RESEARCH ARTICLE

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A Proteomics and Metabolomics-based Approach to Biomarkers Identification for the Early Diagnosis of Cholangiocarcinoma in a Hamster Model: A Preliminary Study

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Abstract

Background: Cholangiocarcinoma (CCA) poses a significant public health challenge, particularly in northeastern Thailand, where its prevalence is the highest worldwide. Early detection and effective treatment remain challenging due to the absence of sensitive and specific biomarkers. This study aimed to identify novel plasma proteins and metabolites as potential biomarkers for the early diagnosis of CCA. **Methods:** Plasma samples from hamsters with *Opisthorchis viverrini* (OV)/dimethylnitrosamine (DMN)-induced CCA and healthy controls (n=3 per group) were analyzed by LC-MS/MS-based proteomics and metabolomics. **Results:** More than 5,000 proteins were identified, including 572 unique to CCA hamsters at week 12. At weeks 8 and 12, 412 and 545 proteins, respectively, were functionally linked to cell proliferation, signal transduction, and metabolic regulation. Metabolomic analysis revealed 273 metabolites, with 59-including cystathionine, putrescine, UDP-N-acetyl-glucosamine, and FMN, upregulated in the CCA group. **Conclusion:** This study provides preliminary insights into candidate biomarkers for the early detection of CCA. These findings are intended to generate hypotheses for future research. Larger studies, including human validation cohorts, are warranted to confirm their clinical applicability.

Keywords: Cholangiocarcinoma- Proteomics- Metabolomics- Biomarkers- Early diagnosis- Hamster model

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Introduction

Cholangiocarcinoma (CCA) is a malignant tumor arising from cholangiocytes, the epithelial cells lining the bile ducts [1]. It is the second most common primary liver cancer, and its global incidence is increasing. CCA is a significant public health concern in Southeast Asia, particularly in northeastern Thailand, where liver fluke infections are highly prevalent [2]. The age-standardized incidence rate is notably high in Khon Kaen province, Thailand, reaching 89.2 per 100,000 males and 37.4 per 100,000 females (ranging from 93.8 to 317.6 per 100,000) [3]. In Thailand, a primary risk factor for CCA is the consumption of raw, undercooked, or fermented freshwater fish, such as "Pla-ra", "Pla-som", or "Koi-pla", which often contain the liver fluke *Opisthorchis viverrini* (OV) and pre-carcinogenic N-nitroso compounds (e.g., dimethylnitrosamine [DMN]) [4, 5]. A possible mechanism for OV-associated CCA involves the production of nitric oxide by inflammatory cells, which facilitates the formation of N-nitroso compounds through reactions between amines and nitrosating agents. These compounds can induce DNA methylation, damaging DNA and promoting carcinogenesis, particularly in the proliferating epithelial cells of the bile ducts [6-11].

The diagnosis of CCA relies on clinical evaluation together with radiological imaging, biochemical tests, and endoscopic brushing [12, 13]. However, imaging modalities such as ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) are often ineffective in detecting CCA due to the tumor's tendency to grow along the bile duct without forming a distinct mass. In addition, currently available serum tumor markers show limited accuracy. Carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) are commonly used in clinical practice, but both exhibit variable sensitivity and specificity, with elevated levels also observed in benign hepatobiliary diseases [14-16]. CCA is highly resistant to chemotherapy, and no effective chemotherapeutic agents are currently available. Surgical resection remains the only curative treatment, but it is feasible only when the disease is detected at an early

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stage. For advanced cases, chemotherapy and radiotherapy may be applied with a palliative intent. Due to the lack of reliable early diagnostic tools, most CCA cases are diagnosed at advanced stages when surgery is no longer possible [12].

Recent advances in proteomics and metabolomics have created new opportunities for biomarker discovery in CCA. Several human studies have reported potential diagnostic proteins and metabolite alterations that reflect cancer-associated metabolic reprogramming [17-19]. While these findings are promising, their clinical application remains limited, and further validation is required. Animal models are therefore essential for understanding disease mechanisms and identifying early molecular changes that precede tumor development. In this study, we employed an *Opisthorchis viverrini* (OV)/dimethylnitrosamine (DMN)-induced cholangiocarcinoma hamster model of CCA to identify plasma proteins and metabolites with potential utility as early biomarkers. Using a combined proteomics and metabolomics approach with LC-MS/MS, we aimed to generate preliminary data to inform future validation studies in high-risk human populations.

Materials and Methods

Chemicals and reagents

DMN and tween-80 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HPLC-grade acetonitrile, methanol and LC/MS grade water were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Internal compounds DL-arabinose alanine, L-alanine, ammonium hydroxide, and ammonium acetate were purchased from Sigma–Aldrich (St.Louis, USA).

Animals

The Ethics Committee approved the use of animals and the experimental protocol for Research in Animals, Thammasat University, Thailand (Reference numbers 013/2556 and 014/2562). Six-week-old male and female Syrian hamsters (100-120 g) were housed under standard environmental conditions (22 ± 3 °C, 30-70% relative humidity, 12-h light-dark cycle) with free access to standard food and water ad libitum.

