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Proteomics-based identification of α -enolase as a potential prognostic marker in cholangiocarcinoma

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Abstract

Objectives—To investigate the association of expression status of α -enolase (ENO1) and clinicopathological outcomes of CCA patients.

Design and methods—Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) were used to compare differential expressed protein profiles of four human CCA cell lines and H69, a non-malignant biliary cell line, as a control. Immunohistochemical analysis was carried out in tissue-microarray of human CCA tissues (n = 301).

Results—We identified ENO1 in all CCA cell lines but not H69 by proteomics based. About 75% of patients with CCA showed over-expression of ENO1 in hyperplastic bile duct and the tumors compared with that in tumor-adjacent normal tissue counterparts. Moreover, over-expression of ENO1 is significantly associated with poor prognosis and tumor invasion of CCA patients.

Conclusions—ENO1 may serve as a prognostic marker to monitor the disease progression of these patients.

Keywords

Cholangiocarcinoma; Proteomics; Prognostic marker; α -enolase; ENO1

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor arising from the malignant transformation of cholangiocytes, the epithelial cells lining the biliary tree. CCA accounts for around 10–25% of the hepatobiliary malignancies in most parts of the world with age-standardized

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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incidence rates (ASRs) between 0.3 and less than 1.5 per 100 000 in Western countries. However, the incidence in Thailand, where the key risk factor - the liver fluke *Opisthorchis viverrini* (*O. viverrini*) is still endemic, is exceedingly high with ASRs of 33.4 per 100 000 in men and 12.3 per 100 000 in women [1]. Despite wide-spread treatment with praziquantel, the prevalence of *O. viverrini* in some endemic areas approaches 70% [2]. People become infected by eating raw or undercooked freshwater fish in regional dishes that contain metacercarial stage of *O. viverrini*. It has long been known that infection with *O. viverrini* associate with the tumor initiation in the development of CCA [3–5]. This has led the World Health Organization's International Agency for Research on Cancer to classify *O. viverrini* as a Group 1 carcinogen to humans [6]. Although mass anthelmintic therapy and intensive health education can protect individuals from being infected by *O. viverrini*, the early detection of CCA in those who have been infected by the fluke remains a challenge. CCA is characterized by a poor prognosis, with median survival of less than 24 months [7] and treatment options are limited. Surgical excision remains the mainstay of long-term survival for CCA but, unfortunately, the majority of patients are diagnosed at advanced stage, when surgical treatments are excluded [8]. Hence there is a tremendous interest and urgency to identify novel CCA biomarkers for early diagnosis and prognostication.

In recent years, more and more attention has been drawn to the study of this deadly disease. Two-dimensional gel electrophoresis (2-DE) combining with mass spectrometry (MS) is still the method of choice for the analysis of proteins above approximately 20–30 kDa. Although the technique itself is time consuming, difficult to reproduce and quantification, it can however be utilized in CCA research for the discovery of biomarkers as demonstrated by several groups [9,10]. Recently, we have reported on the membrane proteome analysis of four human *O. viverrini* associated CCA cell lines with a non-tumor H69 biliary cell line as a control using 2-DE and matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Annexin A2 (ANXA2) was identified as a prognostic marker for predicting the poor outcome of CCA patients [11]. In this study, we not only attempt to characterize cytosolic protein profiles from both CCA cell lines with different tumor forming capabilities and a non-tumor H69 biliary cell line, but also the prognostic significance of candidate proteins in the human liver, under normal conditions and in CCA.

Material and methods

Cell lines and cell culture

Four human CCA cell lines, M156, K100, M139 and M213, were isolated from CCA patients from north eastern Thailand as described elsewhere [12]. In all cases, CCA was associated with chronic *O. viverrini* infection. Approval for use of the tissue was obtained from the Human Research Ethics Committee of Khon Kaen University, Thailand. CCA tissues were histologically characterized based on their marginal zones as follows: moderately differentiated adenocarcinoma (M156), poorly differentiated adenocarcinoma (K100), squamous cell carcinoma (M139), and adenosquamous cell carcinoma (M213). CCA cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 100 U/mL penicillin and 100 µg/mL streptomycin with 10% FBS (Hyclone Laboratories). H69 cells, an 'immortalized' non-malignant human cholangiocyte cell line, were cultured as previously described [13]. Cell growth was performed at 37 °C under 5% CO₂ and 95% humidified air. During this experiment mycoplasma contamination was periodically checked and no contamination was present in any cell line.

