# PROTEIN EXPRESSION PROFILE OF GLYCITEIN-TREATED CHOLANGIOCARCINOMA KKU-M213 CELL LINE

Charin Thawornkuno[1], Onrapak Reamtong[1], Thitiluck Swangsri[1], Poom Adisakwattana[2], Nathamon Ngaosuwankul[3], Sopit Wongkham[4], Polkit Sangvanich[5], Kozo Asano[6] and Songsak Petmitr[1]

[1]Department of Molecular Tropical Medicine and Genetics, [2]Department of Helminthology, [3]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok; [4]Department of Biochemistry, Liver Fluke and Cholangiocarcinoma, Faculty of Medicine, Khon Kaen University, Khon Kaen; [5]Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand; [6]Graduate School of Agriculture, Hokkaido University, Hokkaido, Japan

**Abstract.** Cholangiocarcinoma (CCA), a malignant epithelial neoplasm originating from the biliary tract, is a serious health problem of northeastern Thailand. Currently, there are no effective drugs against this cancer. Among isoflavones daidzein, genistein and glycitein, and equol, a derivative of daidzein, glycitein demonstrated the highest inhibitory effect of on the proliferation of human cholangiocarcinoma cell line KKU-M213 with an $IC_{50}$ (50% inhibitory concentration) of 27 $\mu$M. As a first step towards understanding its mechanism of action, differential protein expression profiling of glycitein-treated and -untreated KKU-M123 cells were conducted using mass spectrometry (MS). Two hundred and fifteen proteins were expressed in both test cells; 129 and 114 proteins only in glycitein-treated and untreated cells respectively; 39 up-regulated and 14 proteins down-regulated in treated compared to untreated cells. The majority of up-regulated proteins were classified as those involved in metabolic and cellular processes, while that of down-regulated proteins also included transport, cell cycle, developmental process, and cellular component organization. These relative fold differences obtained from MS analysis were confirmed by western blotting of two representative proteins. These results provide baseline proteomics data that should be of assistance in identification of isoflavones anti-proliferative mechanisms against CCA and help guide future discovery and development of chemotherapeutic agents from natural products.

**Keywords:** cholangiocarcinoma cell line, glycitein, isoflavone, mass spectrometry, proteomics

Correspondence: Prof Songsak Petmitr, Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Ratchathewi, Bangkok 10400, Thailand.
E-mail: songsak.pet@mahidol.ac.th

## INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant epithelial neoplasm originating in the biliary tract, a region draining bile from liver into small intestine. CCA can occur in any portion of the biliary tract and

is classified as intrahepatic or extrahepatic, depending on the anatomical location of the tumor. Intrahepatic CCA develops within the liver parenchyma while extrahepatic CCA is located between the biliary tract and hepatoduodenal ligament (Cardinale *et al*, 2010). CCA prevalence is geographically heterogeneous, rarely occurring in the population in Western countries but with a higher prevalence among people in East and Southeast Asia (Patel, 2001; Patel, 2002) and highest in northeastern Thailand (Khuntikao, 2005).

The majority of CCA patients in Thailand present with the intrahepatic type (Shin *et al*, 2010). Development of CCA has been linked to infection with liver fluke *Opisthorchis viverrini*, native to Laos, Malaysia and Thailand (Sonakul *et al*, 1978; Thamavit *et al*, 1978) or *Clonorchis sinensis*, native to China, Japan and Korea (Chapman, 1999), but no risk factors so far have been identified for biliary tract cancers in Western countries (Ben-Menachem, 2007). CCA is considered a lethal malignancy and remains incurable, unless both primary and any metastasis tumors are fully resected (Watanapa, 1996; Tsao *et al*, 2000; Zervos *et al*, 2005). However, in Thailand the majority of cases are diagnosed at an advanced stage of the disease upon presentation and are inoperable (Butthongkomvong *et al*, 2013). Patients are, in general, managed with palliative chemotherapy as there is no single chemotherapy or drug regimen, which is universally applicable for patients in advanced stages of the disease, although randomized controlled trials have reported chemotherapy improves quality of life and extends survival in patients with inoperable CCA (Butthongkomvong *et al*, 2013). Nevertheless, patients with advanced stage CCA generally succumb to the caner within 24 months of diagnosis (Glimelius *et al*, 1996) and, thus, new effective drugs are needed to combat this malignancy.

Isoflavones are a major class of phytoestrogens, naturally occurring plant-derived substances structurally or functionally similar to 17β-estradiol, which can serve as a natural product estrogen agonist or antagonist (Barnes and Peterson, 1995; Makela *et al*, 1995; Shemesh *et al*, 1972; Verdeal *et al*, 1980). High concentrations of isoflavones are found in many diets, especially in Asia where food contains large amounts of soy (Messina *et al*, 2006). Studies have suggested isoflavones can play a role in prevention of various estrogen-related cancers, diseases and symptoms, *viz* breast (Key *et al*, 1999; Dai *et al*, 2001; Beral, 2003; Chlebowski *et al*, 2003) and prostate cancer (Shimizu *et al*, 1991; Pienta *et al*, 1996), cardiovascular diseases (Nagata *et al*, 1998; Vanharanta *et al*, 1999; de Kleijn *et al*, 2002; van der Schouw *et al*, 2002), osteoporosis (Lauderdale *et al*, 1997; Tsuchida *et al*, 1999; Horiuchi *et al*, 2000; Kritz-Silverstein and Goodman-Gruen, 2002), and menopausal symptoms (Adlercreutz *et al*, 1992; Nagata *et al*, 1999; Nagata *et al*, 2001)