Experimental induction of OV/DMN-induced CCA

The OV/DMN-induced CCA experiment's development has been described in detail [20]. In brief, metacercariae of OV were collected from naturally occurring cyprinoid fish obtained from a freshwater reservoir in an endemic area of Khon Kaen province, Thailand. Fish were minced and digested with pepsin-hydrochloric acid for 1 hours at 37 °C, then filtered and washed with normal saline. The metacercariae were identified and collected under the light microscope. The initial feeding of hamsters with 50 metacercariae of OV-induced CCA was followed four weeks later by drinking water containing 12.5 ppm of DMN for eight weeks. The occurrence and development of CCA were measured and confirmed by ultrasonography and histology examination [21].

Experimental design

Hamsters were randomly divided into two groups, with each group containing three hamsters, as follows: i) Group 1 (control): healthy hamsters fed with distilled water for 12 weeks, and ii) Group 2: OV/DMN-induced CCA hamsters without any treatment (group 2). Body weight, food and water consumption were recorded daily for 30 days. Survival time and survival rate were the primary endpoint parameters for evaluating the CCA development.

Blood specimen collection and histopathological examination

The hamsters of each group underwent anesthesia, and blood samples were collected into ethylenediamine tetra-acetate (EDTA) plastic tubes from the lateral saphenous vein every 2 weeks. Plasma sample from each group was separated through centrifugation at 3,000 x g for 10 minutes (4 °C) and stored at -80 °C until analysis. After the completion of the experiment, vital organs were immediately removed, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm thick and followed by hematoxylin-eosin staining [22]. The sections were examined under a light microscope and scanned at 40× magnification (20 microscopic fields).

Samples preparation for proteomics

Plasma samples were purified by C18 ZipTip (Merck Millipore, Darmstadt, Germany), precipitated in cold acetone, and then the pellet was collected by centrifugation at 12,000 x g at 4 °C for 15 minutes. The protein pellet was dissolved in 0.5% SDS, and the concentration was evaluated using the Lowry method [23]. Samples were digested with trypsin and incubated at 37 ˚C for 12 hours for further mass spectrometric analysis.

Protein Identification and Analysis

Peptides in the prepared samples were separated by Ultimate 3,000 LC system (Dionex) coupled to ESI-Ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik) with electrospray at a flow rate of 20 μL/min to μ-precolumn (Monolithic Trap Column, 200 μm i.d. x 5 mm). The proteins were separated on a nano column (Monolithic Nano Column, 100 μm i.d. x 5 cm) with a solvent gradient (solvent A: H2O, 0.1% formic acid; solvent B: 50% H2O, 50% ACN, and 0.1% formic acid) running at a flow rate of 1 μL/min for 20 minutes.

The LC-MS/MS data were analyzed using DeCyderTM (Amersham Bioscience AB, Uppsala, Sweden) and MASCOT (http://www.matrixscience.com) programs for protein identification. PANTHER (http://www.pantherdb.org) [24] and STITCH 5.0 database (http://stitch.embl.de) [25] were respectively applied for the identification of gene ontology and protein-protein interactions.

Samples preparation for metabolomics

Plasma samples were centrifuged at 14,000 x g (4-8 °C) for 5 minutes. The supernatant was collected, mixed with methanol (cooled to -80 °C) and incubated at -80 °C for 8 hours. The supernatant was collected through centrifugation at 14,000 x g (4-8°C) for 10 minutes, dried (SpeedVac) and stored at -80 °C until use [26].

Metabolite identification and analysis

The dried metabolites were dissolved in LC/MS-grade water and filtered through a Phenix-RC filter (0.2 µm pore size). The metabolites were isolated using HPLC (Agilent Technologies, 1200 Infinity Series) and identified by QTRAP® 5500 (AB SCIEX). The system consisted of a security universal HPLC guard cartridge (Phenomenex, CA, USA), an amide XBridge HPLC column (3.5 µm x 4.6 mm i.d. × 100 mm length (Waters, MA, USA). The mobile phase consisted of 50% buffer A (95% water, 5% acetonitrile, 20 mM ammonium hydroxide, and 20 mM ammonium acetate adjusted to pH 9.0) and 50% buffer B (100% acetonitrile). The flow rate was 400 µL/min with a run time of 23 minutes. For MS/MS, the spray voltage was set at 3,200 V.

Data was analyzed using XcalibarTM software (Thermo Scientific, Massachusetts, USA). The metabolites which were up- and down-regulated were selected for the identification of possible signaling pathways, including the correlation between the identified metabolites and proteins, using the free online web-based MetaboAnalyst 4.0 (http://old.metaboanalyst.ca/MetaboAnalyst/faces/ModuleView.xhtml).

Statistical analysis

All quantitative variables are presented as median and mean with 95% CI (confidence interval). Fold change analysis was performed to measure the changes of identified proteins between the CCA group and the control with P values ≤ 0.05.