Cholangiocarcinoma tissue samples

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 [11]. The experimental protocol was approved by the Human Research Ethics committee of Khon Kaen University (IRB No. HE42075). Of the 301 liver fluke-associated CCA samples analyzed, 203 were from males and 98 were from females at a ratio of 2:1. The mean ($\pm$ SD) age in years was 55 ± 9.4 years (range, 31–75 years). Most of the patients were at an advanced CCA stage, 73.9% ($n = 210$) with lymphatic invasion, 53.1% ($n = 152$) with vascular invasion, and 39.6% ($n = 112$) with perineural invasion. The histopathologic grade of differentiation of the tumors was assessed to be well-differentiated histophotological type in 53 (35%) patients. The majority of patients (63.5%) possessed a tumor size > 5 cm.

Cytosolic protein preparation

The cell lines were examined under a phase-contrast microscope to ensure that they were 70% confluent before lysis. The culture medium was discarded and the cells washed with 0.25 M sucrose in thrice on ice. Cells were scraped thoroughly with a scraper in 0.25 M sucrose containing 1% Protease Inhibitor Mix (GE healthcare). The cells were collected and centrifuged at $1,500 \times g$ for 5 min at 4°C . The pellets were resuspended in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPs (zwitterionic detergent commonly used in isoelectric focusing), 2% Immobilized pH gradient (IPG) buffer pH 3–10 nonlinear (GE healthcare), 40 mM Dithiothreitol (DTT), and 1% Protease Inhibitor Mix and allowed to lyse on ice for 15 min. Cell lysates were sonicated according to the manufacturer's instructions (Sonics & Materials Inc. VCX 400, USA) and incubated for 2 h at 4°C . The lysate was then centrifuged at $600 \times g$ for 10 min to remove the nuclei and unlysed cells. The supernatant containing cytosolic protein was transferred to a clean tube and centrifuged at $10,000 \times g$ for 15 min to remove the mitochondrial fraction. The supernatant was collected again and centrifuged at $100,000 \times g$ for 2 h to generate a pellet containing the enriched microsomal fraction and the supernatant representing the cytosolic fraction. The cytosolic protein concentration was determined using Bradford Assay using a VersaMaxTM absorbance microplate reader with SOFTMax pro (Molecular Devices Corporation California, USA) at 595 nm.

Two-Dimensional gel electrophoresis of cytosolic proteins

Total protein (100 μg) from CCA cell lines and H69 as a control were separated by 2-DE. Isoelectric focusing was performed using IPG strips 7 cm, pH 3–10 non-linear on an IPGphor isoelectric focusing cell (GE healthcare) and second-dimension SDS-PAGE using a Hoefer system (GE healthcare) as described previously [11]. After electrophoresis, the protein spots were visualized by CBR-250 (GE healthcare) staining. Stained gels were scanned using an ImageScanner (GE healthcare) and the 2-DE images of each cell line were compared using ImageMaster 2D Platinum 6.0 software (GE healthcare).

Protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

Protein spots that were unique to or stained more intensely in CCA cell lines compared with H69 cells were excised from the gel and transferred to V-bottom 96-well microtitre plates. Tryptic digestions were performed on an EttanTM Spot Handling Workstation robot (Amersham Biosciences) according to the manufacturer's specifications. The 10 mg of matrix solution, α -cyano-4-hydroxycinnamic acid (Bruker Daltonik GmbH, Germany), was prepared by dissolving to saturation in 50% acetonitrile/water with 0.1% trifluoroacetic acid and then mixed with equal volumes of tryptic peptide with matrix solution. The mixture (1 μL) was spotted onto a steel target surface (MTP 384 ground steel, Bruker Daltonik) and dried at room temperature. Mass spectra were recorded on an Autoflex MALDI-TOF-mass spectrometer (Bruker Daltonik) at a maximum accelerating potential of 19 kV and in reflector mode. The m/z range was from 400 to 4000. The MALDI-TOF-MS was calibrated

