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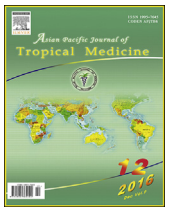

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Glycoproteomics analysis of plasma proteins associated with *Opisthorchis viverrini* infection-induced cholangiocarcinoma in hamster model

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ABSTRACT

Objective: To apply lectin affinity chromatography and glycoproteomics-based LC-MS/MS to preliminarily investigate the possible potential plasma biomarkers of *Opisthorchis viverrini* (OV)-associated CCA in OV/dimethylnitrosamine (DMN)-induced CCA hamster model.
Methods: Nine Syrian hamsters were divided into 3 groups as follows (n = 3 each): normal (healthy control group); OV group; and OV/DMN group (CCA group). Pooled plasma samples collected from animals in each group at the 6th month post-infection with OV metacercarae were subjected to glycoproteomics analysis. Glycoproteins in the pooled sample from each group were initially isolated by concanavalin A (ConA)-based affinity chromatography. The expression of glycoproteins isolated by both enrichment methods were determined using LC-MS/MS.
Results: Among the 24 ConA-binding glycoproteins isolated, two proteins, N-myc downstream regulated gene 1 (NDRG1) and fetuin-B (FETUB) were found up-regulated only in the samples from the OV and control groups, but not in the OV/DMN (CCA) groups. On the other hand, one protein, i.e., NSFL1 cofactor p47 isoform ×3 (NSFL1C) was found only in the samples from OV/DMN (CCA) and control groups, but not in the OV group. The remaining 21 proteins were upregulated in the samples from all groups.
Conclusions: NDRG1, FETUB and NSFL1C glycoproteins isolated by ConA-based affinity chromatography could be potential biomarkers for CCA. Plasma samples with negative for NDRG1 and FETUB proteins but positive for NSFL1C are likely to be OV-associated CCA. Nevertheless, this conclusion remains to be confirmed whether this battery test can discriminate OV-associated CCA from other risk factors.

1. Introduction

Cholangiocarcinoma (CCA) is a devastating cancer with an incidence and mortality progressively increasing worldwide [1]. In Thailand, the high incidence rate is reported in the northeastern region, where is the high prevalence of *Opisthorchis viverrini* (OV) infection [2–4]. The risk factor of CCA in this region is the consumption of improperly cooked and fermented fresh water cyprinoid fish call "Pla-ra", which contains the OV and the pre-carcinogen nitrosamine [5]. At present, the diagnosis of CCA is based on clinical findings with radiological investigations, biochemical measurements, and endoscopic brushing [6,7]. Current imaging techniques including ultrasonography, computed

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tomography (CT), and magnetic resonance imaging (MRI) are not efficient to reveal this lesion since the tumor typically grows along the bile duct without protruding outward as a forming mass. Sensitivity and specificity of laboratory assessments are often not adequate. CCA has been characterized as highly chemoresistant. Currently, there is no effective chemotherapeutics; the only curative treatment is surgical resection. Nevertheless, it is applicable and effective only when CCA is diagnosed at an early stage. Postoperative 5-year survival rate is very low, with a recurrence rate of 60% and a median disease-free survival of 26 months [8]. Treatment with radiotherapy and chemotherapy also carries a poor overall survival rate [9]. Effective early diagnostic, prognostic, and therapeutic biomarkers are required to improve the prognosis and prolong survival rates in CCA patients. Identification of new tumor markers in the plasma/serum would therefore, be beneficial in the clinical management of this disease.

The aim of the study was to apply lectin affinity chromatography and glycoproteomics-based LC-MS/MS to preliminarily investigate the possible potential plasma biomarkers of *Opisthorchis viverrini* (OV)-associated CCA in OV/dimethylnitrosamine (DMN)-induced CCA hamster model.

