microscopic visualization of the cells permits the selection of normal, premalignant and malignant cells, or disease and disease-free cells, as distinct cell populations from the heterogeneous tissue. For instance a tissue section comprised of 80% tumor, 10% stroma and 10% infiltrating lymphocytes. In this example the 10% lymphocyte population may contribute more than 10% of the overall signal. A two-fold signal difference is considered significant for quantitative proteomic analysis. There may be greater than two-fold differences in cell numbers between different tissue sections from the same tumor, making comparisons of molecular profiles invalid for heterogeneous, non-micro dissected samples (Imbeaud et al., 2005). The molecular analysis of tumors requires the isolation of specific population of cells due to the presence to contaminating cells within a sample remains a major obstacle to meaningful biological analysis. Laser capture microdissection (LCM) is a recently developed technique that permits the rapid and reliable procurement of a specific type of cell from a tissue section, in one step under direct microscopic visualization.

In this context, one of the areas of a tumor tissue where protein expression has been observed to be dynamically adapted is the invasive front, as a principal component of the interaction between a tumor and the surroundings. This has been evidenced not only on those tumor modifications that are induced by the microenvironment as cytokines or inflammatory mediators, but also on those

associated with the phenotype of the epithelial cells that become invasive. All this has led tumor invasion to become an active and promising field of research. Good examples of the power of LCM are provided by the elegant dissections of areas of tissue that represent discrete stages in breast, oesophageal and prostate tumor development (Palmer-Toy et al., 2000; Paweletz et al., 2000; Simone et al., 2000a), giving researchers the ability to profile minor cell populations and thereby chronicle cancer progression. In this study, we here in combined LCM and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify a suite of proteins up-regulated in invasive front tumor compared to non-invasive area using samples of cholangiocarcinoma (CCA) patients archived at Srinagarind Hospital, Khon Kaen, Thailand where the liver fluke *Opisthorchis viverrini* is endermic.

Aim

To identify a suite of up-regulated proteins from invasive front in 10 paired invasive and noninvasive CCA tissues of confirmed *O. viverrini*-induced CCA patients, laser capture micro-dissection (LCM) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were employed in this study. Differential protein expression analysis was obtained using the isobaric tag for relative and absolute quantitation (iTRAQ) labeling system.

6.2 Materials and Methods

6.2.1 Patients and specimens

In this study, CCA tissues were obtained after informed consent from the patients who underwent hepatectomy at Srinagarind hospital, Khon Kaen University, Thailand as described elsewhere (Yonglitthipagon et al., 2010). Of the 10 liver fluke-associated CCA samples analyzed, 4 were from males and 6 were from females. Patients ranged from 50 to 80 years of age. Most of the patients were at an advanced CCA stage. The protocol was previously approved by the Human Research Ethics committee of Khon Kaen University (IRB No. HE42075), and informed consent was obtained from all of the patients involved in the study. After surgery, each tissue sample was immediately dissected by a pathologist, with superficial and deep tumor sections being collected form each patient, embedded in cryostat mounting medium and frozen in liquid nitrogen before storage at -80°C. Eight-

micrometer sections were cut at -20°C using a cryostat (Leica CM3050S) and mounted onto a sterilized polyethylene-naphthalene membrane on a microscope slide (P.A.L.M. Microlaser Technologies, Germany). The slides were then stored at -80°C until microdissection.

6.2.2 Laser capture microdissection

Staining consisted of sequential incubations in 70% ethanol (1 min), hematoxylin (30 s), Scott's tap water (10 s), 70% ethanol (30 s) and 100% ethanol (2x30 s) with brief water washes between most steps, followed by two final rinses in xylene (5 min each). The 70% ethanol contained complete protease inhibitor cocktail. After 5 min of air-drying, microdissection was performed with a PALM microbeam laser catapult microscope (P.A.L.M. Microlaser Technologies). CCA cells in invasive front and deep tumor of the same patients were dissected from the slides using 1.8 ms duration time. The power was adjusted to the lowest value possible to allow efficient capture (between 70-100 mW). An area of approximately 24 million squared mm (approximately 60,000 cells of CCA) was collected separately from each of the CCA tissues onto 500 ml opaque adhesive caps (P.A.L.M. Microlaser Technologies). The time of microdissection was less than 45 min for each slide to avoid proteolysis. The opaque adhesive caps were kept in -80°C until protein extraction was done (Figure 6.1).