using trypsin auto-digestion peptide signals and matrix ion signals. Typically, 100 shots were accumulated from three to five different positions within a sample spot. The mass spectra were analyzed using MALDI evaluation software (Amersham Biosciences; release version 2.0). Proteins were identified by peptide mass fingerprinting using Mascot (<http://www.matrixscience.com>) in searches against the NCBI nr (NCBI nr 2009.04.03). The parameters used in the Mascot searches were as follows: (1) taxonomy was restricted to *Homo sapiens*, (2) trypsin specified with the allowance for one missed cleavage, (3) peptide mass tolerance fragment mass tolerance were set to 100 ppm and ± 0.5 Da, respectively and (4) carbamidomethyl and oxidized methionine were chosen as the fixed and variable modifications, respectively. The proteins were considered identified if they had resulting Mascot scores > 40 (significance level, $p < 0.05$) and at least two peptides with score > 20 . If peptides matched to multiple members of a protein family, proteins with shared peptides were grouped and the highest scoring protein was selected as the representative protein.

Tissue microarray and immunohistochemistry

Tissue microarrays (TMAs) were constructed by the Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand. Following TMA construction [14], an H&E stained section of the TMA recipient block was prepared and reviewed to confirm the presence of intact neoplasm. The arrays contained 301 CCA cases and 4 cases of adjacent normal liver tissues as negative control. Each core was taken from each formalin-fixed paraffin-embedded CCA sample by using punch cores that measured 0.9 mm in greatest diameter from the non-necrotic area of tumor foci. Prior to TMA construction, all tissue slides were histopathologically re-evaluated by experienced pathologist (Chawalit Pairojkul). The extent of invasion by the cancer was determined in both the interface of the growing tumor border and the adjacent liver tissue. Pathologic vascular invasion was defined as the presence of a tumor cell emboli within a vessel space, which were identified by associated fibrin clot and/or an endothelial cell lining. Lymphatic invasion was defined as being present when cancer cells were detected floating within an endothelium-lined space. Perineural invasion was defined as tumor invading the perineural sheath or endoneurium.

Immunohistochemical (IHC) reactions were performed on 4 μ m-thick sections of TMA silane-coated slides (Sigma, St. Louis, MO, USA) by an immunoperoxidase method. TMA sections probed with rabbit polyclonal anti-enolase 1 (Abcam Inc., USA) diluted 1:500 (v/v) diluted 1:400 (v/v) in PBS and incubated overnight at 4°C. After rinsing for 3 $\times$ 5 min with PBS the sections were incubated with horse radish peroxidase-conjugated goat anti-rabbit immunoglobulin (Zymed Labs, Carlsbad, CA) diluted 1:300 (v/v) in PBS and incubated at RT for 1 h. Sections were rinsed with PBS for 2 $\times$ 10 min, after which the sections were developed with DAB (Sigma Chemical Co.). The sections were counterstained with Mayer's haematoxylin, dehydrated, cleared in xylene and mounted in Permount.

Assessment of immunohistochemistry and statistical analysis

Immunoreactivity was evaluated independently by three researchers (Ponlapat Yonglitthipagon, Banchob Sripana and Chawalit Pairojkul) who were blinded to patient status and outcome evaluated immunoreactivity independently. The percentage of positive tumor cells was determined using interactive stereological immunoscore based on systematic random sampling [15], and the mean score calculated. More specifically, within a defined area of the lesion, we randomly selected five high-power fields (magnification, 400 $\times$; 100 cells/high-power field) and approximately 500 tumor cells were counted. In this study, the percentage of positive cells expressing ENO1 was categorized as follows: $<10\%$ (–), 10% (+) as described elsewhere [16].

