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Effective enrichment of cholangiocarcinoma secretomes using the hollow fiber bioreactor culture system

Churat Weeraphan [a,1], Penchatr Diskul-Na-Ayudthaya [a,1], Khajeelak Chiablaem [a], Amnart Khongmanee [b], Daranee Chokchaichamnankit [a], Pantipa Subhasitanont [a], Jisnuson Svasti [a,c], Chantragan Srisomsap [a,*]

[a] Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok, Thailand
[b] Applied Biological Sciences Program, Chulabhorn Graduate Institute, Bangkok, Thailand
[c] Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand

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ABSTRACT

The Northeastern region of Thailand is well known to have high incidence of bile duct cancer known as cholangiocarcinoma. So there is a continued need to improve diagnosis and treatment, and discovery of biomarkers for early detection of bile duct cancer should greatly improve treatment outcome for these patients. The secretome, a collection of proteins secreted from cells, is a useful source for identifying circulating biomarkers in blood secreted from cancer cells. Here a Hollow Fiber Bioreactor culture system was used for enrichment of cholangiocarcinoma secretomes, since this culture system mimics the dense three-dimensional microenvironment of the tumor found *in vivo*. Two-dimensional fluorescence difference gel electrophoresis using a sensitive Fluor saturation dye staining, followed by LC/MS/MS, was used to compare protein expression in the secretomes of cells cultured in the Hollow Fiber system and cells cultured in the monolayer culture system. For the first time, the 2D-patterns of cholangiocarcinoma secretomes from the two culture systems could be compared. The Hollow Fiber system improved the quality and quantity of cholangiocarcinoma secreted proteins compared to conventional monolayer system, showing less interference by cytoplasmic proteins and yielding more secreted proteins. Overall, 75 spots were analyzed by LC/MS/MS and 106 secreted proteins were identified. Two novel secreted proteins (C19orf10 and cystatin B) were found only in the Hollow Fiber system and were absent from the traditional monolayer culture system. Among the highly expressed proteins, 22 secreted soluble proteins were enriched by 5 fold in Hollow Fiber system compared to monolayer culture system. The Hollow Fiber system is therefore useful for preparing a wide range of proteins from low-abundance cell secretomes.

1. Introduction

Cholangiocarcinoma (CCA) is an aggressive malignant tumor with especially high incidence in Northeast Thailand, where it is associated with liver fluke (*Opisthorchis viverrini*) infestation and nitrosamine ingestation [1,2]. The incidence and mortality rates of CCA continue to increase worldwide [3]. Treatment of CCA includes radical surgery that is effective only at early stages of CCA. Current diagnosis is limited by the lack of sensitivity and specificity of currently available biomarkers to detect early signs of the disease. Continued efforts have been made to identify better tumor markers for early diagnosis, including the search for novel markers in serum or bile fluid for the diagnosis and prognosis of CCA [4–6]. Thus, interleukin 6, trypsinogen, mucin-5AC, soluble fragment of CK19 and the platelet–lymphocyte ratio levels in serum have all been reported to be useful in the diagnosis of CCA [4].

Work on secretomes or secreted proteins have provided valuable results in discovering biomarkers using proteomics [7–10]. In addition, more proteomic studies of secreted proteins from cell culture media have been reported recently [11–13]. We have reported studies on both the proteome and secretome of CCA [14,15], comparing the cholangiocarcinoma cell line HuCCA-1 to four hepatocellular carcinoma cell lines, with the result that each cell line showed distinct secreted proteins [16]. Forty-nine proteins were secreted only in HuCCA-1 and after validation using twelve homogenate samples from pairs of normal and cancer tissues from CCA patients, we concluded that lipocalin 2 (NGAL) may be a potential biomarker.

The monolayer culture system (MNC) is generally used to prepare secreted proteins, by changing the serum-containing

* Corresponding author. Tel.: +66 2 5740622 33x3715.
E-mail address: chantragan@cri.or.th (C. Srisomsap).
[1] Equal contribution.