6.2.3 iTRAQ labeling

Each frozen tissue sample was microdissected and samples from each morphological grouping (non-invasive tumor area and invasive front tumor area) were pooled and reduced with tris (2-arboxyethyl) phosphine and alkylated with methyl methanethiosulfonate prior to proteolytic cleavage with trypsin (Figure 6.2). Non-invasive and invasive pooled samples were then labeled with 119 and 121 iTRAQ reagents (4-plex kit, Applied Biosystems), respectively, and subjected to first dimension fractionation on the basis of charge using either strong cation exchange (SCX) chromatographic separation or isoelectric focusing (IEF) off-gel fractionation (Agilent) regarding the protein concentration. Second dimension LC was performed with a C18 reverse-phase (RP) HPLC nano-column.

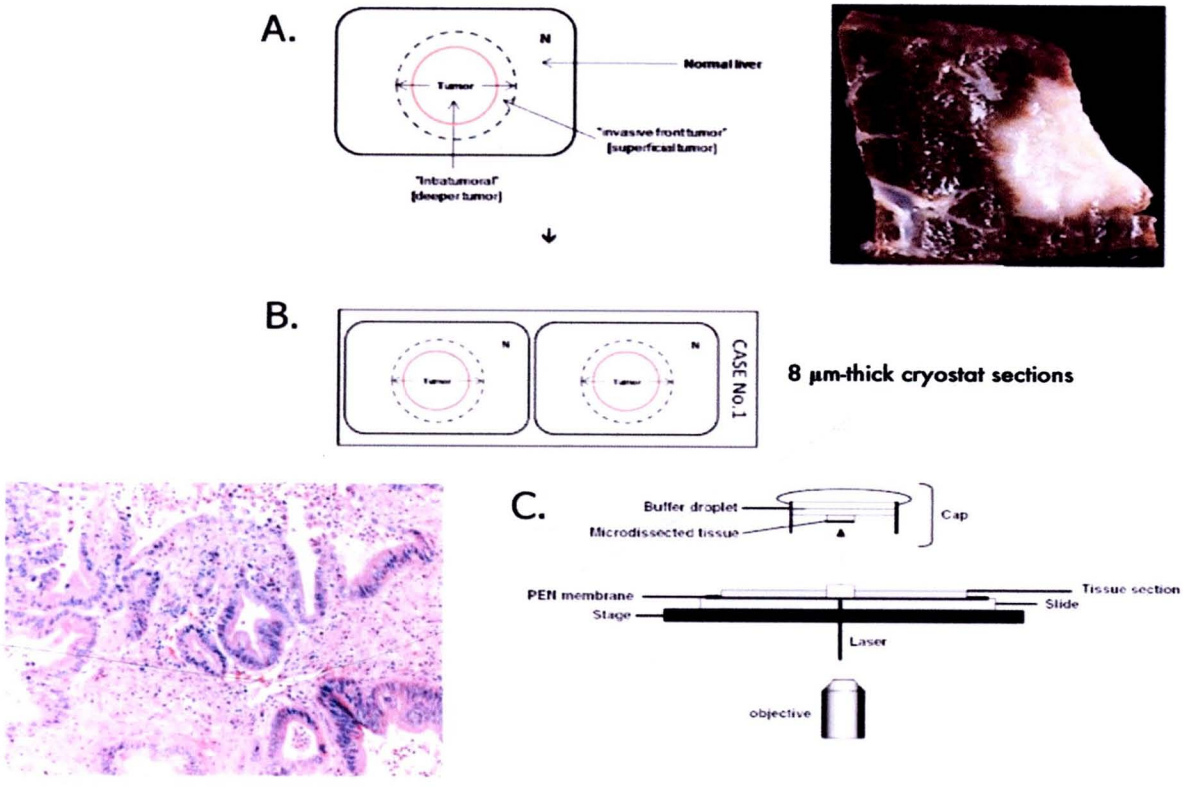

Figure 6.1 Overview of the study design for laser capture microdissection (LCM). (A) For the purposes of this study, two different areas of CCA tissue will be defined; intratumoral and invasive front areas. (B) For microdissection at least two, and in most cases more, 8 µm-thick sections were taken. (C) A PALM microbeam laser catapult microscope was employed to take multiple microdissected elements from each of the defined areas before iTRAQ labeling and MS/MS analysis. In set pictures of whole resected liver and histologic section (hematoxylin stained) of CCA tumor showing cancer cells and other fibrotic and necrotic tissue.