For cross-sectional analyses, the chi-square test was utilized to analyze the relationship between ENO1 expressions and categorical variables regarding clinical pathology parameters (age group, gender, gross type, tumor size, vascular invasion, lymphatic invasion, perineural invasion and histologic subtype). The Kaplan–Meier method was used to calculate cumulative survival. Differences in survival between low expression and high expression groups were analyzed for significance by the log-rank method. The Cox-regression model was used to perform multivariate analysis, and values of $P < 0.05$ were considered statistically significant. The statistical analysis was performed using SPSS 16.0 (SPSS, Inc, Chicago, IL).

Results

2-DE of cytosolic proteins in H69 and CCA cell lines

In order to investigate the differential expression profile of cytosolic proteomes from cholangiocyte cell lines under non-tumor condition (H69) and in CCA (M156, KKU100, M139, three biological replicates for each cell type were generated. The separated protein spots were visualized on 2D gels by CBR-250 staining, and displayed good reproducibility for protein spot quantification and comparative analysis. Figure 1 shows representative 2-DE maps of each CCA cell lines (respectively M156, K100, M139 and M213) with spots subjected to MALDI-TOF MS and their identification numbers; the identified spots are listed in Table 1. The experimental pI and Mr values of identified proteins correlated with the theoretical values reported in the non-redundant National Center Biotechnology Information (NCBI) database. Some observed differences between the experimental and theoretical pI/Mr values and the presence of different isoforms of the same protein could reflect post-translational modifications or splice variants, as reported previously [11]

MALDI-TOF MS analysis of the differentially expressed spots

Using 2D gel replicates, identical and differential protein spots in 4 CCA cell lines subtracted from H69 were identified. In Table 1, the proteins, corresponding to 48 spots, which were expressed only in CCA cell lines but not in H69 are shown, together with the MS identification parameters. To classify the biological significance of the differentially expressed proteins, MS-identified proteins were classified into molecular and biological functional groups using the PANTHER database. Several of the identified proteins have been associated with tumorigenesis in previous studies, such as heat shock proteins (HSP) including HSP60 [17], HSP70/HSP90 [18], ezrin [19], moesin [20] and heterogeneous nuclear ribonucleoproteins (hnRNPs) [21]. As shown in Figure 2, MALDI-TOF MS analysis and peptide mass fingerprinting of the tryptic digests subsequently identified protein spot 17 as α -enolase (ENO1) which was found in all CCA cell lines but not in H69. ENO1 was then selected for further verification, as its over-expression correlates with tumor transformation and invasion/metastasis in other cancers [22,23], however, its prognostic implication in human *O. viverrini*-associated CCA tissues has not been explored.

Immunolocalization of ENO1 in CCA tissues

IHC studies were carried out on the TMAs (n=301) to examine whether the expression status of ENO1 changes during the development of bile duct cancer. The IHC result revealed a weak cytoplasmic staining of ENO1 in cholangiocytes and hepatocytes of adjacent non-cancerous tissue (Figure 3A) as well as 15 normal liver tissues as controls (data not shown). In contrast, strong intensity of cytoplasmic staining of ENO1 was observed in hyperplastic bile duct (Figure 3B) and the majority of corresponding CCA tissues. Interestingly, IHC staining also showed not only cytoplasmic but also membranous immunoreactivity. Moreover, identification of perineural invasion (Figure 3C) and tumor emboli within lymphatic vessel (Figure 3D) detected by anti-ENO1 antibody were also observed.

Association of ENO1 expression with clinical outcomes

To evaluate the relevance of ENO1 expression with clinicopathological outcomes of the patients, its expression status was quantified by interactive stereological immunostaining based on systematic random sampling [15]. The IHC showed that ENO1 was frequently overexpressed in CCA tissues, 74.75% (226/301) of biopsies stained positively. Statistical analysis showed that overexpression of ENO1 was significantly correlated with an age-related effect in young CCA patients (< 56 years; $P=0.0001$), a presence of lymphatic ($P=0.006$) and an absence of perineural invasion ($P=0.006$) shown in Table 2. No statistical differences in gender, histological subtype, gross type, tumor size or tumor emboli observed within vascular vessel were found between patients with low or high ENO1 expression.