2. Materials and methods

2.1. Preparation of parasite

Naturally infected fresh water cyprinoid fish were captured from a water reservoir in an endemic area of opisthorchiasis. Fish were minced and digested with pepsin-HCl, then incubated in a shaking water bath at 37 °C for 1 h. The digested fishes were filtered through sieves (pore sizes 1 000, 425 and 106 μm, respectively). The OV metacercariae sediments were isolated and examined under a stereomicroscope [10,11].

2.2. Preparation of dimethylnitrosamine

Dimethylnitrosamine (DMN) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, JPN). DMN was diluted in distilled water under biohazard cabinet to make a 12.5 ppm final concentration. The solution was stored in a dark container until use. Diluted DMN was administered to hamster every day for 2 months in order to induce cholangiocarcinogenesis.

2.3. Animals and experimental design

Approval of the study protocol was obtained from the Ethics Committee for Research in Animals, Thammasat University, Thailand. Randomly nine bred female golden hamsters, (6–8 weeks of age, supplied by the Animals Care Unit, Faculty of Medicine, KhonKaen University) were allocated into three groups ($n = 3$ each) as follows: Control group: hamsters were fed with normal saline (200 μL) at 1st month post-infection, OV group: hamsters were fed with 50 OV metacercaria in normal saline (200 μL) (0 month), OV/DMN (CCA) group: hamsters were fed daily with 50 OV metacercaria (0 month), together with DMN in normal saline (200 μL) daily for 2 months (from 1st to 2nd months post-infection) [12].

2.4. Blood specimen collection and histopathological examination

The hamsters of each group will be sacrificed under deep anesthesia, and blood samples were collected into ethylenediamine tetra-acetate (EDTA) plastic tubes, from the hearts and the livers will be removed of each hamster in all groups at 6th months post-infection. Pooled plasma sample from each group was separated through centrifugation at 3 000× *g* for 10 min (4 °C) and stored at −80 °C until analysis. The livers were examined for gross pathology and histopathology. Then, the livers were fixed in 10% buffer formalin, serially sliced in a 4–5 mm thickness, embed in paraffin. Sections were cut as 4 μm thickness and stained with hematoxylin-eosin [13].

2.5. Isolation of glycoprotein from pooled plasma samples using concanavalin A (ConA)-based affinity chromatography

Isolation of glycoproteins in the pooled plasma sample collected at 6th months post-infection in each group was performed using ConA lectin resin kit (Thermo Scientific Inc) according to the manufacturer's recommendations. In brief, the ConA lectin resin column was prepared using 200 μL of 50% resin. The column was initially equilibrated with 600 μL of the binding buffer. The pooled plasma sample (800 μL) was loaded onto the equilibrated column. After incubating for 10 min at room temperature (25 °C) with end-over-end mixing using a rotator, the column was centrifuged for 1 min at 1 000 × g and the flow-through was discarded. The unbound proteins in the column were removed by washing four times with the binding buffer. The glycoproteins bound ConA were eluted twice with 200 μL of elution buffer at room temperature for 5 min with end-over-end mixing using a rotator. The column was centrifuged for 1 min at 1 000 × g and the eluate was collected.

2.6. LC-MS/MS analysis

2.6.1. Peptides preparation for LC-MS/MS analysis

The eluted glycosylated protein fractions from the pooled plasma sample of each group were directly applied onto a ZebaTM Spin Desalting columns (Thermo Scientific Inc) and protein concentration in each desalted fraction determined by Lowry assay using BSA as a standard protein. The Sub-10 kDa protein fraction (>2 μg) was reduced and alkylated with dithiothreitol (DTT) and Iodoacetamide, respectively. The sample was digested with trypsin and kept at −80 °C for further mass spectrometric analysis.