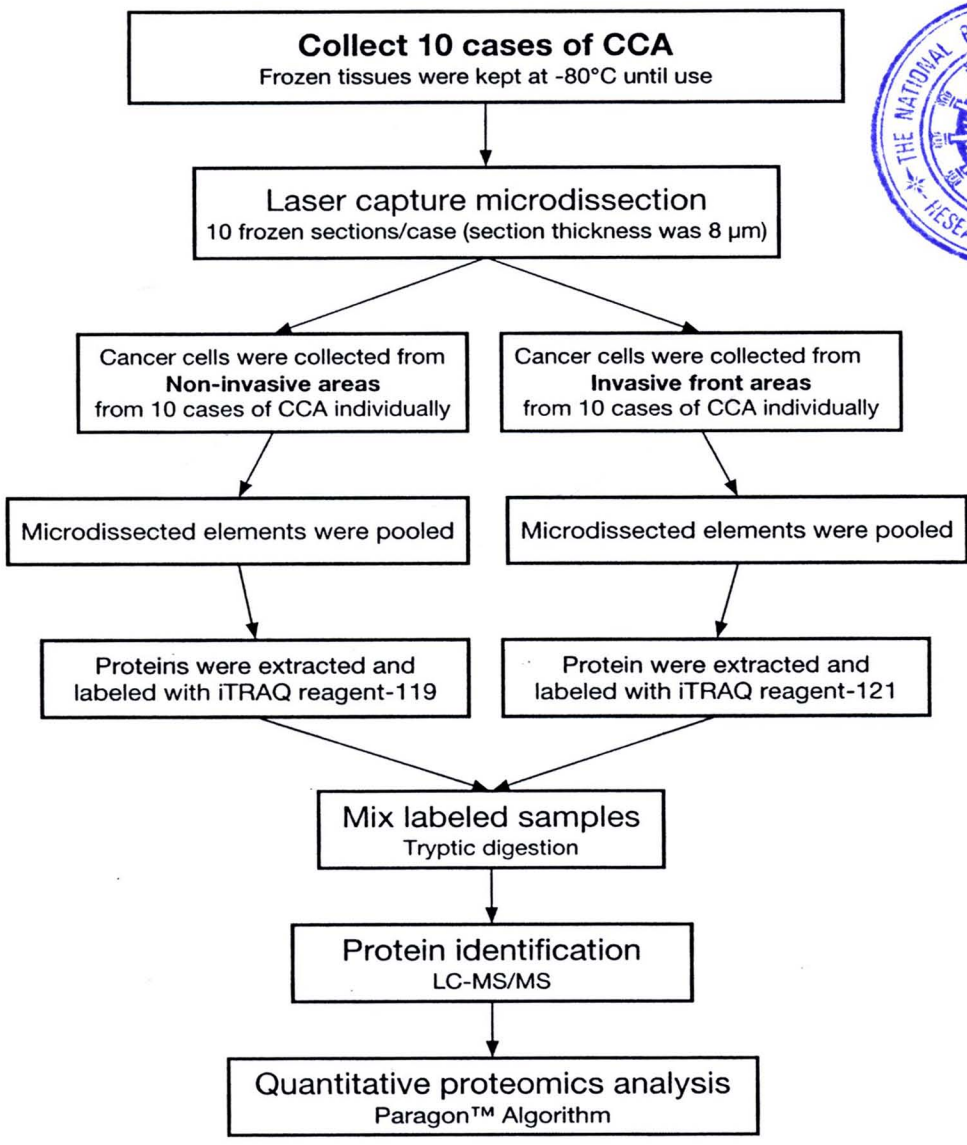

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Figure 6.2 Outline of the strategy used to develop cell specific protein expression analysis integrating laser capture microdissection and proteomics. iTRAQ, isobaric tag for relative and absolute quantitation.

6.2.4 Mass spectrometry and data analysis

Proteins were identified and characterized using MS and MS/MS on a QStar Elite QqTOF and a 4000 QTRAP MS/MS for protein identification. The use of both MS instruments provided complementary data which were combined to produce a more complete proteome map. Data analysis was performed using the ProteinPilot (Applied Biosystems) and MASCOT software packages and the identity

and function of differentially expressed proteins were further investigated by bioinformatic analysis (MASCOT and PANTHER) and review of the literature. Peptides shared among related but distinct proteins were not used for quantitation. Peptide MS/MS for protein identifications inferred from single peptide hits were manually inspected. In all cases except two, protein identifications made from single peptide hits in one-depletion experiment were made with two or more peptides in at least one of the alternative depletion experiments. Proteins were considered over- and under-expression in invasive front of tumor (I) relative to non-invasive area (N) if the iTRAQ® ratio of I/N was >1.6 and < 0.6, respectively. Additionally, the corresponding I/I and N/N ratio was between 0.8 and 1.2 in at least one experiment (Boylan et al., 2010).

6.3 Results and Discussion