Expression of ENO1 and cumulative survival

When the statistically significant variables in the univariate analyses were included in a Cox proportional hazard analysis, over-expression of ENO1 (hazard ratio, 1.733; $P=0.001$) and less differentiated histological type of CCA (hazard ratio, 1.47; $P=0.036$) were identified as independent predictive factors of patient survival (Table 3). The median length of survival for patients with high and low ENO1 expression was 28.14 weeks (95% CI, 22.74–33.54 weeks) and 44 weeks (95% CI, 26.72–61.28 weeks) as shown in Table 3, respectively. Survival analysis according to Kaplan-Meier method revealed significant correlation between ENO1 expression status and the survival in all patients as displayed in Figure 4. With log-rank test, we test the association between patients with high and low expression of ENO1. We found that patients with high ENO1 expression had much poorer survival than patients with low ENO1 expression ($P=0.004$). Taken together, these data strongly support expression status of ENO1 to be a potential prognostic marker for survival outcomes of CCA patients.

Discussion

In this study, we applied a proteomics-based approach to identify differentially expressed tumor proteins in CCA cell lines. However, a drawback in using cell lines is that the cells are not representative of the tissue of origin because they have been maintained and propagated *in vitro* for extended time periods. Furthermore, each cell line was established from a few cells of a single patient biopsy, so the *in vitro* model might not accurately reflect the dynamic carcinogenesis process [24]. Accordingly, validation of the cell line results in patient biopsies is essential.

We identified 47.35 kDa protein as human alpha-enolase (ENO1), which was identified in all CCA cell lines but not H69. About 75% of patients with CCA, we have examined thus far consistently show up-regulation of ENO1 in hyperplastic bile duct and the tumors compared with that in tumor-adjacent normal tissue counterparts. Moreover, the statistical analyses correlating ENO1 expression in the patients with their clinicopathological outcomes further reveal that the expression status of ENO1 is highly associated with survival and tumor invasion of CCA patients.

Enolase is a glycolytic enzyme of approximately 47 kD and acts as a dimer containing Mg^{2+} responsible for catalyzing the conversion of 2-phosphoglycerate (2-PGE) to phosphoenolpyruvate (PEP) in the penultimate step of Embden Meyerhoff Parnas pathway [25]. Vertebrates exhibit three tissue-specific enolase isozymes including α , β and γ -enolase [26]. α -enolase (enolase 1; ENO1) is found mainly in the liver although it is also present in other tissues, while β -enolase is abundant in muscles and γ -enolase is localized in neural tissues, respectively [27].

Over-expression of ENO1 is a common scenario in several cancers [28–30], a bioinformatics study using gene chips and ESTs databases further supports a correlation between ENO1 expression and tumorigenicity [31]. In response to a number of stimuli, such as hypoxia, cytokine (interferon $\alpha_2\alpha_1$; $\text{INF}\alpha_2\alpha_1$) [32] and signals mediated by the MEK/ERK 1/2 pathway [33], normal cells will increase gene expression of ENO1 to adapt environmental stress through activation of hypoxic-inducible transcription factor. However, this cellular process is irreversible for cancer cells. Additionally, ENO1 may act as a stress protein that promotes hypoxic tolerance in tumor cells by increasing anaerobic metabolism (Warburg effect) [34]. ENO1 also functions as a strong plasminogen-binding receptor on the cell surface of non-small cell lung cancer and cancer of head and neck enhancing local fibrinolysis and contribute to tumor invasion [29,22]. Consistent with this possibility, we found that ENO1 was also detected on the cell surface of CCA, in addition to its presence in the cytosol and its expression was also associated with established features of tumor aggressiveness including positive tumor invasion in lymphatic vessels and perineural tissues. Hence, its dual location in cytosol and on cell surface indicated its pivotal role in glycolysis and tumor progression, respectively. However, the factors that affect its export and membrane association remain a mystery and require further investigation. In addition, the expression pattern we reported are accordance with previous findings that the patients with well differentiated histopathological type of CCA had longer survival times after surgery compared to the less differentiated type [35].