Results

Development of cholangiocarcinoma in OV/DMN-induced CCA hamster model

In our previous study [20], the development of cholangiocarcinoma (CCA) was observed 12 weeks after hamsters were infected with *Opisthorchis viverrini* (OV) and treated with dimethylnitrosamine (DMN). Gross autopsy of the liver in the CCA group revealed opaque common hepatic bile ducts accompanied by marked diffuse proliferation, cystic formations, and fibrosis. Mass-forming lesions were also visible on the liver surface during gross examination. In contrast, the livers of the control group appeared normal, exhibiting red coloration and a smooth surface. The median survival rate of the CCA group was significantly shorter, with survival limited to 17 days after CCA confirmation at week 12. This was significantly contrasted to the control group, as demonstrated during the 2-6 month observation period ($p < 0.01$).

Identification of proteins by LCMS/MS

Comparative analysis of protein patterns between CCA and healthy hamsters at various stages of CCA development revealed over 5,000 proteins (Table 1). At 12 weeks post-induction of CCA using *Opisthorchis viverrini* (OV) and dimethylnitrosamine (DMN), 5,090 proteins were detected in the control group, whereas 4,974 proteins were identified in the CCA group, as illustrated in the Venn diagram. Notably, 572 proteins were uniquely expressed in the CCA group, while 4,402 proteins were common to both groups (Figure 1).

The 572 CCA-specific proteins were classified based on protein class, biological process, and molecular function using the GO and PANTHER databases (Figure 2). They were predominantly associated with cellular processes (27.9%), metabolic processes (16.6%), localization (9.1%), biological regulation (8.9%), and

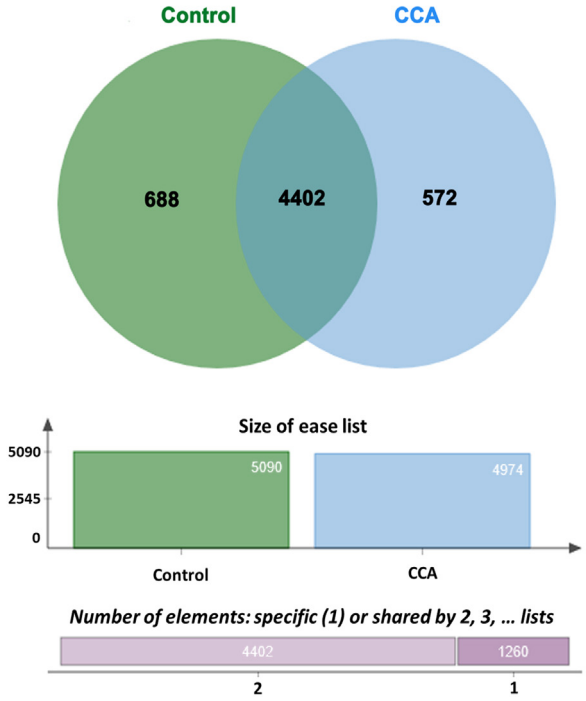

Figure 1. Venn Diagrams showing the number of proteins identified in the plasma of control and CCA groups at 12 weeks after hamsters were induced CCA development with OV/DMN.

Table 1. Number of Proteins Identified in the Plasma of Control and CCA Groups at Different Time Points after Hamsters were Induced CCA Development with OV together with the DMN Administration

Number (n) of proteins in each group	Time points after induction CCA in hamsters with 50 metacercariae of OV				
	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
Control	6,421	5,873	5,513	5,252	3,504
CCA	6,083	5,723	5,261	5,170	3,469
Only found in control	595	588	741	596	883
Only found in CCA	257	438	489	514	848
Overlap CCA & control	4,826	5,285	4,772	4,656	2,621