In plants, isoflavones exist in four forms, namely, aglycone, 7-*O*-glucoside, 6′′-*O*-acetylglucoside, and 6′′-*O*-malonylglucoside (Coward *et al*, 1998). Conjugated isoflavones are biologically inactive, but once ingested, are converted to aglycone by β-glucosidase. Furthermore, during the manufacturing of foods such as miso and tempeh, fermentation processes result in hydrolysis of glycosides to aglycones (Manach *et al*, 2004). The major aglycone isoflavones isolated from soy are daidzein (DAI), genistein (GEN) and glycitein (GLY) (Heinonen *et al*, 2003),

and gastrointestinal tract microflora can convert aglycone isoflavones into various metabolites (Zubik and Meydani, 2003), among which equol (EQL) exhibits stronger estrogenic activity than DAI (Sathyamoorthy and Wang, 1997), and thus, EQL is suggested as being an active metabolite of DAI. At present, the mechanism(s) of action of isoflavones or metabolites against estrogen-related cancers, diseases and symptoms is (are) not well understood. Potential mechanisms of action suggested include interaction with estrogen receptors and other steroid hormones (Kurzer, 2000; Nagata *et al*, 2000; Xu *et al*, 2000), induction of apoptosis (Li *et al*, 1999), inhibition of angiogenesis, aromatase and 17β-hydroxysteroid dehydrogenase enzymes (Adlercreutz *et al*, 1993; Krazeisen *et al*, 2001).

As anti-proliferative effects of soy isoflavones and derivatives against CCA cell lines have hitherto not been reported, here effectiveness of soy isoflavones (DAI, GEN and GLY) and a derivative (EQL) in inhibiting growth of an intrahepatic CCA KKU-M213 cell line isolated from a Thai patient was determined. In order to elucidate the mechanism of action of the most active test compounds, protein expression profile of treated in comparison with control cells were examined using by mass spectrometry. The findings should help guide future development of soy isoflavones as novel chemotherapeutic agents against CCA.

## MATERIALS AND METHODS

### Chemical reagents

Isoflavones (DAI, EQL, GEN and GLY) were from LC Laboratories (Woburn, MA) and acetonitrile, dimethylsulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dithiothreitol (DTT), 17β-estradiol ($E_2$), formic acid, iodoacetamide, sodium dodecylsufate (SDS), and trypsin from Sigma-Aldrich (St Louis, MO).

### Cell lines and culture conditions

Intrahepatic CCA KKU-M213 cell line, kindly provided by Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen, Thailand, was maintained in Ham's F-12 medium (Invitrogen, Carlsbad, CA) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum at 37°C under a humidified atmosphere containing 5% $CO_2$.

### Cell proliferation assay

An MTT assay was used to determine proliferation of CCA cells (Merck Millipore, Darmstadt, Germany). In brief, cells at 70% confluency were removed from culture flask by trypsinization, concentration determined using a hemocytometer, seeded onto a 96-well plate at a density of $1.5\times10^4$ cells/well, and cultured as described above for 24 hours. Then cells were treated with 0-50 μM DAI, $E_2$, EQL, GEN, and GLY for 24 hours. A 40 μl aliquot of 5 mg/ml MTT in DMSO was added into each well and cell suspension further incubated for 3.5 hours. Then supernatant in each well was replaced with 150 μl aliquot of DMSO and plate agitated for 15 minutes before $A_{590nm}$ was measured (Sunrise™, Tecan, Männedorf, Switzerland). Cell proliferation index was calculated using the formula: ($A_{590nm}$ of treated cells/ $A_{590nm}$ of untreated cells) × 100. Half maximal inhibitory concentration ($IC_{50}$) was determined from a dose-response curve.

### Mass spectrometric (MS) analysis

Trypsinized KKU-M213 cells ($7.5\times10^5$/well) were seeded onto a 6-well plate, cultured as described above for 24 hours,

then treated with 27 μM GLY for a further 24 hours. Untreated cells were incubated with vehicle. Following removal of culture medium, attached cells were washed three times with phosphate-buffered normal saline pH 7.4 (PBS), added with 50 μl of PBS and harvested mechanically using a rubber scraper. Cells were lysed with 0.5% (w/v) SDS solution at 60°C for 30 minutes. Protein concentration was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Protein samples (5 μg) was separated by 12% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250 dye (Bio-Rad, Hercules, CA). Each stained protein band was excised and cut into 1 mm pieces, which were subsequently destained with 50% (v/v) acetonitrile, incubated with 10 mM DTT for 15 minutes at 60ºC, followed by 55 mM iodoacetamide (to alkylate reduced cysteine residues) for 30 minutes at 25ºC. Gel pieces were dehydrated by addition of 100% (v/v) acetonitrile and allowed to dry in a fume hood, then incubated with 0.1 mg/ml trypsin solution overnight at 37°C. Peptides were extracted from the gel pieces with 0.5 ml of 50% (v/v) acetonitrile, concentrated in centrifugal concentrator (TOMY, Tokyo, Japan) until completely dry and stored at -20°C until used.

