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High expression of apoptosis-inducing factor, mitochondrion-associated 3 (AIFM3) in human cholangiocarcinoma

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Abstract Prognosis of cholangiocarcinoma (CCA) patients is absolutely poor in spite of extensive efforts for the development of chemotherapy. Mitochondrial proteins play key roles in carcinogenesis of various cancers. Therefore, mitochondria are considered as the target organelles for chemotherapy of several cancers including CCA. The purpose of this study is to identify potential candidate proteins for chemotherapy using mitochondrial proteome analysis for CCA tissues. A shotgun proteomic approach using SDS-PAGE coupled with LC-MS/MS was applied to compare the expression of mitochondrial proteins in CCA and the adjacent non-cancerous tissues. Using the proteomic analysis for the pooled mitochondrial proteins purified from three each of papillary and non-papillary types of CCA and their adjacent tissues, 281 proteins were identified as mitochondrial proteins, and 105 of them have significantly different expression levels compared with the corresponding counterparts. Among the 105 proteins, apoptosis-inducing factor, mitochondrion-associated 3 (AIFM3) was a unique protein commonly over-expressed in both papillary and non-papillary types of CCA tissues but not in the adjacent non-cancerous tissues. In conclusion, AIFM3 was aberrantly expressed only in the mitochondria of CCA tissues. This finding suggests that AIFM3 could be a potential target molecule for CCA chemotherapy.

Keywords Apoptosis-inducing factor · Mitochondrial proteins · Mass spectrometry · Cholangiocarcinoma

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Introduction

Cholangiocarcinoma (CCA) is a cancer originating from the bile duct epithelium (cholangiocytes). It is rare in Western countries, but it is highly prevalent in the Northeast Thailand particularly in Khon Kaen province, where the prevalence is highest in the world. [1]. The main risk factor for CCA in this area is an infection with carcinogenic liver flukes, *Opisthorchis viverrini* (OV) due to dietary habits of eating raw or undercooked freshwater fish and poor sanitation practice. Liver fluke infestation induces chronic inflammation leading to oxidative DNA damage and CCA transformation of the infected bile ducts [1, 2]. At present, opisthorchiasis-associated CCA is a serious public health problem in this region.

Surgery to remove the cancer completely is the only possible curative treatment for CCA. However, most of CCA patients are diagnosed with advanced unresectable tumors. For postoperative treatment or the treatment of unresectable cases, chemotherapy in combination with or without radiotherapy has been used even with poor prognosis. Although several

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attempts have been developed for screening, diagnosis and treatment of CCA, practically none of those attempts brought about apparent improvement of survival of patients. However, targeted cancer therapies are still required to increase the efficacy of cancer treatment and reduce the side effects [3].

Mitochondrial proteins play key roles in pathogenesis, cellular patterns [4], and carcinogenesis of various cancers e.g., renal [5], gastric [6], breast [7], hepatocellular [8], and colon cancer [9]. Thus, mitochondria are considered as target organelles for cancer chemotherapy. In particular, the expression levels of the mitochondrial proteins such as serine hydroxymethyltransferase 2, complement 1q-binding protein, ornithine aminotransferase, mitochondrial uncoupling protein 2, mortalin, succinyl-CoA/3, and ketoacid coenzyme A transferase1 were found to correlate with the progression of various cancers [7–12]. For CCA therapy, combinations of mitochondrial protein induction and chemotherapy or radiotherapy were reported to enhance apoptosis of CCA cells [13–15]. Also, high expression of a mitochondrial protein, superoxide dismutase 2 (SOD2), was observed during CCA genesis in hamster model [16]. Recent study of whole mitochondrial DNA (mtDNA) genome sequence analysis, however, revealed that there is no direct association between mtDNA mutations and CCA [17]. Therefore, differential expression of mitochondrial proteins in CCA and adjacent non-cancerous tissues may provide better understanding of the roles of mitochondrial proteins in carcinogenesis and pave us the way to discover new molecular targets for diagnosis and treatment of CCA. Here, we report that using proteomic analysis of mitochondrial proteins in CCA and adjacent non-cancerous tissues, AIFM3 was overexpressed only in CCA regardless of the tumor types but not in non-cancerous tissues, suggesting its potential target for chemotherapy.

Materials and methods

Tumor samples

A total of 25 CCA samples (11 non-papillary and 14 papillary types) were used in this study. Among those samples, three, each of non-papillary and papillary CCA containing cancerous and adjacent non-cancerous tissues were used for the isolations of mitochondria and subsequently for proteomic analyses. Paraffin-embedded sections of all 25 samples were used for immunohistochemistry.

All samples were kindly provided from the Liver Flukes and Cholangiocarcinoma Research Center (LFCRC), Faculty of Medicine, Khon Kaen University, Thailand. They were immediately snap-frozen in liquid nitrogen and stored at −80 °C until use. For histopathological typing of CCA, frozen sections were collected from each tissue and a couple of sections from each tissue were fixed in a 4 % buffered formalin

and stained with hematoxylin and eosin. This project was approved by the Khon Kaen University Ethics Committee for Human Research (HE571416).

Isolation of mitochondrial protein fractions

The methods for isolation of mitochondrial protein fractions followed to those described previously [18–20]. Approximately, 100–200 mg of frozen human CCA and its adjacent non-cancerous tissues were washed with ice-cold phosphate buffered saline (PBS, 0.1 M Sodium phosphate, 0.15 M Sodium chloride, pH 7.2) to remove any blood clots and connective tissues. Mitochondria were isolated using a Mitochondria Isolation Kit for Tissue (Pierce, Rockford, IL, USA) according to the manufacturer's instructions with some modifications. In brief, human CCA and adjacent non-cancerous tissues were separately minced in each of the 800 μl of the Mitochondria Isolation Reagent 1, supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and carefully homogenized about 40–80 strokes on ice [19]. Each crude homogenate was then returned to the original tube, and 800 μl of the Mitochondria Isolation Reagent 2 supplemented with protease inhibitor cocktail (Roche) was added for further cell lysis. The tube was centrifuged at 700×g for 5 min at 4 °C to spin down unbroken tissues, cell debris, and nuclei. The supernatant was transferred into a new tube and centrifuged at 12,000×g for 10 min at 4 °C to spin down mitochondria [20]. The supernatant was transferred into a new tube as cytosolic lysates. Then, 500 μl of the Mitochondria Isolation Reagent 2 was added to the pellet, resuspended, and the suspension was centrifuged at 12,000×g for 5 min at 4 °C to reduce contamination of other subcellular components. The pellet was lysed in lysis buffer containing 0.5 % SDS and protease inhibitors (Roche). Protein concentrations of both mitochondrial and cytosolic lysates were determined by Lowry's method [21]. Voltage-dependent anion channel (VDAC) protein, a mitochondrial marker, was investigated using western blot analysis.