2.6.2. HCTUltra LC-MS analysis

Peptides in the prepared samples were analyzed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., Germany) coupled to an UltiMate 3000 LC System (Dionex Ltd., UK). Peptides were separated on a nanocolumn (PepSwift monolithic column 100 μm i.d. × 50 mm). Eluent A and B consisted of 0.1% formic acid and 80% acetonitrile in water containing 0.1% formic acid. Peptide separation was achieved with a linear gradient from 10% to 70% eluate B for 13 min at a flow rate of 300 nL/min, followed by a regeneration step at 90% eluate B and an

equilibration step at 10% eluate B. Peptide fragment mass spectrum were acquired in data-dependent AutoMS mode with a scan range of 300–1 500 *m/z*, 3 averages, and up to 5 precursor ions selected from the MS scan 50–3 000 *m/z*.

2.6.3. Protein quantitation and identification

DeCyder MS Differential Analysis software (DeCyder MS, GE Healthcare) was used for quantitation of proteins in the samples. Acquired LC-MS raw data were converted and the PepDetect module was used for automated peptide detection, charge state assignment, and quantitation, based on the peptide ions signal intensities in MS mode. The analyzed MS/MS data from DeCyder MS were submitted for a database search using the Mascot software. The data were as searched against the NCBI database for protein identification. Database interrogation; taxonomy (*Homo sapiens*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da), peptide charge state (1+, 2+ and 3+) and max missed cleavages. The maximum value of each group was used to determine the presence or absence of each identified protein. Data normalization and quantification of the changes in protein abundance between the control and OV or OV/DMN treated samples were performed and visualized using MultiExperiment Viewer (Mev) software version 4.6.1. Briefly, peptide intensities from the LC-MS analyses were transformed and normalized using a mean central tendency procedure. Statistical analysis for comparison of the protein contents in the plasma samples from the three groups was performed using Analysis of Variance (ANOVA) at the statistical significance level of $P < 0.05$. Gene ontology annotation including biological process, cellular component and molecular function were performed using Gocat (http://eagl.unige.ch/GOCat/). Protein-chemical interactions were analyzed according to STITCH 4.0 database (http://stitch.embl.de/).

3. Results

3.1. Gross pathology and histopathology of liver of OV/DMN-induced CCA in hamsters

Based on gross liver autopsy appearance, hamsters infected with *Opisthorchis viverrini* together with the DMN administration (OV/DMN: CCA group) showed opaque common hepatic bile ducts. The mass forming lesions were detected from gross observation of the liver's surface. In the OV-treated group, the gallbladder and common bile ducts were slightly enlarged and opaque, with wall thickening in accordance with the duration time of infection. In the control group, liver appeared normal with a red color and smooth surface. Results obtained from histopathological examination in all groups were found to be in good correlation between gross autopsy and histopathology (Figure 1).

3.2. Identification of ConA-binding glycoproteins by LC-MS/MS

After enrichment, a total of 24 glycoproteins were identified using LC-MS/MS in all the control, OV and OV/DMN (CCA) groups (Table 1). A two-way hierarchical clustering analysis (heatmap analysis) indicates that 24 proteins were differentially expressed in the pooled plasma samples of the three groups (Figure 2). Figure 3 shows the Venn diagram of 24 glycoproteins that were differentially expressed only in the plasma sample from each group (0 glycoprotein in all groups) and those with overlapping existence in either the samples from both the CCA and control groups (1 glycoprotein), the OV/DMN (CCA) and OV group (no glycoprotein), the OV and control group (2 proteins), as well as those which were up-regulated in all groups (21 glycoproteins). Two glycoproteins