Each tryptic-digested sample was added with 0.1% (v/v) formic acid containing 2% (v/v) acetonitrile and analyzed in a MicroTOF-Q II mass spectrometer (Bruker, Billerica, MA) coupled with an Ultimate 3000 nano-LC system (Dionex, Surrey, UK). Separation was carried out at a flow rate of 200 nl/minute, with a mobile phase A (2% (v/v) acetonitrile, 0.1% (v/v) formic acid in HPLC grade water) and mobile phase B (0.1% (v/v) formic acid in HPLC grade acetonitrile) to establish a 58-minute gradient of mobile phase B, commencing for 10 minutes with 2-10% gradient, followed for 45 minutes with 10-40% gradient, then ramped rapidly for 1 minute with 40-95% gradient, before being maintained at 95% for 1 minute. Eluent was introduced into the mass spectrometer using a nano-electrospray source and ionized. Data were acquired using a HyStar software (Bruker, Billerica, MA) and MS and MS/MS spectra were acquired in a mass range of m/z 400-2000 and m/z 50-1500 respectively.

**Data analysis**

MS data were smoothed and centroided, and then converted into a Mascot Generic File (.mgf) using a DataAnalysis software, version 4.0 (Matrix Science, Boston, MA). The .mgf file was searched using a Mascot 2.2 software (Matrix Science, Boston, MA), then cross-referenced against a Swiss-Prot human protein database (https://www.uniprot.org/proteomes/UP000005640). Mass tolerance for precursor and fragment ions was set at 200 ppm and 0.6 m/z respectively. Missed cleavage by trypsin was allowed at 1. Carbamidomethylation of cysteine and oxidation of methionine were set as variable modifications. Peptides identified above 95% confidence were reported. Quantification was performed using an exponentially modified protein abundance index (emPAI), provided by Mascot (Matrix Science, Boston, MA). Calculation of emPAI value was carried out using the following formula:

$$\text{emPAI} = 10^{(\text{Nobserved} / \text{Nobservable})} - 1$$

where $N_{observed}$ represents number of experimentally observed peptides and $N_{observable}$ represents calculated number of observable peptides for each protein (Ishihama *et al*, 2005). All emPAI values are presented as mean of three

independent experiments. PANTHER software (http://www.pantherdb.org/feedback.jsp) was employed to classify identified proteins according to their gene ontology.

**Western blotting**

Protein sample (7.5 μg) was separated by 12% SDS-PAGE, transferred to a polyvinylidene difluoride (PVD) membrane (Millipore, Bedford, MA) by electroblotting at 4°C, incubated with 3% (w/v) bovine serum albumin (EMD Millipore) in phosphate-buffered saline, pH 7.4 with 0.1% (V/V) Tween® 20 (PBST) solution, treated with rabbit anti-uridine phosphorylase 1 or anti-calpain small subunit 1 monoclonal antibody (Abcam, Cambridge, MA) (1:5000 dilution) overnight at 4°C, washed with PBST solution, followed by treatment with goat secondary horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling, Danvers, MA) (1:2000 dilution) for 1 hour at 4°C. Immunoreactive protein bands were visualized using luminol-based substrate, chemiluminescence detected with a SuperSignal West Pico system (Thermo Scientific, Waltham, MA), images recorded with an ImageQuant LAS 4000 Mini (GE Healthcare Bio-Science, Uppsala, Sweden), and quantified using a GelQuant.NET software (http://biochemlabsolutions.com). Signal was normalized using rabbit monoclonal anti-β-actin (Cell Signaling Technology) as an internal standard.

## RESULTS

**Inhibition of CCA cell lines by isoflavones**

CCA cell lines were treated with 0-50 μM isoflavones for 24 hours and then proliferation indices determined using an MTT assay. Lowest half maximal inhibitory concentration ($IC_{50}$) was observed with GLY at 27 μM for KKU-M213 cell line (Fig 1). All other isoflavones tested did not reach an $IC_{50}$ value within the concentration range used, including $E_2$.

**Proteomics profiles of GLY-treated amd untreated KKU-M213 cells**

Following exposure to $IC_{50}$ value of GLY for 24 hours, proteins from KKU-M213 cell lysate were separated by 12% SDS-PAGE, and gel sections containing Coomassie-stained protein band were excised, proteins in gel slices destained, extracted from gel and digested with trypsin before being subjected to MS analysis. Similar procedures were carried with untreated control cells. A total of 344 and 329 proteins were identified from GYL-treated and control KKU-M213 cells (supplement data available upon request to the corresponding author), with 129 and 114 proteins expressed only in GLY-treated and control cells respectively, and 215 proteins were expressed in both test cells. Among the 129 proteins associated specifically with GLY-treated KKU-M213 cells, all proteins were able to be classified according to their gene ontology (GO), except for seven proteins, namely, ubiquitin-conjugating enzyme E2 variant 1 (UB2V1_HUMAN), nucleoside-diphosphate kinase B (NDKB_HUMAN), apoptosis protein 6 (PDCD6_HUMAN), uncharacterized protein C21orf129 (CU129_HUMAN), UPF0732 protein C1orf227 (CA227_HUMAN), coiled-coil domain-containing protein 152 (CC152_HUMAN), and putative UPF0607 protein FLJ37424 (YJ012_HUMAN). Similarly, among the 114 control cell-specific proteins only putative tubulin-like protein alpha-4B (TBA4B_HUMAN) and solute carrier family 12 member 8 (S12A8_HUMAN) were unable to be classified according to GO. In addition, among the 215 proteins present in both treated and