6.3.1 Isolation of CCA cells from invasive front and non-invasive tumor area by laser capture microdissection

There are several factors found to be important to ensure satisfactory and consistent microdissection of cells using LCM including very brief air drying of sections onto a sterilized polyethylene-naphthalene membrane on a microscope slides and complete dehydration of sections after completion of the histological staining. Prolonged air drying of sections before staining appeared to inhibit successful LCM, and even trace amounts of moisture present in the sections appeared to inhibit successful LCM, and even trace amounts of moisture present in the sections appeared to inhibit, either partially or completely, the transfer of cells to the plastic film. Thus, complete dehydration of sections in xylene after histological staining was important. Cancer cells in invasive front and non-invasive tumor area were microdissected successfully from the CCA section (Figure 6.3). In this study, the histological procedures used to prepare tissue for LCM involved alcohol fixation, staining with histological dyes, and dehydration in organic solvents, all of which can potentially result in changes in the proteins. In particular, the binding of the stain (hematoxylin) may exert effects on the charge of some proteins although dissociation of such complexes may occur during the denaturation step (Banks et al., 1999). Therefore, tudiline blue as the histological stain should be used in stead of the hematoxylin

because it is a rapid one step staining method; thus minimizing exposure to organic solvents that can alter protein structure during the proteomic procedures (Lawrie et al., 2001).