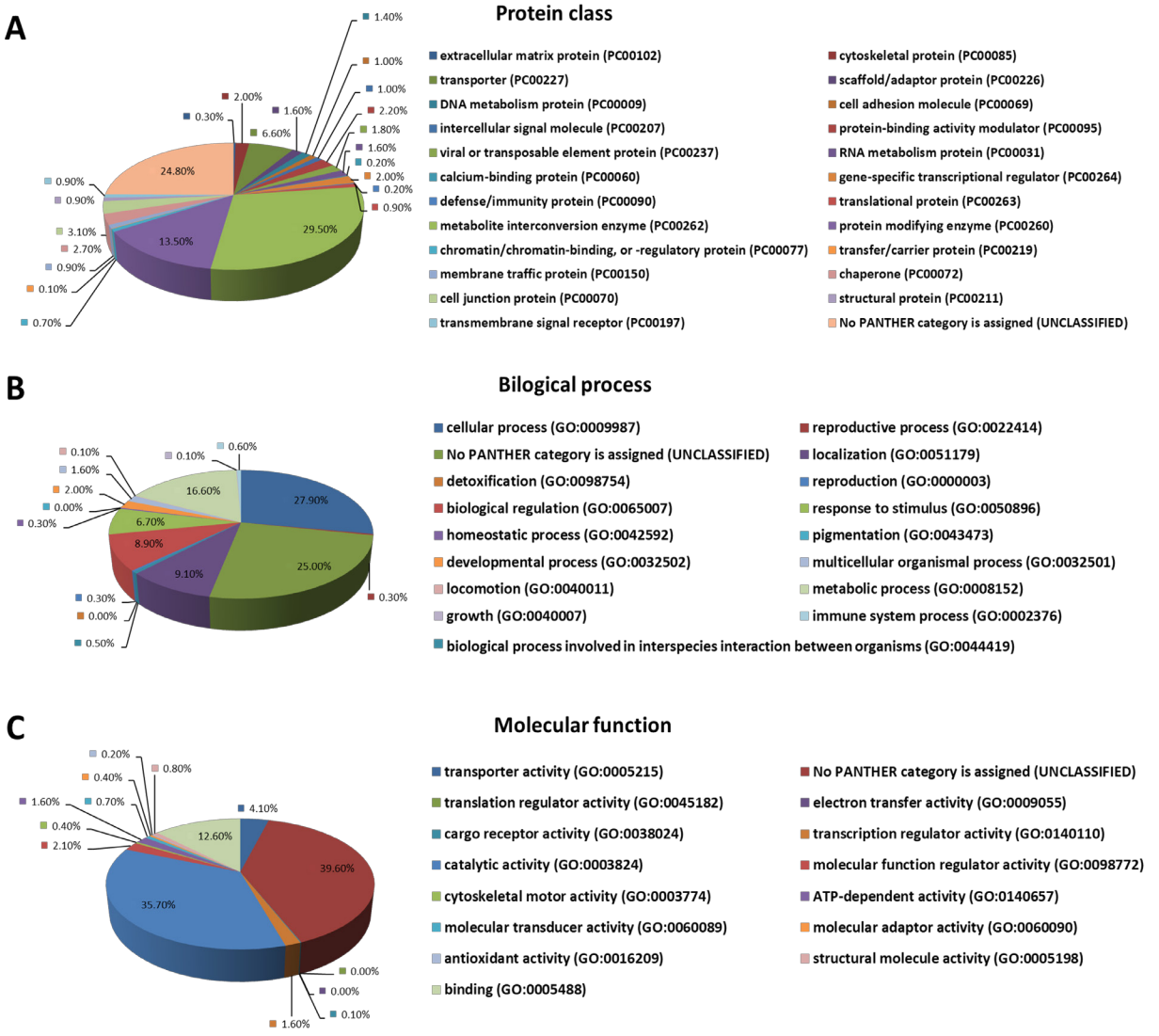

Figure 2. Gene Ontology Analysis by PANTHER. GO analysis of the 572 proteins uniquely identified in plasma from the CCA group at 12 weeks after hamsters induced CCA development with OV/DMN.

response to stimuli (6.7%). Other categories included developmental processes (2.0%), multicellular organismal processes (1.6%), immune system processes (0.6%), interspecies interaction (0.5%), reproduction (0.3%), hemostasis (0.3%), locomotion (0.1%), and growth (0.3%) (Figure 2B).

Proteins were further grouped into classes such as metabolite interconversion enzymes, protein-modifying enzymes, transporters, cell junction proteins, chaperones, protein-binding activity modulators, cytoskeletal proteins, and gene-specific transcriptional regulators (Figure 2A). In terms of molecular function, they were categorized into five main groups: catalytic activity, binding, transporter activity, molecular function regulator activity, and transcription regulator activity (Figure 2C). Among these proteins, several noteworthy candidates were identified, including tyrosine-protein kinase (EC 2.7.10.2) (A0A3L7I725), thrombospondin-2 (A0A3L7I7V8), peroxisomal carnitine O-octanoyl transferase (G3HRY2), DNA topoisomerase II-binding protein 1 (G3GZG7), and FAST kinase domain-containing protein 2 (G3HDK4) (Table 2).

Using STITCH software, the cell signaling pathways associated with the 572 unique proteins in the CCA group were analyzed. Several proteins were linked to pathways involved in cell growth, proliferation, and signal transduction, including protein tyrosine phosphatase (PTPN11), GRB2-associated binding protein 2 (GAB2), and growth factor receptor-bound protein 2 (GRB2). Additionally, von Willebrand factor A domain-containing protein (Fragment) and uncharacterized protein (G3H5S6) were consistently detected across all time points (4, 6, 8, 10, and 12 weeks post-induction). Only five and nine proteins were uniquely identified in the CCA group at four and three different time points, respectively (Table 2).