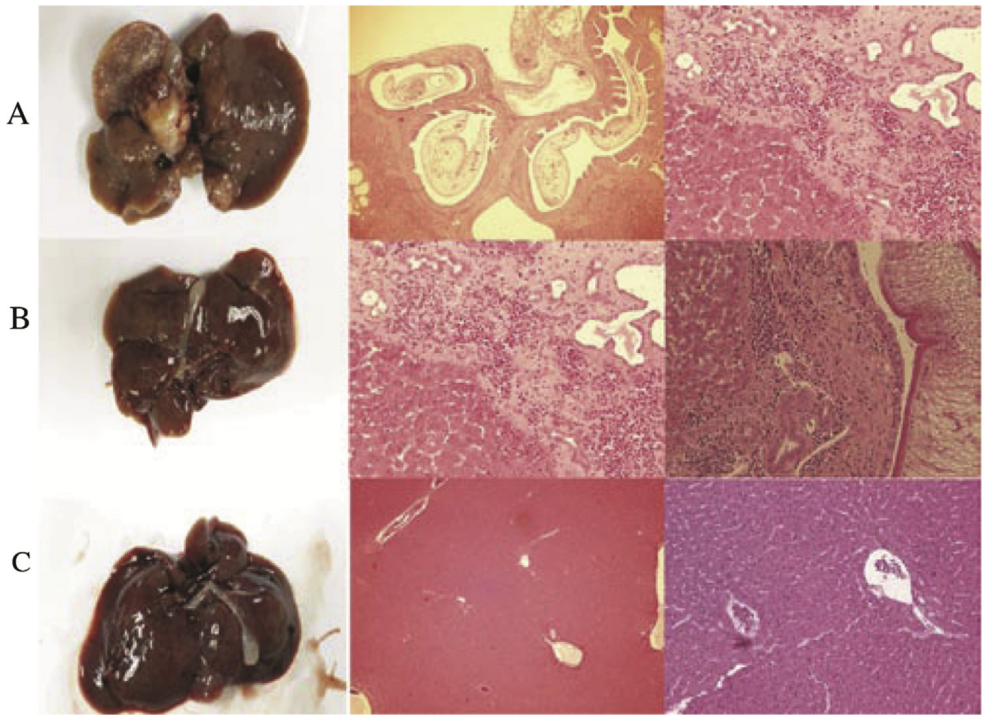

Figure 1. Representative gross and histopathological examination of the livers from (A) OV/DMN (CCA), (B) OV, and (C) control groups.

Table 1
List of glycoproteins isolated by ConA-based affinity chromatography.

Database ID no.	Protein name	Protein ID score	Peptides
gil524938471	Fetuin-B	36.27	LVVLPFPGK
gil537118064	Protein NDRG1	18.65	SRSHTSEDAR
gil354467899	Fatty aldehyde dehydrogenase-like	11.69	CXSRPLR
gil524968551	Alpha-2-macroglobulin-like	53.27	SIGLNVFTNSK
gil524968948	Phosphatidylinositol 4-kinase beta isoform X3	11.92	MAIGK
gil537225446	Transcription factor HIVEP3	7.36	KASSFPPMDR
gil537274398	Glutathione S-transferase theta-1-like protein	18.43	DMPPLMDPTLK
gil524942047	NSFL1 cofactor p47 isoform ×3	17.97	KSPNELVDDLFKGAK
gil537200449	Tripartite motif-containing protein 30	9.35	MASAFMANVK
gil537237353	Glucocorticoid modulatory element-binding protein 1	36.34	EIEELLR
gil537168917	Ig lambda chain V-I region BL2-like protein	34.22	QITVSWLR
gil354495235	Zinc finger protein 394-like	5.27	TDSMK
gil537257512	TSC22 domain family protein 2, partial	8.68	TEDVSSEIFDVSR
gil537169691	Protein Daple	35.72	KAGSLNLQIEK
gil537168406	Serpin B11	23.58	GKSAEQMEK
gil537226277	Glycoside hydrolase, family 31 containing protein	8.51	HVDPEALQK
gil524955568	V-set and transmembrane domain-containing protein 4	13.79	KDSLLAVR
gil537260058	Fibrinogen gamma chain-like protein	52.84	TSTADYAMFR
gil524959813	Ceruloplasmin	19.90	TESSTVVPTLPGETR
gil537200957	ATP-binding cassette sub-family E member 1	11.40	KMCMYK
gil537167905	Zinc finger Ran-binding domain-containing protein 3	9.18	AGAVK
gil524969534	Ubiquitin carboxyl-terminal hydrolase 7	28.67	QVSQNGGQPL
gil537091186	Hypothetical protein H671_21496, partial	20.55	YNYKGQR
gil344249997	Mitochondrial import inner membrane translocase subunit Tim17-B	20.55	GKEDPWNSITSGALTGAVLAAR