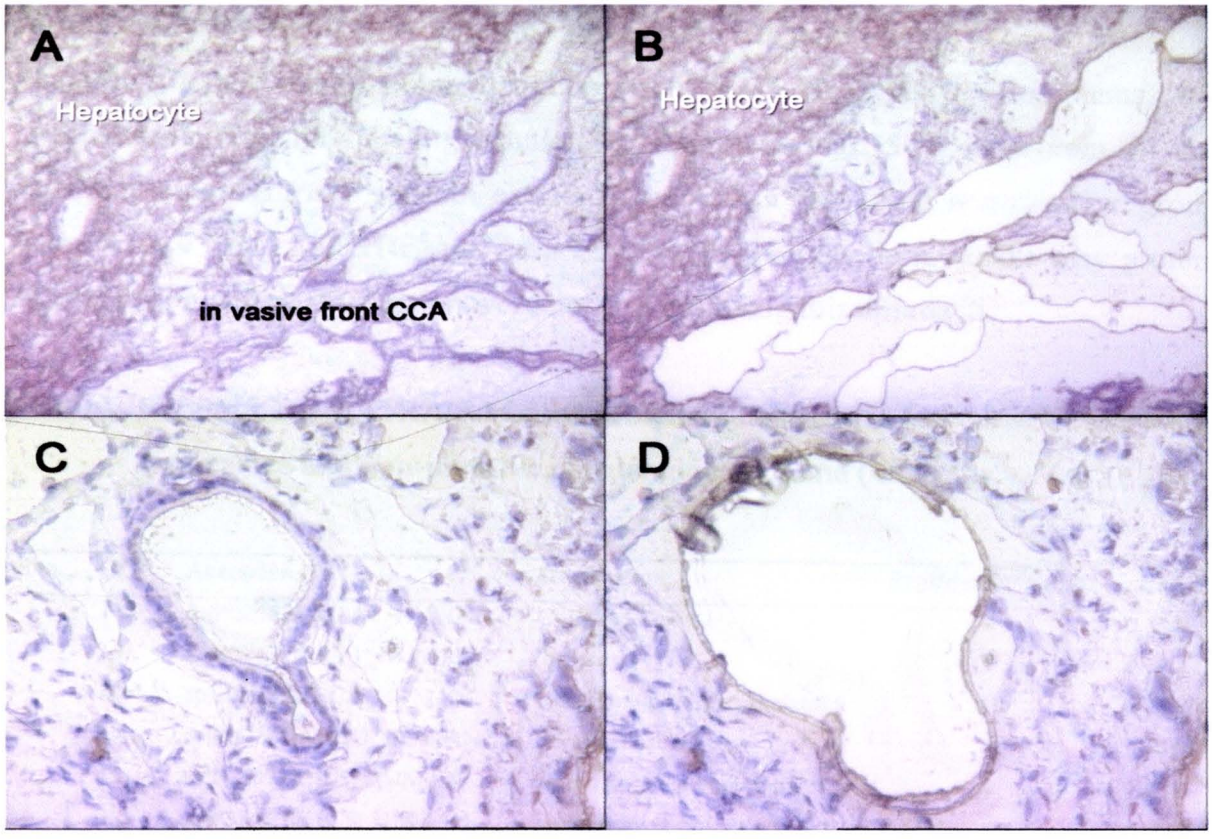

Figure 6.3 Photographs of laser capture microdissection of hematoxylin stained sections of cholangiocarcinoma (CCA). Panels A and C show CCA in invasive front area before microdissection, whereas panels B and D indicate successful microdissection. Original magnification: x100 (A-B) and x400 (C-D).

6.3.2 Quantitative proteomic analysis of CCA cell lysate proteomes by iTRAQ analysis

In this work, a laser capture micro-dissection (LCM) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were used to identify a suite of up-regulated proteins of the invasive front by comparing the invasive area of tumor with the non-invasive area from the same patients represent invaluable

resource for CCA biomarker discovery. Differential protein expression analysis was obtained using the isobaric tag for relative and absolute quantitation (iTRAQ) labeling system. However, using LCM to collect the areas of interest from such frozen CCA tissues provided relatively low amounts of total protein for analysis with LC-MS/MS resulted in valid biomarkers might be present at concentration too low for detection. According to the manufacture instruction of iTRAQ kit (Applied Biosystems), the sufficient material for labeling reaction is required at least 5 μg of protein. In this regard, the concentrated protein extract of pooled invasive and non-invasive group were $6.1x10^{-4}$ and $1.08x10^{-4}$ μg, respectively. This might be a reason why protein identification and quantification were not success using this approach.