Fold-change analysis indicated significant upregulation of proteins in the CCA group compared to controls, with 169, 412, and 545 proteins upregulated at weeks 4, 8, and 12, respectively ($p < 0.05$). The top upregulated proteins at week 4 included DUS4L (fragment), alpha-tectorin,

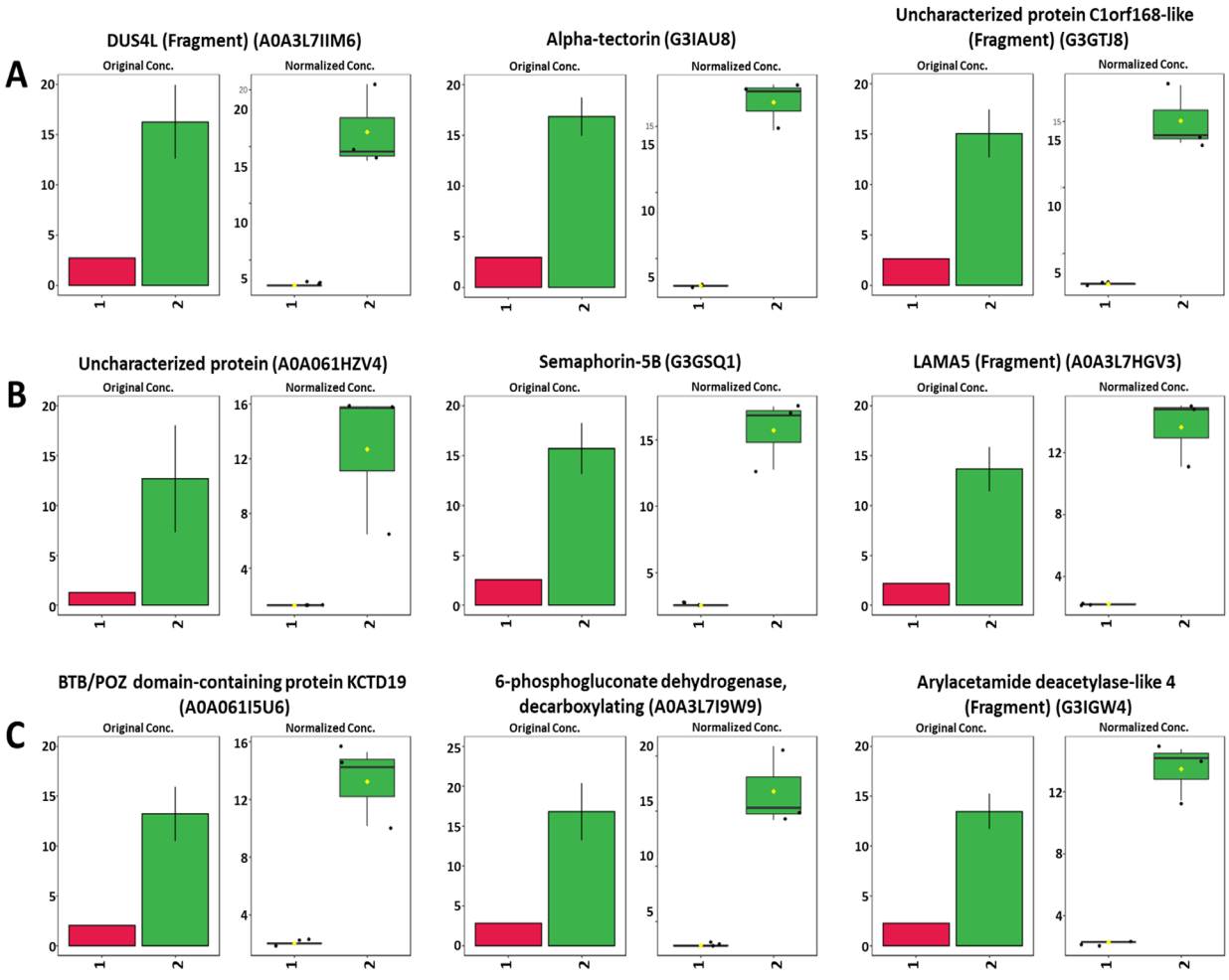

Figure 3. Fold Change Analysis of the Top Three Upregulated Identified Proteins between CCA and Control Group at Weeks 4 (A), 8 (B), and 12 (C). Statistically significant at p-value < 0.05

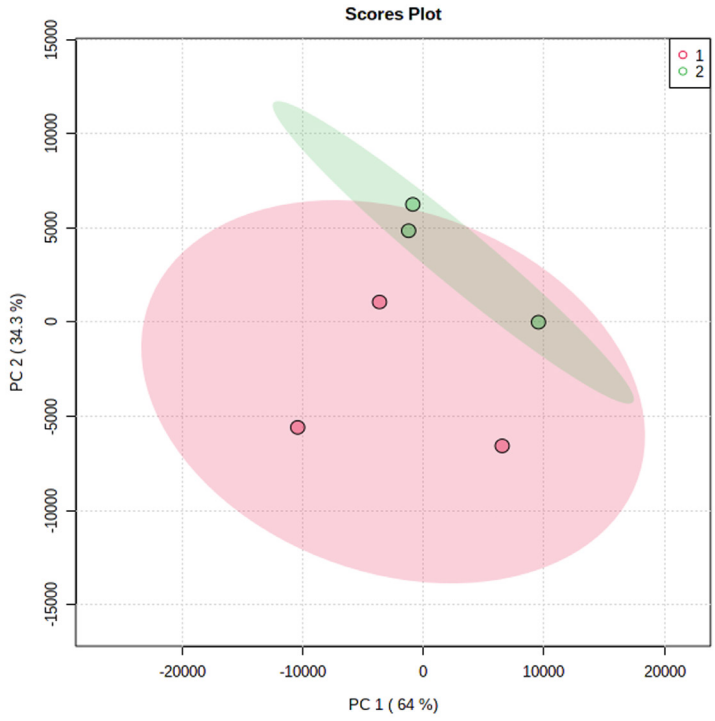

Figure 4. Score Plot of Metabolites between CCA and Healthy Group by Principal Component Analysis (PCA).