Electrophoresis and tryptic digestion

Mitochondrial proteins from each non-papillary and papillary CCA and their corresponding adjacent non-cancerous tissues were separately pooled (50 μg aliquots for each sample). Protein samples (50 μg each) were separated on 12.5 % SDS-PAGE (ATTO AE-6530 system, Tokyo, Japan). After Coomassie Brilliant Blue (CBB) staining, the gel lanes were cut into 12 ranges. Each gel range was chopped into fine pieces (1 mm^3/piece), and transferred to a 96-well plate (12–16 pieces/well). The gel pieces were subjected to in-gel digestion according to the method developed by the Proteomics Research Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC) and the National Science and Technology Development Agency (NSTDA),

Thailand. In brief, the gel pieces were dehydrated twice in 100 % acetonitrile (ACN) for 5 min each with agitation and dried at room temperature for 15 min. Subsequently, disulfide bonds were reduced with 10 mM dithiothreitol (DTT)/10 mM ammonium bicarbonate (NH_4HCO_3) for 1 h at room temperature and alkylated with 100 mM iodoacetamide (IAA)/10 mM NH_4HCO_3 for 1 h at room temperature in the dark. Then, 20 μl of 10 ng/μl trypsin in 50 % ACN/10 mM NH_4HCO_3 was added and incubated for 20 min before adding 20 μl of 30 % ACN. The reaction mixture was incubated overnight at 37 °C. The tryptic peptides were extracted from the gel for three times with 30 μl of 50 % ACN/0.1 % formic acid. Finally, the tryptic peptide mixtures were dried and kept at −80 °C until LC-MS/MS analysis.

LC-MS/MS analysis

The extracted peptides were dissolved in 15 μl of 0.1 % formic acid, centrifuged at 12,000×*g* for 10 min, and separated using an Ultimate 3000 LC System (Dionex Ltd., U.K.) on a nanocolumn PepSwift monolithic column 100 mm-i.d.650 mm, with a flow rate of 300 nl/min using multi-steps gradient of a linear concentration increase from 10 to 90 % of 80 % acetonitrile in 0.1 % formic acid within 20 min. The nanoLC system was connected with an electrospray interface with ESI-Ion Trap MS (Bruker Daltonik GmbH, Bremen, Germany). The collected LC-MS raw data were directly used for quantification based on MS signal intensities of individual analysis using DeCyder MS differential analysis software (DeCyderMS, GE Healthcare, Piscataway). All differential peptide data were submitted to database search against the NCBI human mitochondria database using MASCOT software version 2.2 (Matrix Science, London, UK). Search parameters were as follows: Trypsin was selected as the enzyme, with three potential missed cleavage, carbamido-methylated cysteine as fixed modification, and oxidation of methionine residues as a variable modification. Peptide mass tolerance was 1.2 Da, while fragment mass tolerance was 0.6 Da, and ESI ion trap was selected for the instrument type. Proteins were selected with statistical significance at $P < 0.05$ as shown in maps of protein levels using MultiExperiment Viewer (MeV, version 4.6.1) software. Then, all significantly expressed proteins were analyzed for their intersections among the different sample groups using jvenn (http://bioinfo.genotoul.fr/jvenn/example.html). Finally, the mitochondrial proteins that exclusively expressed in both types of CCA tissues, but not in adjacent non-cancerous tissues, were chosen as candidate proteins.

Western blot analysis

To validate the integrity of harvested mitochondria, immune-blot distribution patterns of human voltage-dependent anion channel (VDA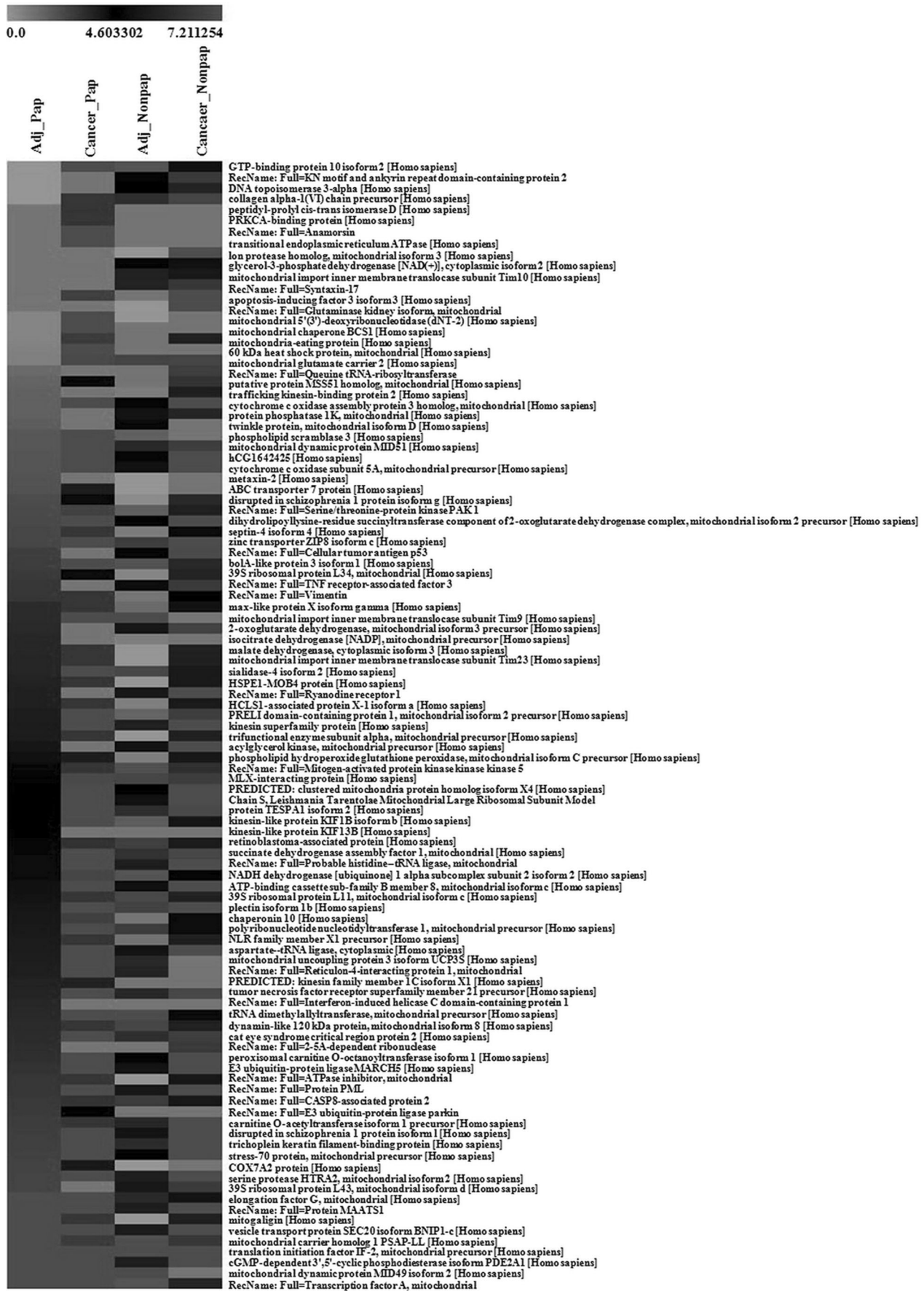C) protein in the isolated mitochondrial and cytosolic fractions were examined using anti-human VDAC antibody (Biorbyt, UK). Fifty micrograms of proteins were size-fractionated by SDS-PAGE using 12.5 % polyacrylamide gels and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, U.K.) for 1 h, and the membrane was blocked with 5 % skim milk in Tris-buffer saline with 0.1 % Tween-20 (TBS-T, pH 7.4) for 1 h at room temperature. Then, the membrane was incubated overnight at 4 °C with rabbit polyclonal antibody against human VDAC. After washing with TBS-T, the membrane was incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody and goat anti-rabbbit IgG (GE Healthcare) diluted in 5 % skim milk in TBS-T. The chemiluminescence was detected using the enhanced chemiluminescence ECL plus system (GE Healthcare) and then visualized using the Image Quant LASmini400.