In conclusion, 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 or distal metastasis. In this study, up-regulation of ENO1 in CCA patients is significantly associated with poorer clinical prognosis for survival. Coincidentally, surface ENO1 also detected in the effusion tumor cells may implicate its potential role in cell invasion. Hence, ENO1 may be used as a good prognostic indicator for CCA patients as its expression could be widely determined on a large number of routinely processed, formalin-fixed paraffin-embedded (FFPE) CCA tissues.

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Highlights

- We analyzed the cytosolic proteome of *Opisthorchis viverrini* (Ov)-associated cholangiocarcinoma (CCA) cell lines
- We have identified different 2-DE maps of cytosolic proteome among CCA cell lines using H69, a non-malignant biliary cell line as a control
- Several differential expressed proteins were identified by MALDI-TOF MS
- α -enolase (ENO1) may contribute potential prognostic marker for CCA.

Fig. 1.

Comparative proteomic analysis of 2-DE gels of CCA cell lines; M156, moderately differentiated (A), KKU-100, poorly differentiated (B), M139, squamous cell carcinoma (C) and M213, adenosquamous cell carcinoma (D). Number of spots on 2-DE showed a total of 405 ± 36 , 316 ± 56 , 368 ± 18 and 245 ± 34 (mean $\pm$ S.E. of three biological replicates) for the M156, KKU-100, M139 and M213, respectively.

Fig. 2.

Peptide mass fingerprint (PMF) of tryptic digests of enolase 1 obtained by MALDI-TOF MS. The spectrum displays m/z ratio (x -axis) and relative intensity (y -axis) of the peptides identified (A). Matched peptides of enolase 1 were 53% protein sequence coverage showed by red and underline (B). Protein identification using PMF data was performed with the Mascot search engine; acquired spectra were processed and search against the NCBI nr database.

Fig. 3. Immunohistochemical staining for enolase 1 (ENO1) in surgical tissue specimens. Weak staining of ENO1 was observed in the cytoplasm of corresponding normal liver tissue (A). In contrast, ENO1 was highly expressed in cytoplasm of hyperplastic bile duct (B). In addition, identification of perineural invasion (C) and lymphatic permeation (D) detected by ENO1 was observed not only in the cytosol but also on the membranous. Immunoperoxidase staining; a brown precipitate in the cell denotes positive signal, original magnification $\times 100$ (A–B), $\times 200$ (C–D). L, lymphatic vessel; N, neural tissue.

Fig. 4. Kaplan-Meier cumulative survival curves of *Opisthorchis viverrini*-induced cholangiocarcinoma (CCA) patients categorized by the expression status of enolase 1 (ENO1). Patients with increased levels of ENO1 expression had significantly poorer survival than patients with low expression levels according to the log-rank test, significance level 0.004 (Table 3).

Table 1
List of differentially expressed cytosolic proteins from 4 CCA cell lines identified by MALDI-TOF MS