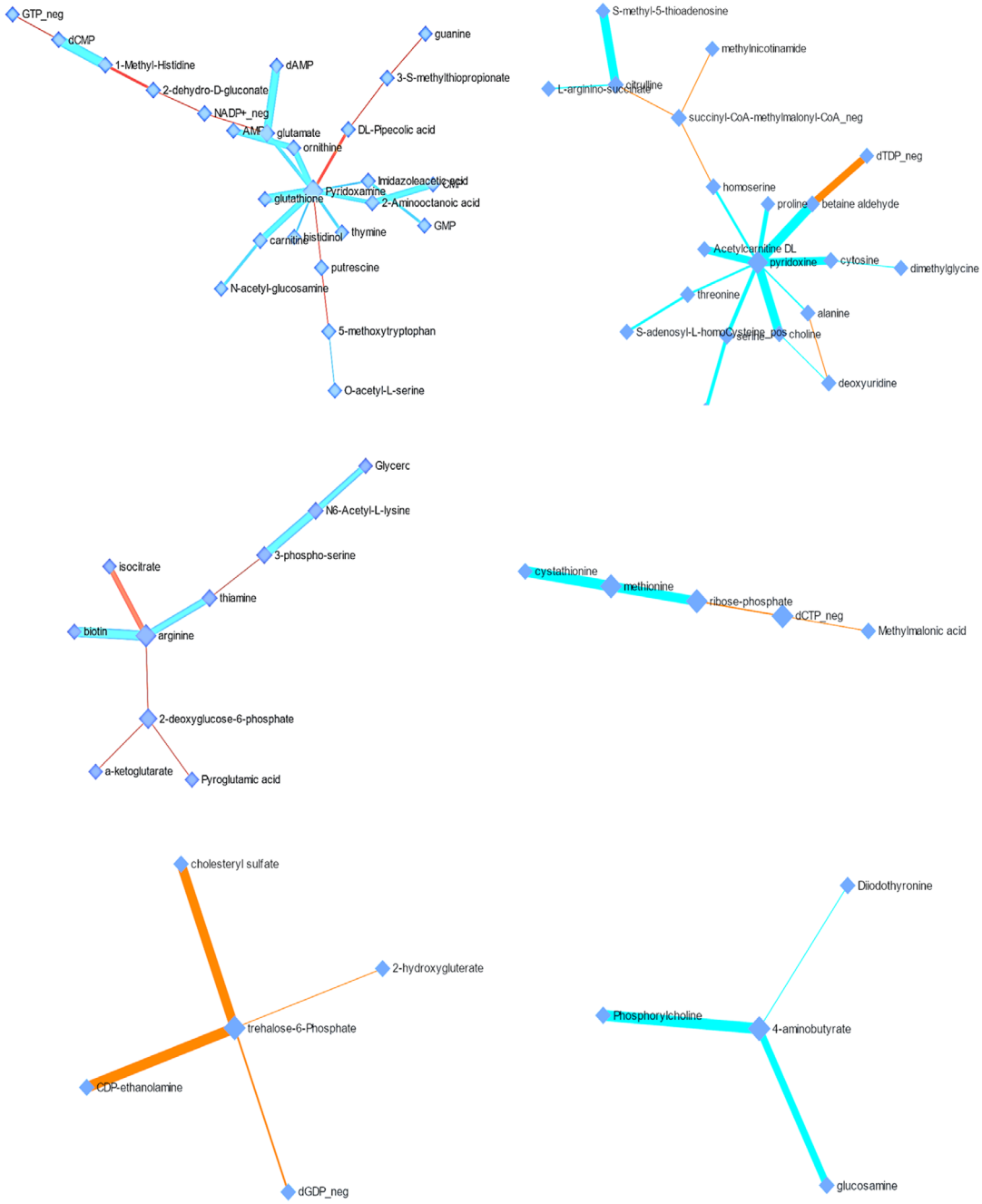

Figure 5. Example of Metabolites Network.

and uncharacterized protein C1orf168-like (fragment). At week 8, semaphorin-5B, LAMA5 (fragment), and another uncharacterized protein were most upregulated. By week 12, the top proteins included BTB/POZ domain-containing protein KCTD19, 6-phosphogluconate dehydrogenase, decarboxylating (EC 1.1.1.44), and arylacetamide deacetylase-like 4 (fragment) (Figure 3).