To validate the candidate mitochondrial protein, AIFM3, its expression level in the individual sample was investigated by western blotting using AIFM3 antibody (Biorbyt, UK). The membrane was incubated overnight at 4 °C with rabbit polyclonal antibody against human AIFM3 (diluted 1:500 in TBS-T) and treated with the secondary antibody labeled with horseradish peroxidase. The antigen-antibody interaction on the membrane was detected as described above.
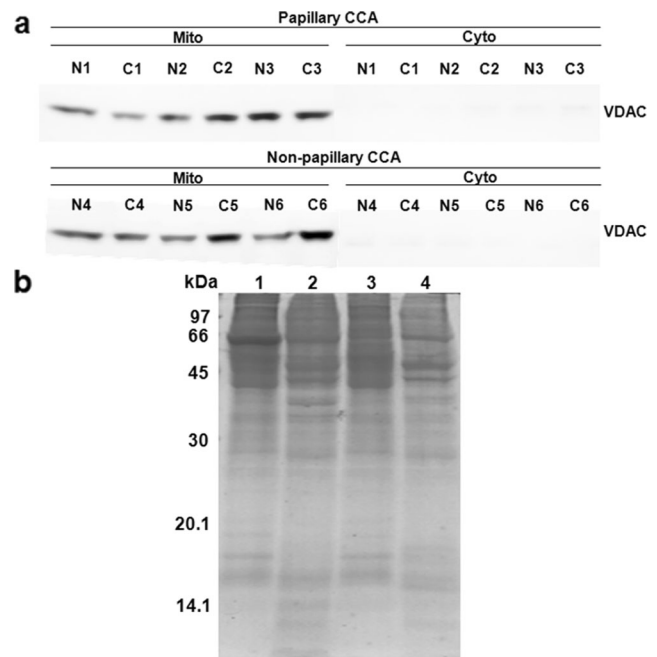
Fig. 1 Proteomic analysis of mitochondrial proteome. **a** Validation of the mitochondrial protein extracts; N, adjacent non-cancerous tissue; C, cancerous tissue of CCA; Mito, mitochondrial protein fraction; Cyto, cytosolic fraction. **b** Mitochondrial proteins (50 μg each) were pooled and 50 μg proteins were loaded onto each well of 12.5 % SDS-PAGE. Protein fractions were visualized by CBB staining; Lane 1: three matched-pairs of adjacent non-cancerous tissue of papillary CCA, Lane 2: three papillary CCA, Lane 3: three matched-pairs of adjacent non-cancerous tissue of non-papillary CCA, Lane 4: three non-papillary CCA