No. ^a	A/N ^b	Description	Biological process	MS ^c	MP/TP ^d	CO ^e	Mr	pI	H69	M156	K100	M139	M213
1	gi 34147630	Tu translation elongation factor	Signal transduction	194	28/38	65	50.19	7.26	-	+	+	-	-
2	gi 1710248	Protein disulfide isomerase	Protein metabolic process	69	7/36	17	46.51	4.95	-	-	+	+	-
3	gi 169404695	Chain A, Pyruvate Kinase M2	Carbohydrate metabolic process	185	22/51	38	57.09	8	-	-	+	+	-
4	gi 544759	Biliverdin-IX beta reductase isozyme I	Unknown	110	10/27	51	21.96	7.31	-	-	+	+	-
5	gi 5031635	Cofilin 1	Cellular component morphogenesis	202	18/55	75	18.72	8.22	-	+	-	-	+
6	gi 4505773	Prohibitin	Nucleic and nucleotide metabolic process	211	17/42	73	29.84	5.57	-	+	-	+	+
7	gi 4758638	Peroxiredoxin 6	Oxygen and reactive oxygen species metabolic process	147	17/50	79	25.13	6	-	+	-	+	+
8	gi 119581639	Proteasome	Protein metabolic process	240	28/53	65	43.22	5.97	-	+	-	+	+
9	gi 4502643	Chaperonin containing TCP1	Protein metabolic process	306	38/80	65	58.44	6.23	-	+	-	+	+
10	gi 999892	Triosephosphate Isomerase	Carbohydrate metabolic process	220	19/42	84	26.81	6.51	-	+	-	+	+
11	gi 18314408	Nucleophosmin	Nucleic and nucleotide metabolic process	78	11/50	27	32.73	4.59	-	+	+	+	-
12	gi 15277503	ACTB protein	Cellular component morphogenesis	164	21/44	67	40.54	5.35	-	+	+	+	-
13	gi 62897681	Calreticulin	Protein metabolic process	222	20/38	58	47.06	4.3	-	+	+	+	-
14	gi 48145549	PGK1	Carbohydrate metabolic process	354	36/46	62	44.97	8.3	-	+	+	+	+
15	gi 90111766	Keratin 19	Cellular component organization	375	34/49	75	44.08	5.04	-	+	+	+	+
16	gi 119582950	Annexin A1	Intracellular protein transport	252	28/45	77	40.48	6.57	-	+	+	+	+
17	gi 203282367	Enolase 1	Carbohydrate metabolic process	174	21/44	53	47.35	6.99	-	+	+	+	+
18	gi 3023905	Glutathione S-transferase	Immune system process	148	12/34	57	23.64	6.89	-	-	+	+	-
19	gi 4502107	Annexin 5	Signal transduction	176	17/27	54	35.97	4.94	-	+	+	-	-
20	gi 197210452	Uridine monophosphate synthetase	Unknown	95	11/53	16	52.71	6.81	-	-	+	-	+
21	gi 4503727	FK506 binding protein 3, 25kDa	Negative regulation of apoptosis	109	12/38	44	25.22	9.26	-	-	+	-	+
22	gi 67464043	Human Liver Gapdh	Carbohydrate metabolic process	185	19/29	57	36.48	8.53	-	+	-	+	-
23	gi 82195535	Gamma-actin	Cellular component morphogenesis	165	22/50	64	42.09	5.3	-	+	-	+	-
24	gi 119602173	Heat shock protein 90kDa	Response to stress	117	16/35	27	57.87	4.92	-	-	+	-	-
25	gi 4507651	Tropomyosin 4 isoform 2	Cellular component organization	146	14/31	43	28.62	4.67	-	-	+	-	-
26	gi 21620034	ZSCAN21 protein	Nucleic and nucleotide metabolic process	43	12/20	24	26.69	9.45	-	-	+	-	-