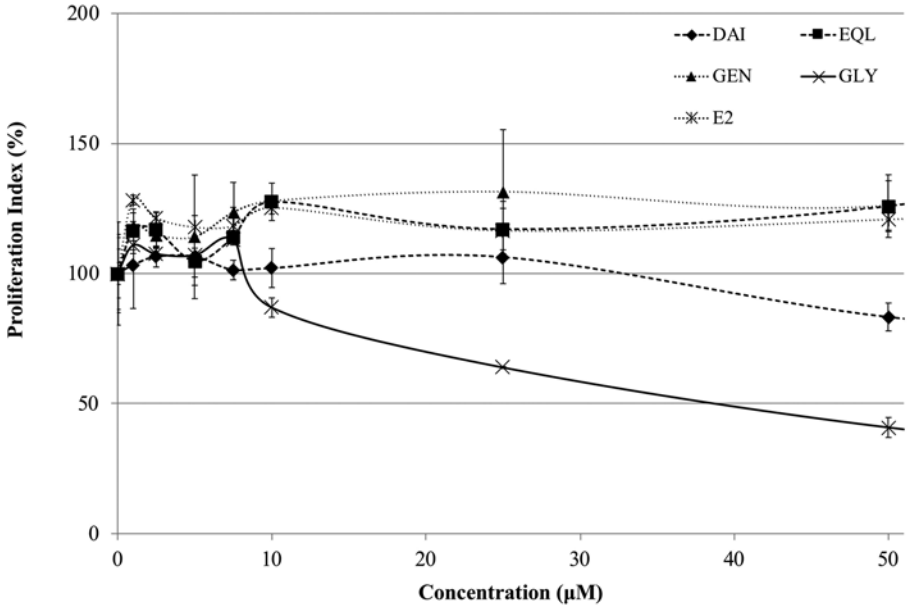


Fig 1-Inhibition of cholangiocarcinoma (CCA) KKU-M213 cell line proliferation by isoflavones.

CCA cells were treated with various concentrations of isoflavones for 24 hours prior to measurement of cell viability using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Proliferation index is shown as mean ± SEM of three independent experiments, each conducted in triplicate.

DAI: daidzein; $E_2$: 17β-estradiol (control); EQL: equol; GEN: genistein; GLY: glycitein.

control cells, only four proteins, namely, transgelin-2 (TAGL2_HUMAN), actin-related protein 2/3 complex subunit 4 (ARPC4_HUMAN), Ras-related protein Rab-6A (RAB6A_HUMAN), and coiled-coil domain-containing protein 110 (CC110_HUMAN) were refractory to GO classification.

Among proteins in control KKU-M213 cells, the majority were classified as involved in metabolic (32.4%) and cellular (17.1%) processes (Fig 2A) not dissimilar to proteins in GLY-treatment (33.2% and 16.5% respectively) (Fig 2B). This suggests GLY treatment did not affect the overall protein profile in KKU-M213 cells. Moreover, 39 and 14 proteins were up- and down regulated respectively in GLY-treated compared to control cells (Table 1). The majority of up-regulated proteins were classified as those involved in metabolic (38.0%) and cellular (14.1%) processes (Fig 3A), while down-regulated proteins were mainly related to cellular (18.4%) and metabolic (15.8%), processes, transport (15.8%), and cell cycle, developmental process, and cellular component organization (13.2% for each category) (Fig 3B). In addition, when all proteins affected by GLY exposure were analyzed for protein-protein interaction using STRING (https://string-db.org), interactions among organonitrogen metabolic processes (Fig 4), as well as in organelle and ribosome assembly (data not shown) were discerned.