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media to serum-free media, and collecting the conditioned media obtained after incubating overnight. However, using this protocol, leakage of cytoplasmic proteins in the system can interfere with analysis of serum-containing media. Such difficulty in using cell culture media from MNC to collect secreted proteins has previously been reported in secretome studies [17–19]. The hollow fiber bioreactor (HFB) culture system is a commercial system that was designed to allow medium to flow continuously [20]. Due to the advantages of HFB system, especially the ability to grow cells continuously in a small volume leading to tissue-like cell densities, the HFB system has been of much interest [21–23]. Additionally, the system has been widely used for many applications including: bioartificial organs [24], monoclonal antibody [25], recombinant protein production [26], screening anticancer activity, and studies on many cell lines [27]. The ability of the HFB system to culture healthy cells at high density continuously while allowing researchers to adapt serum concentration level from low to serum free condition is the main feature that greatly benefits secretome studies. Thus, secretome production has been studied using the HFB system for culture of a nasopharyngeal cancer [28] and hepatocellular carcinoma cell line [29]. The system mimics *in vivo* microenvironment, where cells are grown under dense 3-dimensional conditions, instead of the usual two-dimensional MNC system. Thus, proteins secreted from the HFB culture system should reflect *in vivo* secretion into the bloodstream more accurately, allowing for improved identification of CCA secreted proteins.

In this paper, we have analyzed the secretome of CCA using an HFB culture system compared to conditioned media from a conventional MNC system. Major limitations of analyzing secretomes from conventional monolayer culture are low levels of secreted proteins and presence of cellular proteins from cell lysis, which may mask genuine secreted proteins leading to inconclusive results. With the HFB culture system, we could enrich secreted proteins from CCA, with low levels of contaminant proteins leaking out from cell lysis. Usually the amount of proteins obtained from MNC conditioned media is not enough for analysis using 2-DE. Therefore, most reports on secretomes can only use 1-DE for characterization. However, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) method could be used in our studies. Moreover, the Fluor saturation dye was used instead of the Fluor minimal dye, because the former is more sensitive, since it labels all available cysteine residues, so that only 5 μg protein is sufficient.

The expression of secreted proteins was then studied by two-dimensional DIGE and liquid chromatography mass spectrometry (LC/MS/MS). Comparison of the 2D-patterns of CCA secretomes, showed that the HFB system improved the quality and quantity of the CCA secreted proteins compared to the MNC system, showing less interference by cytoplasmic proteins and increased amounts of secreted proteins. The HFB procedure is therefore a useful method for preparing low-abundance cell secretomes in good yield, and may be helpful for research on biomarker discovery.

2. Materials and methods

2.1. Preparation of serum-free medium cell culture

The bile duct epithelial carcinoma cell line (HuCCA-1) from a Thai patient [30] was grown as a monolayer in tissue culture flask 75 cm^2, in Ham's F-12 culture medium (Gibco, Grand Island NY, USA) containing 10% fetal bovine serum (FBS, HycloneLaboratories, Lagon, UT, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco-Invitrogen, USA). Cells were incubated in a humidified incubator at 37 °C under 5% CO_2. The volume of serum in the medium was then gradually reduced and replaced with CDM-HD serum replacement (FiberCell® Systems Inc., Frederick, USA) by decreasing FBS from 10%, 8%, 6%, 4%, 2% and finally to 0%, at the same time, increasing the percentage of CDM-HD serum replacement step-wise from 0%, 2%, 4%, 6%, 8% and finally to 10% CDM-HD medium. Following the adaptation to serum-free medium, cells were subcultured for 3 passages before experiments were performed.

2.2. Monolayer culture system

The completely adapted serum-free medium HuCCA-1 cells (5×10^6 cells) were cultured in Ham's F-12 medium containing 10% CDM-HD serum replacement and 1% antibiotic in a T75 culture flask. The entire conditioned medium was changed daily and collected for 9 days. Different cell cultures with different passages were performed in triplicate. Twenty flasks were used in total to provide 1×10^8 cells for inoculation into the HFB cartridge.