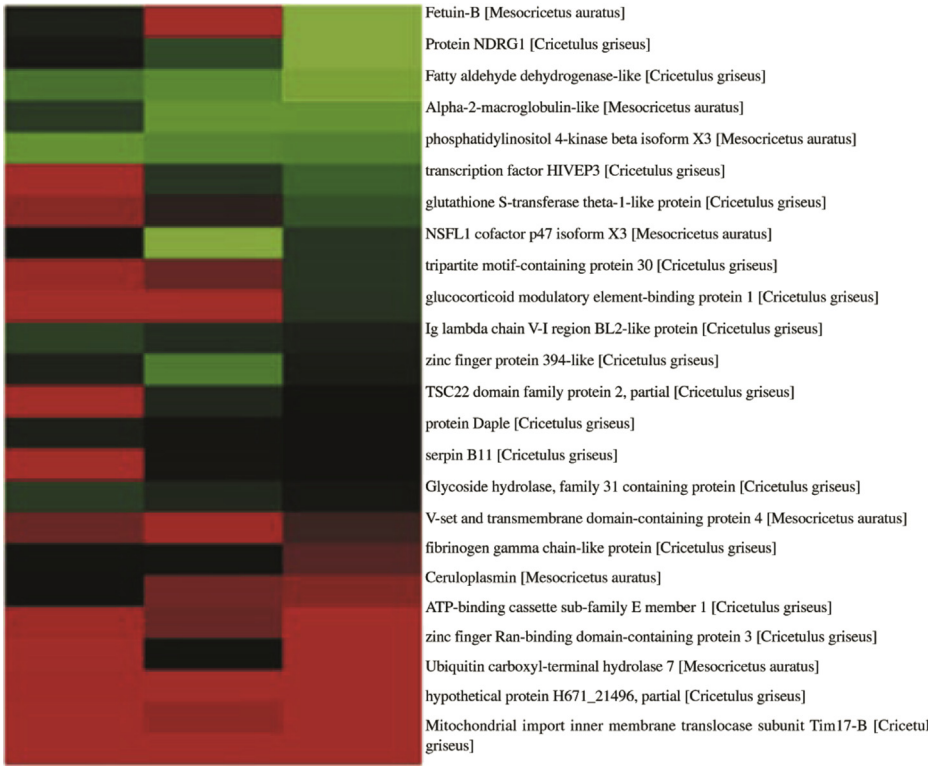

Figure 2. Hierarchical clustering analysis (heatmap analysis) of glycoproteins isolated from the pooled plasma samples of the control, OV, and OV/DMN (CCA) groups, by ConA-based affinity chromatography. The red colors represent up-regulated proteins and the green colors represent down-regulated proteins.

that were up-regulated only in the OV and control groups were Fetuin-B (FETUB) and Protein NDRG1 (NDRG1). One protein, NSFL1 cofactor p47 isoform ×3 (NSFL1C), was found up-regulated only in the samples from the CCA and control. The possible functional interactions and associations between the three glycoproteins with differential expression in plasma samples from the three groups and chemical compounds available in the published articles database were predicted using STITCH 4 software based on the following analysis parameters: species (*Homo sapiens*); medium confidence score (0.0400); and active prediction methods (all and no more than 10). The interactions between NDRG1, FetuB, NSFL1C and