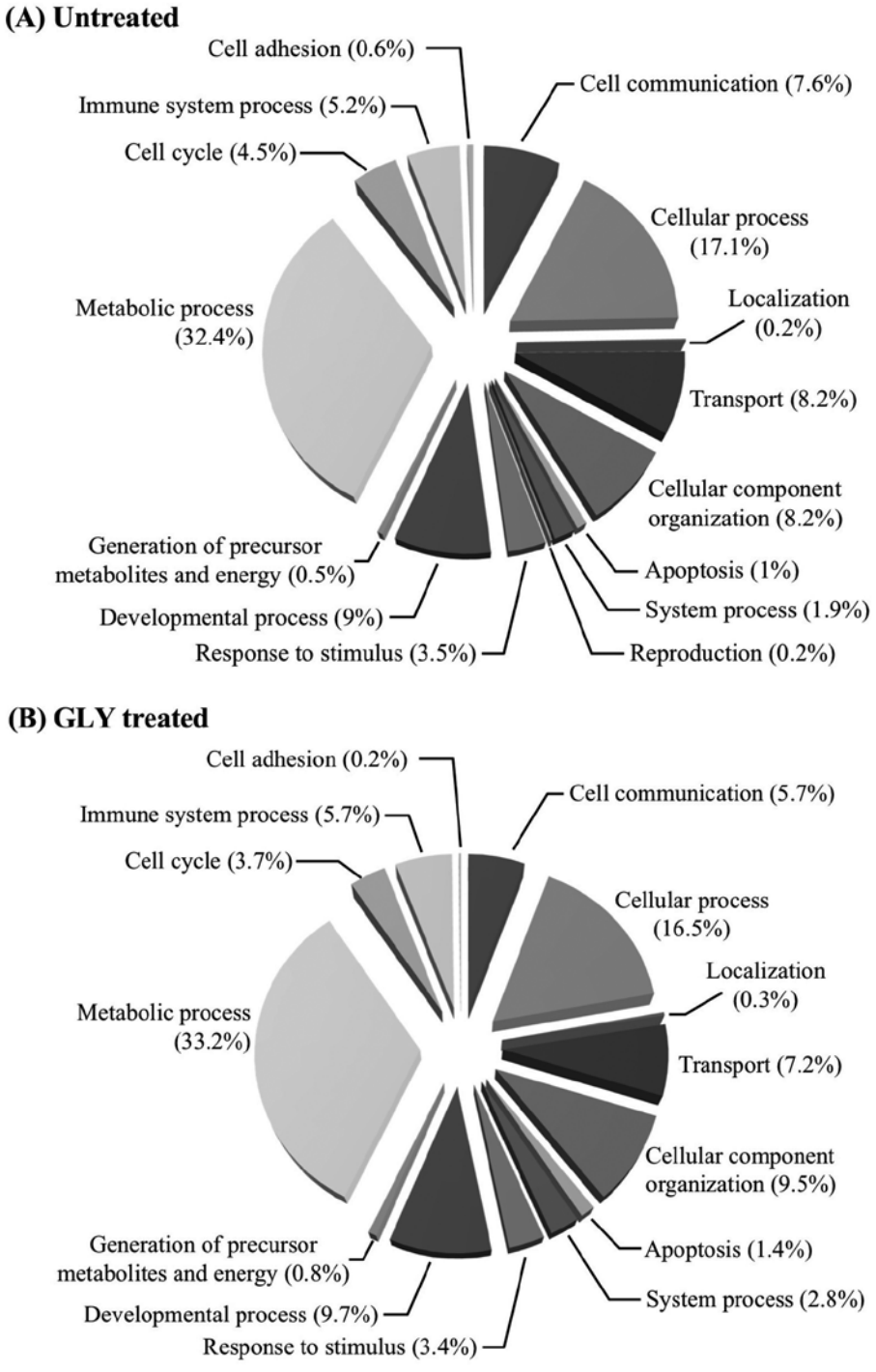


Fig 2-Classification of genistein (GLY)-treated and untreated KKU-M213 cells.

Proteins identified by mass spectrometric analysis were classified according to biological process using a PANTHER software (http://www.pantherdb.org/feedback.jsp).

Table 1
Fold change in protein levels in glycitein (27 $\mu$M)-treated and control KKU-M213 cells based on mass spectrometric analysis.

| Swiss-Prot entry | Swiss-Prot accession number | Protein | Fold change* |
|---|---|---|---|
| PLEC1_HUMAN | Q15149 | Plectin-1 | 11.1 |
| IF6_HUMAN | P56537 | Eukaryotic translation initiation factor 6 | 7.3 |
| UPP1_HUMAN | Q16831 | Uridine phosphorylase 1 | 5.1 |
| HNRPK_HUMAN | P61978 | Heterogeneous nuclear ribonucleoprotein K | 5.1 |
| H2B1B_HUMAN | P33778 | Histone H2B type 1-B | 4.2 |
| HPRT_HUMAN | P00492 | Hypoxanthine-guanine phosphoribosyltransferase | 4.0 |
| PLSI_HUMAN | Q14651 | Plastin-1 | 3.8 |
| TKT_HUMAN | P29401 | Transketolase | 3.7 |
| EZRI_HUMAN | P15311 | Ezrin | 3.7 |
| PEX1_HUMAN | O43933 | Peroxisome biogenesis factor 1 | 3.7 |
| SERC_HUMAN | Q9Y617 | Phosphoserine aminotransferase | 3.7 |
| VDAC2_HUMAN | P45880 | Voltage-dependent anion-selective channel protein 2 | 3.7 |
| RS3_HUMAN | P23396 | 40S ribosomal protein S3 | 3.5 |
| C1TC_HUMAN | P11586 | C-1-tetrahydrofolate synthase, cytoplasmic | 3.4 |
| RS10_HUMAN | P46783 | 40S ribosomal protein S10 | 2.5 |
| PSB3_HUMAN | P49720 | Proteasome subunit beta type-3 | 2.5 |
| LGUL_HUMAN | Q04760 | Lactoylglutathione lyase | 2.5 |
| EF1G_HUMAN | P26641 | Elongation factor 1-gamma | 2.4 |
| ALBU_HUMAN | P02768 | Serum albumin | 2.4 |
| HNRPQ_HUMAN | O60506 | Heterogeneous nuclear ribonucleoprotein Q | 2.4 |
| KU70_HUMAN | P12956 | ATP-dependent DNA helicase 2 subunit 1 | 2.4 |
| NACA_HUMAN | Q13765 | Nascent polypeptide-associated complex subunit alpha | 2.4 |
| RAB7A_HUMAN | P51149 | Ras-related protein Rab-7a | 2.4 |
| RL24_HUMAN | P83731 | 60S ribosomal protein L24 | 2.4 |
| UBE2N_HUMAN | P61088 | Ubiquitin-conjugating enzyme E2 N | 2.4 |
| PDIA1_HUMAN | P07237 | Protein disulfide-isomerase | 2.4 |
| TCPD_HUMAN | P50991 | T-complex protein 1 subunit delta | 2.4 |
| RL11_HUMAN | P62913 | 60S ribosomal protein L11 | 2.4 |
| KPYM_HUMAN | P14618 | Pyruvate kinase isozymes M1/M2 | 2.4 |
| RAC1_HUMAN | P63000 | Ras-related C3 botulinum toxin substrate 1 | 2.3 |
| C1QBP_HUMAN | Q07021 | Complement component 1 Q subcomponent-binding protein, mitochondrial | 2.3 |