0.0   4.603302   7.211254

Adj_Pap
Cancer_Pap
Adj_Nonpap
Cancaer_Nonpap

GTP-binding protein 10 isoform 2 [Homo sapiens]
RecName: Full=KN motif and ankyrin repeat domain-containing protein 2
DNA topoisomerase 3-alpha [Homo sapiens]
collagen alpha-1(VI) chain precursor [Homo sapiens]
peptidyl-prolyl cis-trans isomerase D [Homo sapiens]
PRKCA-binding protein [Homo sapiens]
RecName: Full=Anamorsin
transitional endoplasmic reticulum ATPase [Homo sapiens]
lon protease homolog, mitochondrial isoform 3 [Homo sapiens]
glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic isoform 2 [Homo sapiens]
mitochondrial import inner membrane translocase subunit Tim10 [Homo sapiens]
RecName: Full=Syntaxin-17
apoptosis-inducing factor 3 isoform 3 [Homo sapiens]
RecName: Full=Glutaminase kidney isoform, mitochondrial
mitochondrial 5'(3')-deoxyribonucleotidase (dNT-2) [Homo sapiens]
mitochondrial chaperone BCS1 [Homo sapiens]
mitochondria-eating protein [Homo sapiens]
60 kDa heat shock protein, mitochondrial [Homo sapiens]
mitochondrial glutamate carrier 2 [Homo sapiens]
RecName: Full=Queuine tRNA-ribosyltransferase
putative protein MSS51 homolog, mitochondrial [Homo sapiens]
trafficking kinesin-binding protein 2 [Homo sapiens]
cytochrome c oxidase assembly protein 3 homolog, mitochondrial [Homo sapiens]
protein phosphatase 1K, mitochondrial [Homo sapiens]
twinkle protein, mitochondrial isoform D [Homo sapiens]
phospholipid scramblase 3 [Homo sapiens]
mitochondrial dynamic protein MID51 [Homo sapiens]
hCG1642425 [Homo sapiens]
cytochrome c oxidase subunit 5A, mitochondrial precursor [Homo sapiens]
metaxin-2 [Homo sapiens]
ABC transporter 7 protein [Homo sapiens]
disrupted in schizophrenia 1 protein isoform g [Homo sapiens]
RecName: Full=Serine/threonine-protein kinase PAK 1
dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial isoform 2 precursor [Homo sapiens]
septin-4 isoform 4 [Homo sapiens]
zinc transporter ZIP8 isoform c [Homo sapiens]
RecName: Full=Cellular tumor antigen p53
bolA-like protein 3 isoform 1 [Homo sapiens]
39S ribosomal protein L34, mitochondrial [Homo sapiens]
RecName: Full=TNF receptor-associated factor 3
RecName: Full=Vimentin
max-like protein X isoform gamma [Homo sapiens]
mitochondrial import inner membrane translocase subunit Tim9 [Homo sapiens]
2-oxoglutarate dehydrogenase, mitochondrial isoform 3 precursor [Homo sapiens]
isocitrate dehydrogenase [NADP], mitochondrial precursor [Homo sapiens]
malate dehydrogenase, cytoplasmic isoform 3 [Homo sapiens]
mitochondrial import inner membrane translocase subunit Tim23 [Homo sapiens]
sialidase-4 isoform 2 [Homo sapiens]
HSPE1-MOB4 protein [Homo sapiens]
RecName: Full=Ryanodine receptor 1
HCLS1-associated protein X-1 isoform a [Homo sapiens]
PRELI domain-containing protein 1, mitochondrial isoform 2 precursor [Homo sapiens]
kinesin superfamily protein [Homo sapiens]
trifunctional enzyme subunit alpha, mitochondrial precursor [Homo sapiens]
acylglycerol kinase, mitochondrial precursor [Homo sapiens]
phospholipid hydroperoxide glutathione peroxidase, mitochondrial isoform C precursor [Homo sapiens]
RecName: Full=Mitogen-activated protein kinase kinase kinase 5
MLX-interacting protein [Homo sapiens]
PREDICTED: clustered mitochondria protein homolog isoform X4 [Homo sapiens]
Chain S, Leishmania Tarentolae Mitochondrial Large Ribosomal Subunit Model
protein TESPA1 isoform 2 [Homo sapiens]
kinesin-like protein KIF1B isoform b [Homo sapiens]
kinesin-like protein KIF13B [Homo sapiens]
retinoblastoma-associated protein [Homo sapiens]
succinate dehydrogenase assembly factor 1, mitochondrial [Homo sapiens]
RecName: Full=Probable histidine–tRNA ligase, mitochondrial
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 isoform 2 [Homo sapiens]
ATP-binding cassette sub-family B member 8, mitochondrial isoform c [Homo sapiens]
39S ribosomal protein L11, mitochondrial isoform c [Homo sapiens]
plectin isoform 1b [Homo sapiens]
chaperonin 10 [Homo sapiens]
polyribonucleotide nucleotidyltransferase 1, mitochondrial precursor [Homo sapiens]
NLR family member X1 precursor [Homo sapiens]
aspartate--tRNA ligase, cytoplasmic [Homo sapiens]
mitochondrial uncoupling protein 3 isoform UCP3S [Homo sapiens]
RecName: Full=Reticulon-4-interacting protein 1, mitochondrial
PREDICTED: kinesin family member 1C isoform X1 [Homo sapiens]
tumor necrosis factor receptor superfamily member 21 precursor [Homo sapiens]
RecName: Full=Interferon-induced helicase C domain-containing protein 1
tRNA dimethylallyltransferase, mitochondrial precursor [Homo sapiens]
dynamin-like 120 kDa protein, mitochondrial isoform 8 [Homo sapiens]
cat eye syndrome critical region protein 2 [Homo sapiens]
RecName: Full=2-5A-dependent ribonuclease
peroxisomal carnitine O-octanoyltransferase isoform 1 [Homo sapiens]
E3 ubiquitin-protein ligase MARCH5 [Homo sapiens]
RecName: Full=ATPase inhibitor, mitochondrial
RecName: Full=Protein PML
RecName: Full=CASP8-associated protein 2
RecName: Full=E3 ubiquitin-protein ligase parkin
carnitine O-acetyltransferase isoform 1 precursor [Homo sapiens]
disrupted in schizophrenia 1 protein isoform l [Homo sapiens]
trichoplein keratin filament-binding protein [Homo sapiens]
stress-70 protein, mitochondrial precursor [Homo sapiens]
COX7A2 protein [Homo sapiens]
serine protease HTRA2, mitochondrial isoform 2 [Homo sapiens]
39S ribosomal protein L43, mitochondrial isoform d [Homo sapiens]
elongation factor G, mitochondrial [Homo sapiens]
RecName: Full=Protein MAATS1
mitogaligin [Homo sapiens]
vesicle transport protein SEC20 isoform BNIP1-c [Homo sapiens]
mitochondrial carrier homolog 1 PSAP-LL [Homo sapiens]
translation initiation factor IF-2, mitochondrial precursor [Homo sapiens]
cGMP-dependent 3',5'-cyclic phosphodiesterase isoform PDE2A1 [Homo sapiens]
mitochondrial dynamic protein MID49 isoform 2 [Homo sapiens]
RecName: Full=Transcription factor A, mitochondrial

Fig. 2 Heatmap of protein expression profile pattern of total 105 proteins. Each row represents an individual protein and each column represents matched-pairs of (adj-pap) adjacent non-cancerous tissue of papillary type, (Cancer-pap) CCA papillary type, (adj-nonpap) matched-pairs of adjacent non-cancerous tissue of non-papillary type, and (Cancer-nonpap) CCA non-papillary type, respectively (bar chart represents the signal intensity level as shown in the corresponding shades)

Potential interaction analysis

Potential interaction of the identified proteins was analyzed using public-domain software, the search tool for interacting chemicals known as STITCH 4.0 [22]. In brief, a set of input page icons of the software was shown at the start page. The page icon namely "multiple names" was selected. The list of proteins of interest was keyed in e.g. AIFM3 and/or other specified proteins and an item "*Homo sapiens*" was selected as the organism, and then clicked "Go". The pop-up new page with the list of the protein names of interest for the example, AIFM3 and other proteins were seen and then clicked "Continue". The other pop-up new page showed the confidence view. To interpret the confidence view, stronger associations are represented by the *thicker lines*. Protein-protein interactions are shown in *solid lines,* chemical-protein interactions in *dashed lines* and interaction between chemicals in *dotted lines*. In our study, highly confident interaction was focused with a score greater than 0.7.

