CHAPTER VII
CONCLUSIONS AND SUGGESTIONS

Cholangiocarcinoma (CCA), bile duct cancer, is a fatal disease. While the etiology for most cancers, including CCA in the west, remains obscure, it has long been established that the single most important risk factor for CCA in Thailand is infection with the liver fluke *Opisthorchis viverrini (O. viverrini)*. Early diagnosis of CCA can influence the therapeutic strategy by increasing the chance of cure or, at least, prolonged survival. It is equally important to be able to detect prognostic markers for this fatal disease. However, up to date, there are no specific markers for CCA. Hence, there is an urgent need for molecular diagnostic and prognostic markers of CCA. There are several important reasons for focusing on the analysis of proteins: (1) mRNA expression may not correlate with the amount of active protein in a cell, (2) the gene sequence does not describe posttranslational modifications that may be essential for protein function and activity, and (3) the study of the genome does not provide information on dynamic cellular processes (Anderson et al., 1997). The application of proteomics can be expected to provide an integrated view of an individual disease process at the protein level. This is particularly important for CCA because proteomics can be expected to show changes in the protein expression profile occurring during tumor development and progression, thus leading to the identification of new molecular diagnostic and prognostic markers for CCA.

An older and still commonly used approach for proteomic quantification is the combination of two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). In this study, we employed 2-DE followed by MALDI-TOF MS to identify differentially expressed proteins in four CCA cell lines; K100 (poorly differentiated adenocarcinoma), M156 (moderately differentiated adenocarcinoma), M213 (adenosquamous cell carcinoma) and M139 (squamous cell carcinoma), compared to a control normal biliary cell line, H69. This approach was separately applied to both membrane and cytosolic proteomes of the cell lines. Among 20 up-regulated

membrane proteins identified in the cell lines, the potential of annexin A2 (ANXA2), a participant in tumor invasion and metastasis in several cancers, for facilitating the diagnosis or prognosis of CCA was investigated. ANXA2 was found to associate with one of several tumor progression stages as reflected by lymphatic invasion and metastasis using immunohistochemical (IHC) analysis in tissue microarrays (TMAs) of human CCA (n = 301). Patients with high expression of ANXA2 where found to have a significantly worse prognosis, suggesting that up-regulation of ANXA2 is not particularly effective as a diagnostic marker for CCA, but is an effective marker for poor prognosis.

When the strategy was applied to the cyotosolic proteomes, we identified 48 differentially expressed proteins, three of which including peroxiredoxin 1 (PRX1), ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) and enolase 1 (ENO1) were subsequently selected for further validation using IHC in the TMAs because these proteins had not been previously studied in CCA. Firstly, under-expression of PRX1 whose function has been implicated in regulating cell proliferation, differentiation and apoptosis (Cha et al., 2009) was correlated with perineural and lymphatic invasion and poor prognosis. Secondly, EBP50 has been previously reported to act as tumor suppressor or an oncogene regarding its distribution (Shibata et al., 2003; Cardone et al., 2007). For instance, up-regulation of EBP50 in the cytoplasm and/or nucleus can break up complexes with PTEN or β-catenin resulted in separating signaling molecules away from the plasma membrane accordingly exerting tumor progression (Georgescu, 2008). Consistent with the previous studies, immunoaffinity-based verification of EBP50 revealed high levels of expression in the cytoplasm of cancerous tissue over normal bile duct. Moreover, elevated expression of EBP50 was associated with lymphatic and vascular vessels and worse prognosis. Finally, changes in energy metabolism are fundamental properties of cancer cells by which the cells survive in a state of hypoxic stress followed by promotion of angiogenesis and enhancement of local invasion and/or distant metastasis. ENO1, a glycolytic enzyme involving in tumor progression in several cancers, was exclusively expressed in the cytoplasm and membrane of CCA tissue and bile duct hyperplasia compared to corresponding normal bile duct. In addition, up-regulation of this protein

was significantly associated with poor prognosis. These findings led us to strengthen the prognostic potential of PRX1, EBP50 and ENO1 for poor prognosis.

To quantify protein expression levels, we have employed isobaric tagging for relative and absolute quantitation (iTRAQ), a system that measures the relative abundance of isotope coded tags covalently attached to free amines in peptide mixtures. Using this technique we performed a quantitative profiling in CCA cell lines OCA17 (well differentiated adenocarcinoma), M156 (moderately differentiated adenocarcinoma) and K100 (poorly differentiated adenocarcinoma), compared to the normal control, H69. Three proteins of particular interest were found in the iTRAQ experiments and the differential expression was confirmed for cathepsin D (CATD), catenin delta 1 (CTND1) and transgelin 3 (TNGL3) using immuno-localysation by IHC. Over-expression of CATD and CTND were observed in both bile duct hyperplasia and CCA tissues but not in normal liver. In contrast, the expression of TNGL3 was not detected in bile duct tissues under normal condition and in CCA. Apart from these proteins many other unique proteins identified using this strategy have not previously been reported to associate with CCA. Thus, the results presented here will provide important leads for biomarker discovery in CCA.

Finally, given tumor invasion defines the transition between tissue-restricted CCA, and is related to poor prognosis and a dramatic decrease in survival, in this thesis laser capture microdissection (LCM) was used to characterize specific components of the tumor invasive front, by comparing the invasive area of CCA with the noninvasive tumor area from the same patients. This approach eliminates many of the problems associated with the heterogeneity of clinical tumor tissues by controlling for differences in protein expression between both individual patients and different cell types. Microdissected elements of invasive and non-invasive tumor area were pooled into two groups for protein extraction and tryptic digestion. The peptides were quantified and identified using iTRAQ analysis and LC-MS/MS method, respectively. Unfortunately, protein yields employing LCM approach did not achieve identification and quantification of the proteomes due to the yield did not reach the minimal requirement of iTRAQ manufacture instruction.

In summary, the proteomic techniques developed in this thesis have uncovered ANXA2, PRX1, EBP50 and ENO1 in CCA cell lines that can serve as prognostic

markers for the patients with CCA. Furthermore, the other differentially regulated proteins found in this study provide an important first step towards developing effective biomarkers for diagnosis and/or predicting prognosis of CCA.

Future direction, it will also important to validate candidate biomarkers identified in this thesis in a large population of individual serum samples, and to include samples from patients with early stage CCA, benign and as well as healthy controls using targeted mass spectrometry (Multiple Reaction Monitoring; MRM) of plasma from confirmed CCA patients (paired with tumor samples) to identify peptides derived from the candidate biomarkers to form the basis for a suite of biomarkers putatively specific for CCA. The panel of potential CCA biomarkers identified will be screened against plasma of patients with advanced periductal fibrosis (cases), which is a precursor stage to *O. viverrini*-induced CCA, and matched controls from our longitudinal study of the *O. viverrini*-related pathogenesis.