Table 1 (Continued)

| Swiss-Prot entry | Swiss-Prot accession number | Protein | Fold change* |
|---|---|---|---|
| PRDX3_HUMAN | P30048 | Thioredoxin-dependent peroxide reductase, mitochondrial | 2.3 |
| ANXA3_HUMAN | P12429 | Annexin A3 | 2.3 |
| MOES_HUMAN | P26038 | Moesin | 2.2 |
| HS71L_HUMAN | P34931 | Heat shock 70 kDa protein 1-like | 2.2 |
| ENOG_HUMAN | P09104 | Gamma-enolase | 2.2 |
| STIP1_HUMAN | P31948 | Stress-induced-phosphoprotein 1 | 2.2 |
| 1433E_HUMAN | P62258 | 14-3-3 protein epsilon | 2.2 |
| PLST_HUMAN | P13797 | Plastin-3 | 2.1 |
| PSA5_HUMAN | P28066 | Proteasome subunit alpha type-5 | -2.0 |
| PDIA3_HUMAN | P30101 | Protein disulfide-isomerase A3 | -2.0 |
| TBB6_HUMAN | Q9BUF5 | Tubulin beta-6 chain | -2.0 |
| APT_HUMAN | P07741 | Adenine phosphoribosyltransferase | -2.0 |
| TBB2A_HUMAN | Q13885 | Tubulin beta-2A chain | -2.0 |
| TBB2C_HUMAN | P68371 | Tubulin beta-2C chain | -2.0 |
| UB2L3_HUMAN | P04350 | Ubiquitin-conjugating enzyme E2 L3 | -2.1 |
| TBB4_HUMAN | P68036 | Tubulin beta-4 chain | -2.1 |
| TBB5_HUMAN | P07437 | Tubulin beta chain | -2.1 |
| ARPC4_HUMAN | P59998 | Actin-related protein 2/3 complex subunit 4 | -2.2 |
| IF4A1_HUMAN | Q00839 | Eukaryotic initiation factor 4A-I | -2.3 |
| HNRPU_HUMAN | P60842 | Heterogeneous nuclear ribonucleoprotein U | -2.3 |
| CPNS1_HUMAN | P04632 | Calpain small subunit 1 | -3.2 |
| CLH1_HUMAN | Q00610 | Clathrin heavy chain 1 | -4.5 |

*Exponentially modified protein abundance index (emPAI) from GLY-treated cells/emPAI from control cells (Tables 1 and 2). Negative value = emPAI from control cells/emPAI from GLY-treated cells.

**Validation of mass spectrometry data by western blotting**

In order to validate the relative changes in protein levels between GLY-treated and control KKU-M213 cells obtained from MS analysis, western blotting was carried on two representative proteins, one up- and one down-regulated. Following separation of whole cell lysates by 12% SDS-PAGE and transfer onto a PVD membrane, quantification of immunoreactive uridine phosphorylase 1 (UPP1) and calpain small subunit 1 (CPNS1) demonstrated UPP1 and CPNS1 was 2.3 ± 1.0-fold higher and 4.5 ± 0.2-fold lower respectively in GLY-treated