Immunohistochemistry

To evaluate the AIFM3 expression in CCA tissues, paraffin-embedded tissue sections of 25 CCA cases (including 3 papillomatous and 3 non-papillomatous CCA cases used for mitochondrial proteomic analysis as previously mentioned above) were examined using standard immunohistochemistry protocols. The tissue sections were incubated with anti-AIFM3 antibody at 1:300 in antibody dilution buffer (PBS-T, 0.05 % sodium azide) (Biorbyt, UK) at 4 °C overnight. Then, the sections were incubated with peroxidase-conjugated secondary antibody (Dako Cytomation, Inc., California, USA). Peroxidase activity was visualized using the 3, 3′-Diaminobenzidine (DAB) substrate solution, with hematoxylin counterstaining. The staining was assessed using H-score by recording both the intensity of staining (0 = no staining; 1 + = weak staining; 2 + = moderate staining; and 3 + = strong staining), and percentage of stained tumor cells (0–100 %). The H-score was calculated by the following formula: H-score = (% of positively stained tumor cells at weak intensity × 1) + (% of positively stained tumor cells at moderate intensity × 2) + (% of positively stained tumor cells at strong intensity × 3) as previously described [23].

Statistical analysis

Statistical analysis was performed using SPSS 17.0. The H-scores between matched pairs of non-papillary, papillary CCA, and their corresponding adjacent non-cancerous tissues were compared using Student's *t* test. *P* value < 0.05 was considered statistically significant.

Results

Mitochondria isolation

Mitochondrial and cytosolic fractions were prepared from CCA and adjacent non-cancerous tissues of 3 cases each of non-papillary and papillary CCA patients. Successful separation of both fractions was validated by western blot analysis using antibody against mitochondria-specific marker protein. VDAC (Fig. 1a) shows strongly positive reaction in mitochondrial fractions but negative in cytosolic fractions.

Proteomic analysis of mitochondrial proteins

Mitochondrial protein fraction from the three samples each of non-papillary and papillary CCA and the corresponding adjacent non-cancerous tissues were pooled by tissue type and electrophoresed on 12.5 % SDS-PAGE (Fig. 1b). After dividing into 12 ranges according to the molecular size, each gel plug was digested in gel. The extracted tryptic peptides were subjected to LC-MS/MS analysis. The differentially expressed peptides data obtained from DecyderMS software were searched against the NCBI human mitochondria database for protein identification. In total, 281 mitochondrial proteins were identified. Using the MultiExperiment Viewer (MeV, version 4.6.1) software, 105 proteins were selected based on statistically significant (*P* < 0.05) and protein expression levels as shown in the heatmap (Fig. 2).

Identification of AIFM3 as a candidate protein

A total of 105 significantly different in mitochondrial protein expressions in CCA and the adjacent non-cancerous tissues were analyzed using the "jvenn", interactive Venn diagram viewer software. The results show that five and seven proteins are over-expressed in papillary and non-papillary CCA, respectively. However, only one protein was commonly found in both papillary and non-papillary type CCA tissues, but not in the adjacent tissues (Fig. 3a). This protein was identified as AIFM3. Western blot analysis of the mitochondrial solutions of the 3 cases each of papillary and non-papillary CCA cases revealed that AIFM3 protein was uniquely expressed in all 3 CCA tissues, but not in the corresponding adjacent non-cancerous tissues (Fig. 3b).
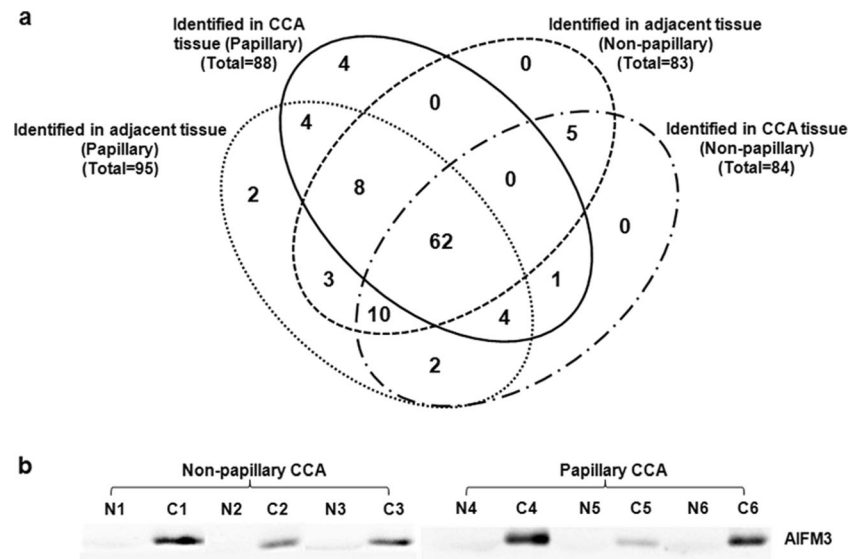
Fig. 3 Identification of candidate protein. **a** Venn diagram presents the number of proteins in each sample and degree of individual overlap between cancerous tissue and adjacent non-cancerous tissue. The *dot line* is total identified proteins in adjacent tissue of papillary CCA and *solid line* is total identified proteins in papillary CCA. The *dash line* is total identified proteins in adjacent tissue of non-papillary CCA and *dash-dot line* is total identified proteins in papillary CCA. **b** Detection of the AIFM3 protein in mitochondrial fractions ($n = 6$) using western blot analysis; N, adjacent non-cancerous tissue; C, cancerous tissue of CCA

To investigate the expression of AIFM3 in clinical specimens, apart from 3 cases each of papillary and non-papillary CCA used for mitochondrial extraction, 25 more human CCA and their corresponding adjacent non-cancerous tissues were examined by immunohistochemistry. The results show that H-scores of the AIFM3 staining were significantly higher in CCA than in the corresponding adjacent non-cancerous tissues (Fig. 4a-d).