2.3. Hollow fiber bioreactor culture system

Before inoculation of cells, the HFB cartridge (#C2011, FiberCell® Systems Inc.) was pre-circulated for 3 days with sterile phosphate buffer saline (PBS), then Ham's F-12 medium for 24 h and Ham's F-12 medium containing 10% CDM-HD for another 24 h. HuCCA-1 cells (1×10^8 cells), which had been completely adapted to serum-free medium, were then inoculated into the HFB cartridge according to the FiberCell Hollow Fiber instruction manual [31]. Briefly, cells were inoculated into hollow fiber cartridge using two sterile syringes followed by gently flushing back and forth through the cartridge five times to ensure a homogeneous distribution of cells. Cells were allowed to attach to the fiber by leaving at 37 °C under 5% CO_2 for 30 min. The cartridge was rotated by 180° and then incubated for another 30 min. After the one-hour attachment period, the cartridge was placed into the pump system (30 pulses per min) and incubated at 37 °C under 5% CO_2 for 9 days. The entire conditioned medium was changed and collected daily for further analysis.

2.4. Determination of glucose consumption

Cell proliferation was determined by glucose consumption. 2 μL of the conditioned medium from both the monolayer and HFB cultures were assayed daily for depletion of glucose with a blood glucose meter (Medisafe-mini GR-102, Terumo Co., Tokyo, Japan).

2.5. Measurement of lactate dehydrogenase release

The lactate dehydrogenase (LDH) assay was used to determine the extent of cell leakage. 100 μL of the conditioned medium from both the monolayer and HFB cultures at days 1–9 were mixed with an equal volume of LDH assay mixture (LDH assay substrate solution: dye solution: co-factor solution). Samples were incubated in the dark at room temperature for 25 min and the reaction stopped by adding 1/10 volume of 1 N HCl. The absorbance was then measured at 450 and 690 nm.

2.6. Isolation of soluble-secreted proteins from HuCCA-1 culture medium

250 mL of conditioned medium from both the monolayer and HFB cultures were harvested by centrifugation at 480*g* for 5 min to sediment non-adherent cells, and the supernatant centrifuged further at 2000*g* for 10 min to remove cellular debris. The

supernatant were lyophilized and concentrated to ~2.5 mL. The concentrated proteins were allowed to precipitate in 10% trichloroacetic acid overnight and resuspended in 2-DE sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 20 mM Tris–HCl buffer, pH 8.0). The concentrations of total proteins were determined by the Bradford protein assay reagent (Bio-Rad Laboratories, CA) [32], and proteins were stored at −80°C for further analysis.

2.7. Preparation of cell lysate proteins from HuCCA-1 monolayer culture system for confirmation of protein leakage by western blot

Approximately 5.6×10^6 cells were grown to 80% confluence. The cells were washed with 25 mM sucrose twice before trypsinization and then centrifuged at 480*g* for 5 min. The cell pellets were suspended in 2-DE sample buffer and then lysed by sonication on ice. Cellular debris was removed by centrifugation at 10,000*g*, 4°C for 10 min. The supernatant was assayed for protein concentration by the Bradford reagent and stored at −80 °C for further analysis.

2.8. Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) analysis using CyDye DIGE fluor saturation dye