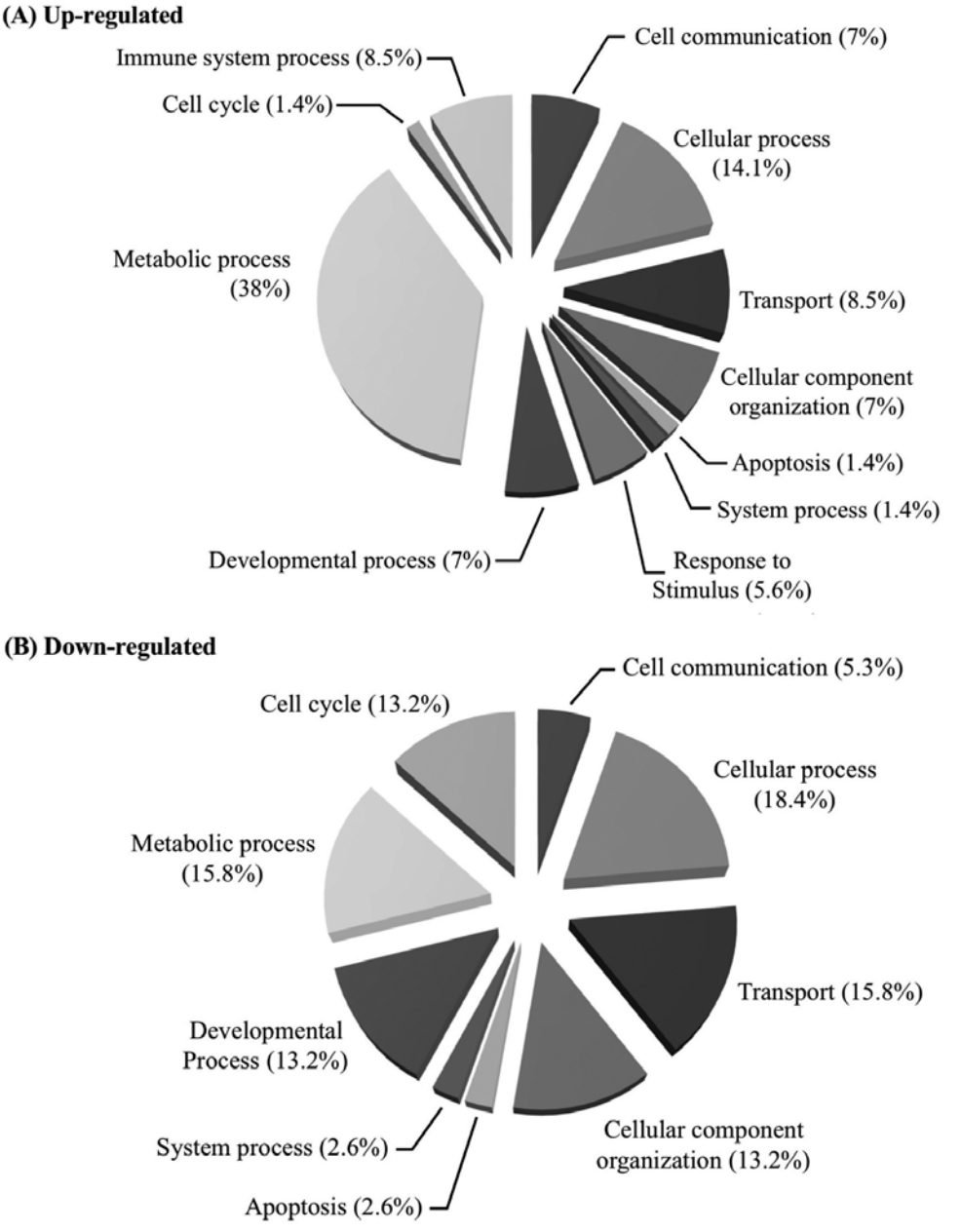


Fig 3-Classification of differentially expressed proteins between genistein-treated and untreated KKU-M213 cells.

Proteins identified by mass spectrometric analysis were classified according to biological process using a PANTHER software (http://www.pantherdb.org/feedback.jsp) and quantified using an exponentially modified protein abundance index (emPAI). (A) EmPAI of proteins from GLY-treated cells/control cells ≥2.1. (B) EmPAI of proteins from control cells/ GLY-treated cells ≥2.0.