AIFM3 protein interaction

To speculate the potential role of AIFM3 in CCA-genesis, possible interaction of AIFM3 and mitochondrial carcinogenesis-related proteins were predicted by STITCH version 4.0 (Fig. 5). The results show that superoxide dismutase 2 (SOD2), NADPH oxidase 1 (NOX1), serine hydroxymethyltransferase 2 (SHMT2), complement component 1q-binding

Fig. 4 Immunohistochemical detection of AIFM3 in cancerous and non-cancerous tissues from CCA patients. **a** Expression of AIFM3 in human tissues was examined by immunohistochemistry (magnification, ×400). **b**–**d** Comparison of AIFM3 levels in adjacent and cancerous tissues ($n = 25$), a level of $P < 0.05$ was considered statistically significant using matched-pairs t test in papillary CCA, non-papillary CCA, and overall CCA; Legend and symbol: Adjacent non-cancerous tissues (*squares*), CCA tissues (*triangles*)
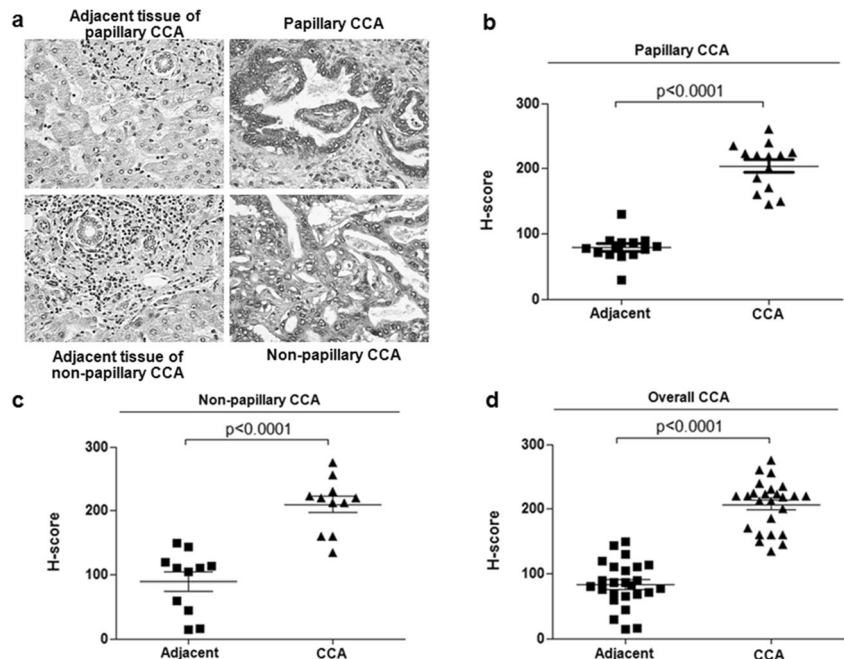
Fig. 5 Protein-chemical interaction network of the AIFM3. Filtering interactions network was according to apoptosis-inducing factor (*in bold circle*) and carcinogenesis processes associated proteins (*in bold box*) predicted by STITCH Version 4.0 (http://stitch.embl.de). AIFM3 was found to be associated with superoxide dismutase 2 (SOD2), NADPH oxidase 1 (NOX1), serine hydroxymethyltransferase 2 (SHMT2), complement component 1q-binding protein (C1QBP), ornithine aminotransferase (OAT), 3-oxoacid CoA transferase 1 (OXCT1), anamorsin (CIAPIN1), heat shock 70 kDa protein 9 (mortalin or HSPA9), and protein kinase C alpha (PRKCA). Stronger associations are represented by the *thicker lines*. Weak associations are represented by *thin lines*. Protein-protein interactions are shown in *solid lines*, chemical-protein interactions in *dashed lines*
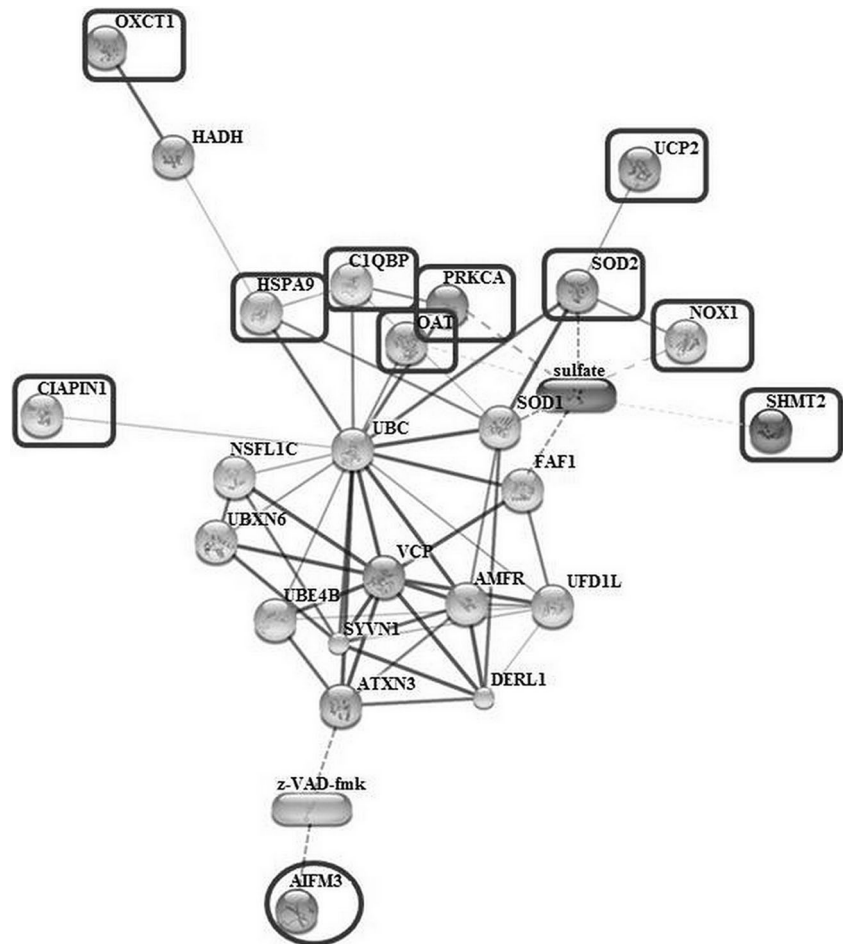
protein (C1QBP), ornithine aminotransferase (OAT), 3-oxoacid CoA transferase 1 (OXCT1), anamorsin (CIAPIN1), heat shock 70 kDa protein 9 (mortalin or HSPA9), and protein kinase C alpha (PRKCA) were identified as proteins interacting with AIFM3.