Soluble-secreted proteins from both monolayer and HFB culture at day 5 of the experiment from 3 different cell cultures were labeled to saturation with fluorescent cyanine dyes (CyDye) according to the manufacturer's instructions (GE Healthcare, NJ, USA). Briefly, 5 μg of each sample were separately incubated with 2 mM Tris-(carboxyethyl) phosphine hydrochloride (TCEP) at 37 °C for 1 h in the dark and then labeled with 40 nmol of Cy5 (GE Healthcare), while the 5 μg internal standard (a mixture of 2.5 μg HFB and 2.5 μg MNC) was labeled with 40 nmol of Cy3. Labeling was performed at 37 °C for 30 min in the dark and then reactions were quenched by addition of 2X sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT and 2% ampholine). The quenched Cy3, and Cy5-labeled samples were combined and incubated overnight with immobilized pH gradient (IPG) strips (13 cm, pH 3–10 non-linear; GE Healthcare). Isoelectric focusing (IEF) was performed using an Ettan IPGphor 3 (GE Healthcare) at 20,000 Vhr, 50 μA per gel strip. The IPG strips were then equilibrated and separated in 12.5% SDS-PAGE using SE 600 Ruby apparatus (GE Healthcare) at a current of 25 mA per gel. Gels were scanned using an Ettan DIGE Imager (GE Healthcare) for excitation and emission (532/580 and 633/670 nm for Cy3 and Cy5, respectively). The Image Master 2-DE platinum software version 7.0 was used for image analysis. Using the internal standard to normalize between gels, protein volume was quantified from fluorescence intensity of Cy3 and Cy5 and then calculating the Cy5/Cy3 ratios (sample/internal standard). Values were then normalized based on the assumption that the amount of protein per image is the same. This approach takes into account gel-to-gel variability over the entire dataset. For this direct analysis, significance levels were determined based on two SDs of the mean volume ratios (95th percentile confidence). One and a half (1.5)-fold increases/decreases of protein expression were classified as significant.

2.9. In-gel digestion

Protein spots that differed significantly were selected and digested using trypsin according to the protocol of Srisomsap et al. [16]. Briefly, spots were excised from the gel and destained with 50% acetonitrile in 0.1 M NH_4HCO_3. Gel pieces were reduced with 10 mM DTT and alkylated with 100 mM iodoacetamide. After removing supernatant, gel pieces were dried and digested with 1 μg of trypsin (Promega Co., WI, USA) in digestion buffer (90 μL 0.05 M Tris–HCl pH 8.5/10% ACN/1 mM $CaCl_2$). The mixture was further incubated overnight at 37 °C and digestion buffer was collected for protein identification.

2.10. Identification of proteins by LC/MS/MS

Nanoflow liquid chromatography (Waters, Milford, MA, USA) coupled with electrospray ionization (nano ESI MS/MS) quadrupole-time of flight tandem mass spectrometry (Q-Tof micro; Micromass, Manchester, UK) was used to identify the trypsinized protein spots following the method described in Srisomsap et al. [16]. A 75 μm id × 150 mm C_{18} PepMap column (LC Packing, Amsterdam, Netherlands) was used to concentrate and desalt all trypsinized peptides. To elute peptides, 0.1% formic acid in 97% water, 3% ACN (solution A) and 0.1% formic acid in 3% water, 97% ACN (solution B) were used, respectively. Six microlitters of sample was injected into the nano-LC system, and then the separation was performed. Nano ESI MS/MS was used to produce MS/MS spectra and automatically processed. Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. An automatic scan rate was 1.0 s with an interscan delay of 0.1 s during analysis. The range from 400 to 1600 m/z was set for parent mass peaks to be picked out for MS/MS analysis. The collision energy used was at 38 eV. The MassLynx 4.0 software (Micromass) was used to process MS/MS data. The file was converted to PKL files by the ProteinLynx 2.2 software (Waters). The MASCOT search engine (http://www.matrixscience.com) was performed using NCBI Database against *Homo sapiens* taxonomy. The search parameters were set as follows: peptide mass tolerance was 1 Da; MS/MS ion mass tolerance was 1 Da, allowance was set to 1 missed cleavage, enzyme set as trypsin, the limit of peptide charges was 2+ and 3+. The protein identification was analyzed by using $p\text{-value} \leq 0.05$ and Mascot score > 35 being considered as promising hits. The criteria from Kristiansen et al. [33] was used, namely for one matched peptide with a length of at least 8 amino acids, a sequence tag of at least 3 amino acids would be selected as a good y-ion series. The peptide score and Mascot score for proteins containing one matched peptide should be greater than 35 and 20 respectively.