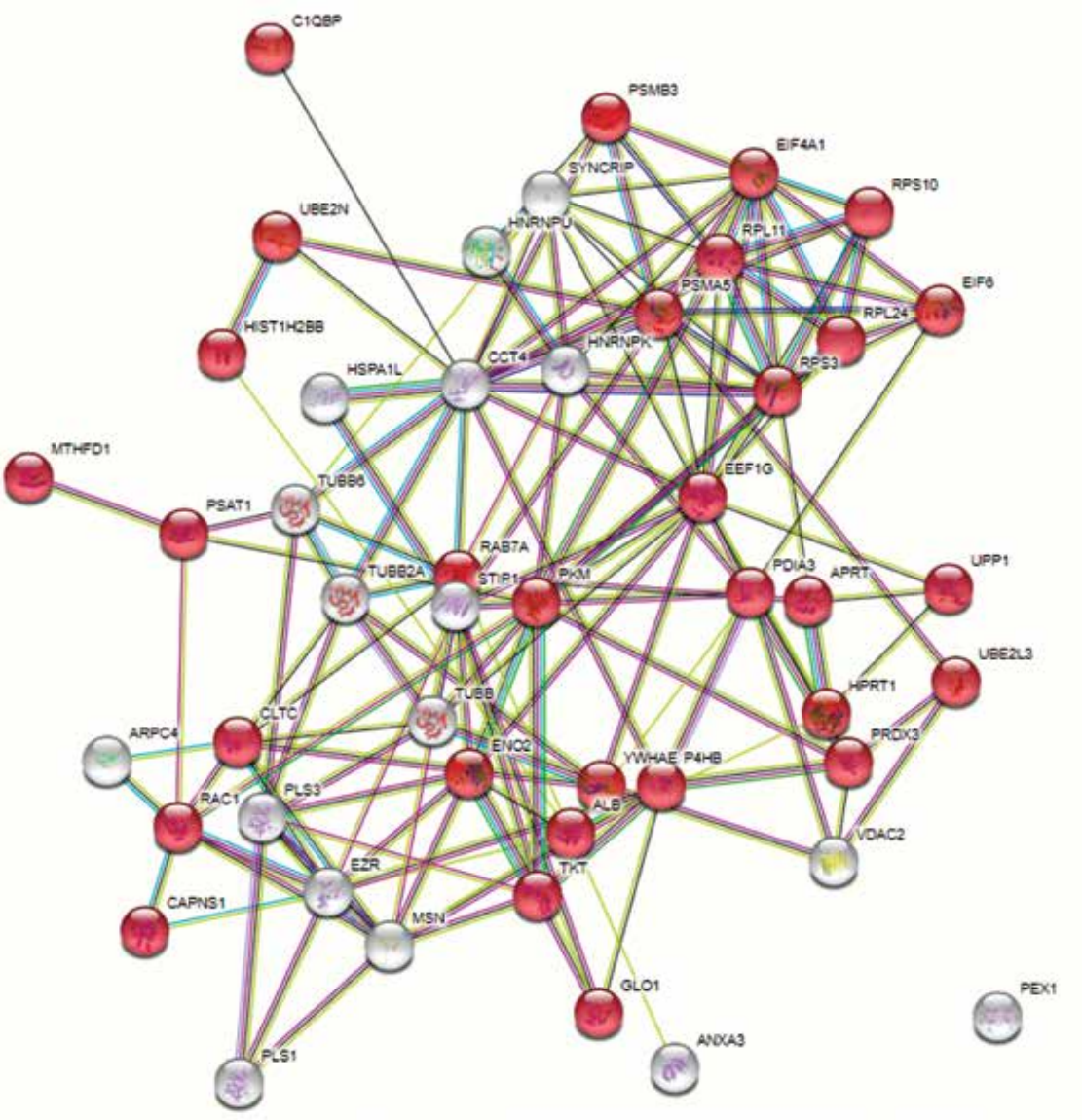


Fig 4-Interactions among proteins involved in organonitrogen metabolic processes differentially expressed between genistein-treated and untreated KKU-M213 cells.

Proteins identified by mass spectrometric analysis and classified according to biological processes as described in legend to Fig 3 were analyzed for their interactions using STRING software (https://string-db.org). Red node represents protein.

compared to control cells (Fig 5), in keeping with 5.1-fold increase and 3.2-fold decrease respectively from MS emPAI values (Table 1).

## DISCUSSION

This is the first report on an anti-proliferative effect of soy isoflavone GLY against a human CCA cell line. As

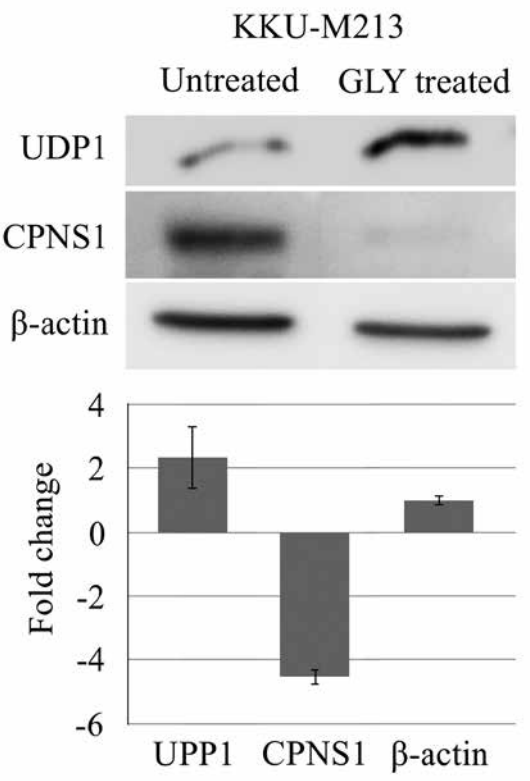


Fig 5-Western blotting analysis of uridine phosphorylase 1 (UPP1) and calpain small subunit 1 (CPNS1) from genistein (GLY)-treated and untreated KKU-M213 cells.

Proteins from cell lysates were separated by 12% SDS-PAGE, transferred onto polyvinylidene difluoride membrane and immunoreactive protein bands were detected using rabbit primary monoclonal antibodies and goat secondary horseradish peroxidase-conjugated anti-rabbit antibodies using an enhanced luminescence assay and quantified relative to β-actin loading control. Results are presented as mean ± SD of triplicate experiments. Upper panel depicts typical immunoreactive bands.

a step towards elucidating GLY anti-proliferative mechanism(s), differences in quality and quantity of proteins were compared between GLY-treated (at $IC_{50}$ value) and control KKU-M213 cells using mass spectrometry. A label-free quantitative proteomics based on emPAI values has been successfully employed in quantification of *Aspergillus fumigatus* secretome profiling at different temperatures (Adav *et al*, 2013) and of mannose-binding proteins from a normal donor and hepatocellular carcinoma patient sera (Yang *et al*, 2013).

A previous study on the induction of apoptosis by treatment with pomiferin, an isoflavone extracted from *Derris malaccensis*, of an intrahepatic CCA HuCCA-1 cell line isolated from a Thai patient, employed two-dimensional gel-electrophoresis to demonstrate upregulation of annexin A1, ATP synthase D, calcyclin, calgizzarin, cofilin, glucose-regulated protein 75, peroxiredoxin-1, ribosomal protein P0, and triose phosphate isomerase; and down-regulation of cytokeratin proteins 7, 18 and 19 compared to control cells (Svasti *et al*, 2005). Similarly, in the present study, GLY-treated KKU-M213 expressed slight down regulation of these latter proteins and slight up-regulation of annexin A1, peroxiredoxin-1, ribosomal protein P0 and triose phosphate isomerase. Although these similarities in protein profiles between pomiferin and GLY exposure of two independently derived intrahepatic CCA cell lines are preliminary, the findings suggest isoflavones might act in the same manner against CCA cells.

In summary, the study demonstrates among four isoflavones evaluated as anti-proliferative agents against a human cholangiocarcinoma cell line glycitein had the lowest 50% inhibitory concentration in the low micromolar range. Mass spectrometric analysis revealed changes in protein profile, both in types and quantity between glycitein-treated and control cells. This information provides

a preliminary baseline set of protein data, which should be useful in further studies to understand anti-cancer mode of action of isoflavones, in particular against cholangiocarcinoma prevalent with high mortality rate in northeastern Thailand, and to stimulate research into the discovery and development of natural products as new drugs against the global rise of cancer.

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