No. ^a	A/N ^b	Description	Biological process	MS ^c	MP/TP ^d	CO ^e	Mr	pI	H69	M156	K100	M139	M213
27	gi 46409504	Hypothetical protein LOC400165	Unknown	46	42/82	38	13.69	8.66	-	-	+	-	-
28	gi 123266507	guanylatecyclase 1, soluble, beta 2	Nucleic and nucleotide metabolic process	44	39/56	19	44.95	8.84	-	-	+	-	-
29	gi 7669492	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolic process	67	42/55	18	36.21	8.57	-	-	+	-	-
30	gi 5174735	Tubulin, beta, 2	Intracellular protein transport	148	23/36	57	50.26	4.79	-	-	+	-	-
31	gi 7108915	Glucocorticoid receptor AF-1	Signal transduction	69	42/85	19	46.58	9.08	-	-	+	-	-
32	gi 49119653	YWHAZ protein	Signal transduction	153	16/37	45	30.11	4.72	-	-	+	-	-
33	gi 189502784	Heat shock protein 60kD	Response to stress	249	30/48	59	60.81	5.83	-	-	-	+	-
34	gi 4504447	hnRNP A2/B1	Intracellular protein transport	89	19/46	44	36.04	8.67	-	-	-	+	-
35	gi 4507669	Tumor protein, translationally-controlled 1	Immune system process	116	16/41	61	19.69	4.84	-	-	-	+	-
36	gi 55960374	Transgelin 2	Muscle contraction	88	10/31	45	21.24	7.63	-	-	-	+	-
37	gi 31542947	Chaperonin	Protein metabolic process	230	26/50	52	61.19	5.7	-	+	-	-	-
38	gi 4505591	Peroxiredoxin 1	Oxygen and reactive oxygen species metabolic process	243	19/33	76	22.32	8.27	-	+	-	-	-
39	gi 46249758	Ezrin	Intracellular protein transport	258	28/31	43	69.31	5.94	-	+	-	-	-
40	gi 4505257	Moesin	Intracellular protein transport	146	26/46	33	67.89	6.08	-	+	-	-	-
41	gi 5803181	Hsp70/Hsp90	Response to stress	129	38/85	55	63.23	6.4	-	+	-	-	-
42	gi 52632385	hnRNP L	Nucleic and nucleotide metabolic process	104	13/28	35	51.16	7.22	-	+	-	-	-
43	gi 38013966	TKT protein	Lipid metabolic process	303	22/26	46	58.74	6.51	-	+	-	-	-
44	gi 167860126	Serine proteinase inhibitor	Protein metabolic process	278	25/42	73	42.53	5.72	-	+	-	-	-
45	gi 12803727	Keratin 7	Cellular component organization	210	22/33	46	51.44	5.42	-	-	-	-	+
46	gi 42476281	Voltage-dependent anion channel 2	Ion transport	147	13/25	54	32.06	7.49	-	-	-	-	+
47	gi 5174447	Guanine nucleotide binding protein	Intracellular protein transport	225	21/32	56	35.51	7.61	-	-	-	-	+
48	gi 54303910	Aging-associated gene 9 protein	Carbohydrate metabolic process	155	14/27	43	36.19	8.57	-	-	-	-	+

Note: The presence of a protein in the relevant study is denoted with a '+', and the absence with a '-'. No., numbers correspond to Figure 1; A/N, NCBI database accession numbers; MS, Mascot score (MS = $-10 \times \log(P)$ where P is the probability that the observed match is a random event from MS analysis; MP/TP, the number of matched peaks/total peaks in MS analysis; CO, percentage of sequence coverage; Mr, relative molecular mass; pI, isoelectric point.

Table 2

Clinicopathological variables and the expression status of enolase 1 (ENO1) in cholangiocarcinoma tissues

Variables	<u>Enolase 1</u>		<i>P</i>
	–ve	+ve	
Age (yr)			<0.0001
56	55	114	
> 56	19	111	
Gender			NS
Male	49	154	
Female	25	70	
Histotype			NS
Less diff.	57	181	
Well diff.	13	40	
Gross type			NS
Mass forming	36	106	
Periductal infiltrating	12	49	
Intraductal	2	7	
Tumor size			NS
5	20	54	
> 5	39	90	
Vascular invasion			NS
Absent	32	102	
Present	38	114	
Lymphatic invasion			0.006
Absent	27	47	
Present	43	167	
Perineural invasion			0.006
Absent	52	119	
Present	18	94	

Note: When the sum of subset numbers does not match patient totals, data were missing or unavailable.

Table 3

Clinical risk factors for overall survival in cholangiocarcinoma patients

Risk factors	Overall survival				
	Univariate analysis (Log-rank)			Multivariate analysis (Cox regression)	
	Median time, wk± SE (95% CI)	P	Relative risk	95%CI	P
Enolase 1		0.004			0.001
Absent	44±8.82 (26.716–61.284)		1	0.417–0.798	
Present	28.14±1.42 (22.74–33.54)		1.733		
Sex		0.03			NS
Male	27.14±1.74 (23.74–30.54)		1	0.572–1.016	
Female	40.14±4.22 (31.86–48.42)		1.312		
Histology group		0.03			0.036
Less diff.	29.00±2.63 (23.85–34.15)		1.47	0.473–0.975	
Well diff.	41.14±6.47 (28.47–53.81)		1		

Abbreviations: CI, confident interval; NS, not significant.