Identification of metabolite by LCMS/MS

Due to limited plasma availability, samples collected at weeks 4, 8, and 12 were pooled for metabolomics analysis. The area under the peak for each metabolite, identified by LC-MS/MS, was calculated and compared to the controls. Metabolites showing at least a 2-fold change in peak area were selected for further investigation. Of 273 identified metabolites, 59 were upregulated in pooled plasma from the CCA group, including cystathionine, putrescine, UDP-N-acetyl-glucosamine, FMN, malate, 3-S-methylthiopropionate, dATP_neg, flavone, glycolate, thymine, urea, and pyridoxine. Principal Component Analysis (PCA) successfully differentiated the metabolite profiles of the CCA and healthy groups (Figure 4). The identified metabolites were further analyzed to construct a 13 networks, highlighting potential pathways and interactions relevant to CCA development (Figure 5).

Table 2. Proteins Uniquely Identified in Plasma from CCA Group at Different Times after Hamsters Induced CCA Development with OV/DMN.

Protein ID	Protein name	Detected at week
A0A3L7I725	Tyrosine-protein kinase (EC 2.7.10.2)	12
A0A3L7H3Q1	Transcription Elongation Factor A Like 3 (TCEAL3)	12
G3H2B9	Growth/differentiation factor 9	12
G3GZG7	DNA topoisomerase II-binding protein 1	12
G3IG85	Tyrosine-protein kinase transforming protein Abl	12
G3HRY2	Peroxisomal carnitine O-octanoyltransferase	12
A0A061II21	Coiled-coil domain-containing protein 11	12
A0A3L7HKK0	Glutamyl-tRNA synthetase (EC 6.1.1.15) (EC 6.1.1.17) (Prolyl-tRNA synthetase) (Fragment)	12
G3ICC8	Zinc finger protein 91	12
A0A3L7HJT8	BTBD7	12
A0A3L7ILB4	CYP51A1 (Fragment)	10, 12
A0A3L7HAD2	Serine protease 27 (PRSS27)	10, 12
A0A3L7I7V8	Thrombospondin-2 (THBS2)	10, 12
A0A061HWD9	Ubiquitin carboxyl-terminal hydrolase (EC 3.4.19.12)	10, 12
G3HVM1	Plexin-D1	10, 12
G3GWD3	Alpha-mannosidase (EC 3.2.1.-)	10, 12
A0A3L7HKC0	Actin-like protein 3 (Actin-related protein 3)	10, 12
A0A3L7H248	NAD(P)-dependent steroid dehydrogenase-like (NSDHL)	10, 12
A0A3L7HA16	MYO15B (Fragment)	10, 12
G3HRB4	ATP synthase subunit gamma, mitochondrial (ATP5C1)	10, 12
G3IJU2	Rho GTPase-activating protein 18	8, 10, 12
A0A3L7IAW0	THEMIS2	8, 10, 12
G3HIX8	WD repeat-containing protein 25	8, 10, 12
A0A3L7GWM5	SYT15 (Fragment)	8, 10, 12
A0A061HY55	RING finger protein (RNF113A)	8, 10, 12
G3HQU3	Vomeronasal type-2 receptor 26	8, 10, 12
G3GWX2	Forkhead box protein F2	8, 10, 12
A0A3L7HJN1	Dimethylaniline monooxygenase [N-oxide-forming] 1 (EC 1.14.13.8) (Dimethylaniline oxidase 1) (Fragment)	8, 10, 12
A0A3L7IF87	ANP32A	8, 10, 12
G3I307	Uncharacterized protein	6, 8, 10, 12
A0A3L7GVR2	Homeobox domain-containing protein	6, 8, 10, 12
A0A061I7B4	Talin-2-like protein	6, 8, 10, 12
A0A3L7I7H6	ICE1	6, 8, 10, 12
A0A061II47	Actin-like protein 6A	6, 8, 10, 12
G3H5S6	Uncharacterized protein	4, 6, 8, 10, 12
A0A061I2G3	von Willebrand factor A domain-containing protein 5A-like protein (Fragment)	4, 6, 8, 10, 12

Note: Representative proteins detected at week 12 and at both weeks 10 and 12 are presented; the complete list is not included.

Discussion

Early diagnosis of cholangiocarcinoma (CCA) remains challenging due to the lack of reliable biomarkers. Biomarkers such as carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) are commonly used in clinical practice, but their specificity and sensitivity are suboptimal [14-16]. CA 19-9 shows a wide range of sensitivity (50–90%) and specificity (54–98%) [27, 28], with elevated levels also found in benign biliary tract diseases [29]. Similarly, CEA and cancer antigen 125 (CA125) demonstrate low specificity and sensitivity for CCA screening [30]. Recent proteomic and metabolomic studies in human CCA have identified potential markers, but clinical application remains limited [17-19]. Our *Opisthorchis viverrini* (OV)/dimethylnitrosamine (DMN)-induced hamster model complements these studies by identifying novel plasma proteins and metabolites that emerge prior to overt CCA, aligning with cancer-associated metabolic and proteomic pathways.