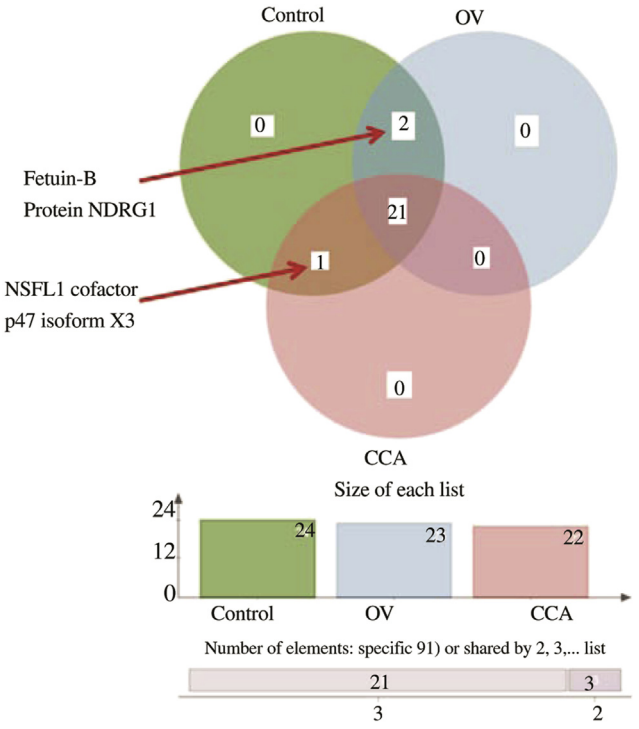

Figure 3. Venn diagrams showing 24 ConA-binding glycoproteins in the control, OV, and OV/DMN (CCA) groups. The numbers indicate glycoproteins which were present in each group, two of the three groups, and all of the three groups.

anticancer drugs: gemcitabine and cisplatin are presented in Figure 4. Gemcitabine and cisplatin were predicted to directly interact with EGFR, and TP53, but indirectly interact with MYC and NDRG1.

4. Discussion

The OV/DMN-induced CCA hamster model was used as an animal model for CCA in the present study as it closely mimics the pathogenesis of human CCA [14–20] OV infection is proposed as a promotor and the carcinogen DMN as an initiator of CCA carcinogenesis [14]. At the 6th month post-infection with OV metacercarae, tumor masses were found to develop in the livers of all the OV/DMN-induced CCA hamsters, but not in the control and OV groups. In the previous study, CCA tumor was detected as early as three months after induction [21]. Confirmation of CCA by histopathological examination is underway.

The isolated glycoproteins enriched by both ConA and WGA affinity chromatography were found to be differentially expressed in the pool plasma samples from the OV, OV/DMN (CCA) and control groups. Among the 24 ConA-binding glycoproteins isolated, two proteins, N-myc downstream regulated gene 1 (NDRG1) and fetuin-B (FETUB) were found up-regulated only in the samples from the OV and control groups, but not in the OV/DMN (CCA) groups. On the other hand, one protein, i.e., NSFL1 cofactor p47 isoform ×3 (NSFL1C) was found only in the samples from OV/DMN (CCA) and control groups, but not in the OV group. The remaining 21 proteins were upregulated in the samples from all groups.

NDRG1 is a cytoplasmic protein involved in stress responses, hormone responses, cell growth, and differentiation. The encoded protein is necessary for p53-mediated caspase activation and apoptosis [22]. The role of NDRG1 is controversial for various cancers. NDRG1 expression was found to be associated with good prognosis in colon cancer, and pancreatic cancer. On the other hand, its expression was