Table 6.1 Summary of iTRAQ-labeled proteins identified in ten cases of pair wise invasive and non-invasive choalangiocarcinoma (CCA).

No.	CO[a]	Accession No.[b]	Description	Peptides (95%CI)	I/N[c]
1	13.9	sp|P02452|CO1A1_HUMAN	Collagen alpha-1	6	0.7248
2	19	sp|P07477|TRY1_HUMAN	Trypsin-1	1	-
3	10.4	sp|P05787|K2C8_HUMAN	Keratin, type II cytoskeletal 8	1	0.8188
4	3.4	sp|P02768|ALBU_HUMAN	Serum albumin	1	1.1455
5	15	sp|P68871|HBB_HUMAN	Hemoglobin subunit beta	1	0.6536
6	3.5	sp|P35527|K1C9_HUMAN	Keratin, type I cytoskeletal 9	1	0.7605

Note: [a]Percentage of sequence coverage protein expression in invasive:non-invasive ratio. [b]Swiss prot database accession numbers (http://ca.expasy.org/sprot). [c]I/N = protein expression ratio between invasive and non-invasive areas, when the ratio was absent, reporter tag was unable to be detected by a 4000 QTRAP MS/MS.

In addition, major problem encountered during LCM procedures and proteomic analysis is contamination of the samples with keratin from dust, hair, wool and fingerprints. If the samples are contaminated with human keratin then the database will predominantly identify cytokeratin 1, which was not found in our study as shown in Table 6.1. Furthermore, the identification of cytokeratins 8 and 9 in our preliminary findings is consistent with their known expression in the previous study of CCA using immunohistochemical analysis (Shimonishi et al., 2000).

In the example provided here of invasive front area from 1 case, dissection took approximately 16 h to collect 60,000 CCA cells. We have tried to dissect each tissue section less than 2 h but still it was too long; the proteins began to degrade after approximately 1 h at room temperature (Bonner et al., 1997; Simone et al., 2000b). Therefore, to identify the specific protein components from the areas of interest of CCA tissue, optimal sample collection and sample preservation techniques have been designed to suit the needs of this study. Questions that must be considered include to what the other preservation techniques of the tissue samples also provide enough and high quality of the proteins.

Even though evaluations of protein separation studies from frozen tissues have been demonstrated to be more feasible, formalin-fixed paraffin embedded (FFPE) tissues which are a largely unexplored archive in MS-based proteomics due to the effects of formalin fixation and long-term storage on the samples, has been demonstrated that a significant amount of proteomic information can be extracted from these samples as compared with fresh frozen tissue samples (Hood et al., 2005). Additionally, the identified peptides contained a formylated lysine residue, and the percentage of oxidized methionine residues or peptides containing missed tryptic cleavages were not significantly different than that observed in proteomic studies of fresh cells (Peng et al., 2003; Yu et al., 2004). In this regard, a large number (>300 confirmed CCAs) of FFPE CCA samples are available at the division of experimental pathology. These samples (similarly resected from confirmed CCA cases) can be microdissected and analyzed in the manner described in the methodology section as pooled samples; i.e. samples gathered from the invasive front and intratumoral areas (non-invasive tumor area) of 300 FFPE CCA samples. Although the pooling of samples may result in the loss of sample discriminatory power with regards to differences in the protein expression of individual patients, the availability of paired plasma samples for these samples will allow us to verify biomarkers common to all sampled tumors and hence provide general biomarkers for downstream verification.

In summary, the combination of the technologies of LCM and proteomics may facilitate the systematic analysis of protein expression profiles in specific CCA cells represent in invasive front and non-invasive tumor area. However, further refinements of these technologies to permit the analysis of smaller numbers of cells are required.