Discussion

Proteomic analysis revealed that the expression patterns of mitochondrial proteins are different between cancerous and their adjacent non-cancerous tissues of both types of CCA. The significant differences of the expression patterns of mitochondrial proteins are also seen between both non-papillary and papillary types of CCA. The concordant findings the difference between mitochondrial protein expression in cancerous and non-cancerous tissues have also been reported in renal carcinoma, breast cancer, and gastric cancer [5, 6, 24].

In this study, five and seven proteins are over-expressed in papillary and non-papillary CCA, respectively. However, only AIFM3, which is also known as AIF-like protein (AIFL), was commonly over-expressed in both papillary and non-papillary types of CCA. AIFM3 consists of 598 amino acids. It is a

mitochondrial flavoenzyme that arises from alternative splicing of human AIF-like gene during transcription [25]. NADH oxidase-dependent complex I function of AIF is important for oxidative phosphorylation and tumorigenicity [26]. AIF has been reported to act as a free radical scavenger that suppresses oxidative stress in neuronal cells and play a role in anti-apoptogenic function [27]. In addition, AIFM3 is a direct target of miR-210 that is related to proliferation of human hepatoma cells [28]. In fact, AIFM3 expression level was increased in miR-210 knocked-down hepatoma xenograft [29]. Some studies demonstrated that AIF induced apoptosis [25, 30]. Thus, the precise involvement of AIF in regulating life-death balances may be rather specific for the cell type and death inducer [26]. Overexpression of AIFM3 was found in human CCA tissues, which represented alteration of mitochondrial proteins in CCA. This high expression of AIFM3 in CCA was consistent with previous finding that showed high expression of SOD2 during CCA genesis in hamster model [16]. SOD2 is antioxidant enzyme that regulates ROS level occurring during OV infection. SOD2 expression seemed to be increased during cholangiocarcinogenesis, although decreased expression when the tumor has fully developed. Thus, high expression of SOD2 was found in CCA-induced

hamsters, when tumors were developed and an expression of this enzyme was impaired. Taken together, AIFM3 was classified into AIF family members that were considered as free radical scavenger, apoptotic protein, and non-apoptotic protein. In the present study, we speculated that AIFM3 may be a potential mitochondria protein in CCA.

Interactions of AIFM3 and mitochondrial carcinogenesis-related proteins have been reported not only in CCA but also in several other cancers such as breast cancer, hepatocellular carcinoma, gastric cancer, and colon cancer [6, 9]. As the predicted counterparts to interact with AIFM3, the following molecules have been listed; superoxide dismutase 2 (SOD2), NADPH oxidase 1 (NOX1), serine hydroxymethyltransferase 2 (SHMT2), complement component 1q-binding protein (C1QBP), ornithine aminotransferase (OAT), 3-oxoacid CoA transferase 1 (OXCT1), anamorsin (CIAPIN1), heat shock 70 kDa protein 9 (mortalin or HSPA9), and protein kinase C alpha (PRKCA) [7, 8, 10–12, 16, 31–33]. Interestingly, 2 out of 4 mitochondrial proteins, namely PRKCA and CIAPIN1, were uniquely expressed in papillary CCA in this study. Their roles in association with AIFM3 should be explored in the future.

In this study, protein-chemical interaction networks analysis revealed that AIFM3 linked to various key molecules in cancer progression through z-VAD-fmk, which is a pan-caspase inhibitor having been widely used to block apoptosis [34]. In addition, z-VAD-fmk can also inhibit cysteine proteases such as calpain and cathepsin B in vitro [35]. This AIFM3 mediated pathway may also be associated with carcinogenesis of CCA. Since the molecular structure and function of AIFM3 have not been fully understood, further studies are required to elucidate its role in carcinogenesis and metastasis.

In terms of technical point of view, we have isolated mitochondria from cancerous and adjacent noncancerous tissues at the first step of proteomic analysis to avoid the complexity of the proteins of the starting material [36, 37]. Although a special reagent kit is commercially available and ready to use, however, few steps for optimization were required to obtain the optimum conditions for each tissue type because of biological variance of human tissues also affected the yield of protein samples [38]. So far, our methods in this study were suitable for proteomic analysis of mitochondrial proteins of CCA.

In conclusion, among mitochondrial proteins, AIFM3 was aberrantly expressed in CCA tissues and could be a potential target molecule for CCA chemotherapy.

Compliance with ethical standards

Conflicts of interest None

References

1. Sripa B, Pairojkul C. Cholangiocarcinoma: lessons from Thailand. Curr Opin Gastroenterol. 2008;24(3):349–56.
2. Sripa B, Tangkawattana S, Laha T, Kaewkes S, Mallory FF, Smith JF, et al. Toward integrated opisthorchiasis control in Northeast Thailand: the Lawa project. Acta Trop. 2015;141(Pt B):361–7.
3. Woradet S, Promthet S, Songserm N, Parkin DM. Factors affecting survival time of cholangiocarcinoma patients: a prospective study in Northeast Thailand. Asian Pac J Cancer Prev. 2013;14(3):1623–7.
4. Jiang Y, Wang X. Comparative mitochondrial proteomics: perspective in human diseases. J Hematol Oncol. 2012;5:11.
5. Yusenko MV, Ruppert T, Kovacs G. Analysis of differentially expressed mitochondrial proteins in chromophobe renal cell carcinomas and renal oncocytomas by 2-D gel electrophoresis. Int J Biol Sci. 2010;6(3):213–24.
6. Kim HK, Park WS, Kang SH, Warda M, Kim N, Ko JH, et al. Mitochondrial alterations in human gastric carcinoma cell line. Am J Phys Cell Physiol. 2007;293(2):C761–71.
7. McGee AM, Douglas DL, Liang Y, Hyder SM, Baines CP. The mitochondrial protein C1qbp promotes cell proliferation, migration and resistance to cell death. Cell Cycle. 2011;10(23):4119–27.
8. Miyasaka Y, Enomoto N, Nagayama K, Izumi N, Marumo F, Watanabe M, et al. Analysis of differentially expressed genes in human hepatocellular carcinoma using suppression subtractive hybridization. Br J Cancer. 2001;85(2):228–34.
9. Kuai XY, Ji ZY, Zhang HJ. Mitochondrial uncoupling protein 2 expression in colon cancer and its clinical significance. World J Gastroenterol. 2010;16(45):5773–8.
10. Jain M, Nilsson R, Sharma S, Madhusudhan N, Kitami T, Souza AL, et al. Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. Science. 2012;336(6084):1040–4.
11. Wadhwa R, Yaguchi T, Hasan MK, Taira K, Kaul SC. Mortalin-MPD (mevalonate pyrophosphate decarboxylase) interactions and their role in control of cellular proliferation. Biochem Biophys Res Commun. 2003;302(4):735–42.
12. Sawai M, Yashiro M, Nishiguchi Y, Ohira M, Hirakawa K. Growth-inhibitory effects of the ketone body, monoacetoacetin, on human gastric cancer cells with succinyl-CoA: 3-oxoacid CoA-transferase (SCOT) deficiency. Anticancer Res. 2004;24(4):2213–7.
13. Kongpetch S, Kukongviriyapan V, Prawan A, Senggunprai L, Kukongviriyapan U, Buranrat B. Crucial role of heme oxygenase-1 on the sensitivity of cholangiocarcinoma cells to chemotherapeutic agents. PLoS One. 2012;7(4):e34994.
14. Okaro A, Fennell D, Corbo M, Davidson B, Cotter F. Pk11195, a mitochondrial benzodiazepine receptor antagonist, reduces apoptosis threshold in Bcl-XL and mcl-1 expressing human cholangiocarcinoma cells. Gut. 2002;51:556–61.
15. Giris M, Erbil Y, Depboylu B, Mete O, Turkoglu U, Abbasoglu SD, et al. Heme oxygenase-1 prevents hyperthyroidism induced hepatic damage via an antioxidant and antiapoptotic pathway. J Surg Res. 2010;164(2):266–75.