CCA developed 12 weeks after infection. Proteomic analysis revealed 572 proteins uniquely identified in the CCA group, primarily associated with cellular processes, metabolic pathways, localization, and biological

regulation. Many of these proteins were linked to cell growth, proliferation, and signal transduction pathways. Notable proteins included tyrosine-protein kinases, which are involved in intracellular signaling and are often overexpression during carcinogenesis [31]. For example, protein tyrosine kinase-7 (PTK7) is associated with invasiveness and poor prognosis in intrahepatic CCA [32]. Other promising proteins included thrombospondin-2 (THBS2), regulating tumor angiogenesis [33, 34], peroxisomal carnitine O-octanoyltransferase (enhancing oxidative metabolism), DNA topoisomerase II-binding protein 1 (TopBP1, maintaining genomic integrity), and FAST kinase domain-containing protein 2 (essential for mitochondrial ribosome assembly). Early-detected proteins, such as Rho GTPase-activating protein 18, Plexin-D1, and THBS2, may serve as potential early diagnostic biomarkers [35-37].

Fold-change analysis showed 169, 412, and 545 proteins upregulated at weeks 4, 8, and 12, respectively, and 59 metabolites upregulated in pooled plasma during the same period. Key proteins included alpha-tectorin (week 4), DUS4L, semaphorin-5B (week 8), LAMA5, LAMA3, BTB/POZ domain-containing protein KCTD19, 6PGD, and AADACL4 (week 12). Many of these proteins are implicated in cancer progression, metabolic reprogramming, and cell proliferation [38-44]. Interestingly, several early markers, including Rho GTPase-activating protein 18 and Plexin-D1, were detected before the full onset of CCA, suggesting their potential utility for early detection. Such findings pave the way for longitudinal studies to validate these markers in pre-symptomatic populations or in individuals at risk, such as those in regions endemic for liver flukes.

Metabolomics analysis identified 59 upregulated metabolites, critical for cancer metabolism in glycolysis, nucleotide synthesis, and redox balance. Principal Component Analysis (PCA) distinguished CCA and healthy metabolite profiles, supporting their potential diagnostic value. Notable metabolites included cystathionine, putrescine, UDP-N-acetyl-glucosamine, FMN, malate, 3-S-methylthiopropionate, dATP_neg, flavone, glycolate, and thymine. These metabolites are involved in oxidative stress, glycosylation, and proliferation pathways, which are relevant to tumor development.

The identification of early biomarkers has significant implications for CCA management, particularly in liver fluke-endemic regions. Early detection improves surgical outcomes and offers opportunities for interventions such as targeted therapies and immunotherapy. Importantly, this pilot study provides preliminary data for hypothesis generation and identification of candidate biomarkers. The findings suggest that combining proteomic and metabolomic data could enhance the sensitivity and specificity of diagnostic tools. While promising, this study has limitations. The use of animal models offers valuable insights but requires further validation in human cohorts. Because of the limited sample size, plasma samples were pooled for metabolomic analysis, which may have obscured inter-individual variations. Additionally, some identified proteins and metabolites, such as alpha-tectorin and AADACL4, have not been extensively studied in cancer, warranting further functional investigations to elucidate their roles in CCA. Despite these limitations, the findings provide a strong foundation for hypothesis-driven research. Follow-up studies with larger sample sizes and human validation cohorts are planned to strengthen the statistical power and clinical relevance of these results. Future research should focus on i) confirming the diagnostic value of the identified biomarkers in larger and more diverse populations, including human cohorts; ii) monitoring biomarker expression over time to evaluate their utility in predicting disease onset and progression; iii) developing non-invasive diagnostic assays, such as blood tests, that integrate proteomic and metabolomic markers for improved accuracy; and iv) investigating the functional roles of less-characterized biomarkers in CCA pathogenesis to explore potential therapeutic targets. By addressing these gaps, the study findings could be translated into practical tools for early diagnosis and intervention, ultimately improving outcomes for patients with CCA.

In conclusion, this pilot study highlights potential proteomic and metabolomic biomarkers for the early detection of CCA. These preliminary results are intended to guide hypothesis generation. Future studies involving larger sample sizes, longitudinal follow-up, and validation in human cohorts are essential to determine their diagnostic and clinical utility.

Author Contribution Statement

Conceptualization: KB and KN; methodology: KB, WC, MT and TP; Qualitative data collection and analysis: KB; investigation: KB, WC, MT and TP; writing, review and editing: KB and KN; supervision: KN. All authors have read and approved the final version of the manuscript.

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Scientific Body Approval

This study was conducted under the oversight and approval of the Chulabhorn International College of Medicine, Thammasat University.

Ethical Approval

All animal procedures were reviewed and approved by the Ethics Committee for Research in Animals, Thammasat University, Thailand (Reference numbers 013/2556 and 014/2562).

Availability of Data

The datasets generated and analyzed in this study

are not publicly available due to institutional restrictions but are available from the corresponding author upon reasonable request.

Availability of Data

This study did not involve clinical trials, guidelines, or meta-analyses and therefore was not registered in any public registry.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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