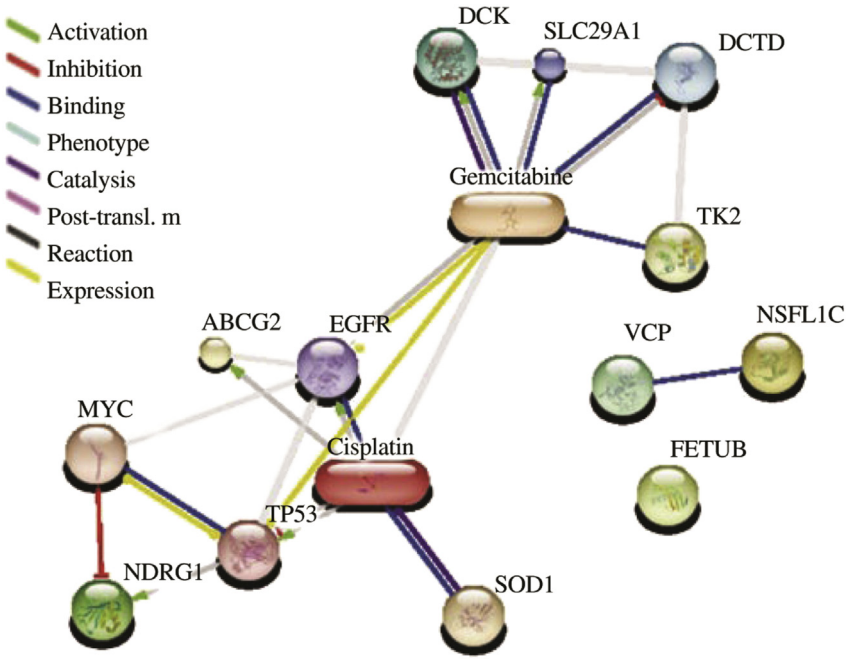

Figure 4. The networks of protein-chemical interactions of the three selected glycoproteins isolated by ConA-based affinity chromatography (red rounded rectangle) in plasma samples from the control, OV, and OV/DMN (CCA) groups, and anticancer drugs; gemcitabine and cisplatin (green rounded rectangle) predicted using STITCH 4 software. The interactions are shown in different color lines. (FETUB = Fetuin-B, NDRG1 = Protein NDRG1, and NSFL1C = NSFL1 cofactor p47 isoform ×3).

found to be associated with poor prognosis in non-small cell lung cancer, hepatocellular carcinoma, and gallbladder cancer. The discordant correlation between NDRG1 and disease prognosis has been reported for many types of cancer [23]. In our study, NDRG1 was found to be down-regulated in plasma sample of the CCA group.

Results obtained from STITCH 4 analysis (Figure 4) predicted the interaction of NDRG1 and anticancer drugs gemcitabine and cisplatin. Gemcitabine and cisplatin activate the expression of TP53 that improved expression of NDRG1. Currently chemotherapy combining gemcitabine and cisplatin is the recommended first-line treatment regimen for advanced biliary tract cancer. The combination of gemcitabine and cisplatin has been shown to improve overall survival and progression-free survival by 30% over gemcitabine alone [24]. The expression of drugs targeting on NDRG1 may provide novel therapeutic approach for CCA.

Taken together, results suggest that NDRG1, FETUB and NSFL1C glycoproteins isolated by ConA-based affinity chromatography could be potential biomarkers for CCA. Plasma samples with positive expression of NSFL1C, with no expression of NDRG1 and FETUB are likely suggestive of OV-associated CCA. These potential biomarkers may be exploited as tools for diagnosis, monitoring of disease prognosis and progress of CCA. This conclusion remains to be confirmed whether the battery test based on these biomarkers can discriminate OV-associated CCA from other risk factors. One limitation of the current study is that the biomarker analysis was performed using plasma samples from animals, not humans. Therefore, results may not totally represent the glycoprotein biomarkers in humans. Nevertheless, the advantage of using this animal model is that, it allows for possibility to investigate biomarkers for early diagnosis of CCA as it is not possible to obtain plasma samples from patients with early diagnosis. Further validation of the assay performance of this battery test in the human CCA is essential before application of the test as a diagnostic tool for CCA and OV infection.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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