16. Loilome W, Kadsanit S, Namwat N, Techasen A, Puapairoj A, Dechakhamphu A, et al. Impaired antioxidant enzyme activity and increased DNA repair enzyme expression in hamster liver tissues related to cholangiocarcinoma development. Asian Pac J Cancer Prev. 2012;13(Suppl):59–64.

17. Muisuk K, Silsirivanit A, Imtawil K, Bunthot S, Pukhem A, Pairojkul C, et al. Novel mutations in cholangiocarcinoma with low frequencies revealed by whole mitochondrial genome sequencing. Asian Pac J Cancer Prev. 2015;16(5):1737–42.

18. Hoegger MJ, Lieven CJ, Levin LA. Differential production of superoxide by neuronal mitochondria. BMC Neurosci. 2008;9:4.

19. Lee JH, Hong CS, Lee S, Yang JE, Park YI, Lee D, et al. Radiating amyloid fibril formation on the surface of lipid membranes through unit-assembly of oligomeric species of a-synuclein. PLoS One. 2012;7:e47580.

20. Fernandez-Vizarra E, Lopez-Perez MJ, Enriquez JA. Isolation of biogenetically competent mitochondria from mammalian tissues and cultured cells. Methods. 2002;26(4):292–7.

21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265–75.

22. Kuhn M, Szklarczyk D, Pletscher-Frankild S, Blicher TH, von Mering C, Jensen LJ, et al. STITCH 4: integration of protein-chemical interactions with user data. Nucleic Acids Res. 2014;42(Database issue):D401–7.

23. Ma HY, YN L, Marchbanks PA, Folger SG, Strom BL, McDonald JA, et al. Quantitative measures of estrogen receptor expression in relation to breast cancer-specific mortality risk among white women and black women. Breast Cancer Res. 2013;15(5):R90.

24. Singh R, Avliyakulov NK, Braga M, Haykinson MJ, Martinez L, Singh V, et al. Proteomic identification of mitochondrial targets of arginase in human breast cancer. PLoS One. 2013;8(11):e79242.

25. Xie Q, Lin T, Zhang Y, Zheng J, Bonanno JA. Molecular cloning and characterization of a human AIF-like gene with ability to induce apoptosis. J Biol Chem. 2005;280(20):19673–81.

26. Urbano A, Lakshmanan U, Choo PH, Kwan JC, Ng PY, Guo K, et al. AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells. EMBO J. 2005;24(15):2815–26.

27. Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, et al. The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature. 2002;419(6905):367–74.

28. Yang W, Sun T, Cao J, Liu F, Tian Y, Zhu W. Downregulation of miR-210 expression inhibits proliferation, induces apoptosis and enhances radiosensitivity in hypoxic human hepatoma cells in vitro. Exp Cell Res. 2012;318(8):944–54.

29. Yang W, Wei J, Sun T, Liu F. Effects of knockdown of miR-210 in combination with ionizing radiation on human hepatoma xenograft in nude mice. Radiat Oncol. 2013;8:102.

30. Delettre C, Yuste VJ, Moubarak RS, Bras M, Lesbordes-Brion JC, Petres S, et al. AIFsh, a novel apoptosis-inducing factor (AIF) pro-apoptotic isoform with potential pathological relevance in human cancer. J Biol Chem. 2006;281(10):6413–27.

31. Wang R, Dashwood WM, Nian H, Lohr CV, Fischer KA, Tsuchiya N, et al. NADPH oxidase overexpression in human colon cancers and rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP). Int J Cancer. 2011;128(11):2581–90.

32. Li X, Pan Y, Fan R, Jin H, Han S, Liu J, et al. Adenovirus-delivered CIAPIN1 small interfering RNA inhibits HCC growth in vitro and in vivo. Carcinogenesis. 2008;29(8):1587–93.

33. Magnifico A, Albano L, Campaner S, Campiglio M, Pilotti S, Menard S, et al. Protein kinase c alpha determines HER2 fate in breast carcinoma cells with HER2 protein overexpression without gene amplification. Cancer Res. 2007;67(11):5308–17.

34. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J Biol Chem. 1999;274(8):5053–60.

35. Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS, et al. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. Blood. 1999;94(5):1683–92.

36. Zhang C, Feng YS, SL Q, Wei X, Zhu HL, Luo Q, et al. Resveratrol attenuates doxorubicin-induced cardiomyocyte apoptosis in mice through SIRT1-mediated deacetylation of p53. Cardiovasc Res. 2011;90(3):538–45.

37. YR F, Yi ZJ, Yan YR, Qiu ZY. Proteomic analysis of mitochondrial proteins in hydroxycamptothecin-treated SMMC-7721 cells. Zhonghua Gan Zang Bing Za Zhi. 2007;15(8):572–6.

38. Diz AP, Truebano M, Skibinski DO. The consequences of sample pooling in proteomics: an empirical study. Electrophoresis. 2009;30(17):2967–75.