2.11. Western blot

Proteins from each condition were resolved in 10% SDS-PAGE at a constant 10 mA/gel for 2–2.5 h. The separated proteins were electrotransferred onto PVDF membranes (Immobilon-P; Millipore, MA, USA), followed by probing membranes with monoclonal antibody against human C19orf10 (1:1000, Abcam Inc., USA), Cystatin B (1:200, Abcam Inc.), S100A6 (1:1000, Abcam Inc.), S100A11 (1:1000, Proteintech Group, Inc., USA), annexin A2 (1:5000, Abcam Inc.), protein DJ-1 (1:2500, Abcam Inc.), serpin B5 (1:1000, Abcam Inc.), stathmin 1 (1:50,000, Abcam Inc.), heat shock cognate 70 (1:5000, Abcam Inc.), Proprotein convertase subtilisin kexin type 9 (1:1000, Abcam Inc.), Cytokeratin 19 (1:2000, Millipore, MA, USA), ß-actin (1:5000, Sigma, USA) and α-tubulin (1:5000, Cell Signaling technology Inc., MA, USA). The membranes were incubated with secondary antibody (Dako Cytomation, Glostrup, Denmark), followed by washing and incubating for 5 min with enhanced chemiluminescent reagent, followed by detection with high-performance film (GE Healthcare).

The loading control images were prepared by using deep purple staining method on the membrane. Briefly, membranes were probed with antibodies and washed with milliQ water for 5 min, followed by incubating with 1:400 deep purple stain solutions (GE Healthcare) in the dark for another 30 min. The membranes were washed with washing solution (30% methanol

and 7.5% acetic acid) and rinsed with 100% methanol until the green background was removed. Membranes were scanned using an Ettan DIGE Imager (GE Healthcare) for excitation and emission at 532 nm and 560 nm, respectively.

2.12. Statistical analysis

Statistical analysis was performed with SPSS 10.0 and data were analyzed using unpaired t-test to determine the difference between HFB and monolayer condition. Values were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. The HuCCA-1 cell line grew very well in protein-free medium, CDM-HD

To reduce serum protein contamination [34], the HuCCA-1 cell line was adapted to serum-free medium by gradually reducing FBS and proportionally increasing CDM-HD supplement before inoculation into the HFB system. CDM-HD protein-free medium is optimized for use in HFB system by FiberCell Systems [20]. During the adaptation process, the morphology of HuCCA-1 cells was continuously monitored. While there were minor morphological differences between HuCCA-1 cells in FBS compared to those in CDM-HD (serum-free) medium, no cell lysis was observed (data not shown). The growth rate of HuCCA-1 cells in serum containing and serum-free conditions were compared. After the third passage of 100% serum-free culture, 2×10^6 of the original HuCCA-1 cells and adapted cells were separately cultured for 55 h, followed by counting cell numbers. No significant differences were observed in cell growth rates after culture in CDM-HD supplement. The average number of cells obtained from serum cultures and CDM-HD cultures were 3.86×10^6 and 3.90×10^6, respectively.

3.2. Comparison of cell growth and protein enrichment in conditioned media from hollow fiber bioreactor and monolayer culture system

Since cell number cannot be directly counted within HFB system, monitoring of glucose consumption rate of the culture was used as an indirect method to determine cell growth rate in the HFB culture system [35]. Therefore, cell growth of HuCCA-1 within the HFB system was monitored by measuring glucose consumption level within the medium. Initially, 1×10^8 adapted cells were inoculated into the HFB system (covering approximately 50% of the surface area in the cartridge). Glucose consumption level showed a steady increase from days 1–7 in HFB, reaching a plateau at day 7, but glucose consumption in MNC increases for days 1–5, but then declines (Fig. 1A). Glucose consumption on the seventh day was about 340 mg, about three-fold that of the first day (Fig. 1A), so it seems that 3.7×10^9 cells may be reached within 7 days after inoculation and be maintained in the HFB cartridge.

Additionally, since the doubling time of HuCCA-1 is 55 h [30], the rate of cell growth should reach plateau at day 5. However, cells grown in HFB remain healthy for more than 1 month (data not shown). This agrees with the reported growth of cultivated cultures in hollow fibers of LNCaP cell line [36], and other tumor cell lines [37]

Total protein extracted from HFB and MNC system were compared from days 1–9 as shown in Fig. 1B. The protein concentrations obtained from MNC show a steady increase over time while those obtained from HFB were maintained at a

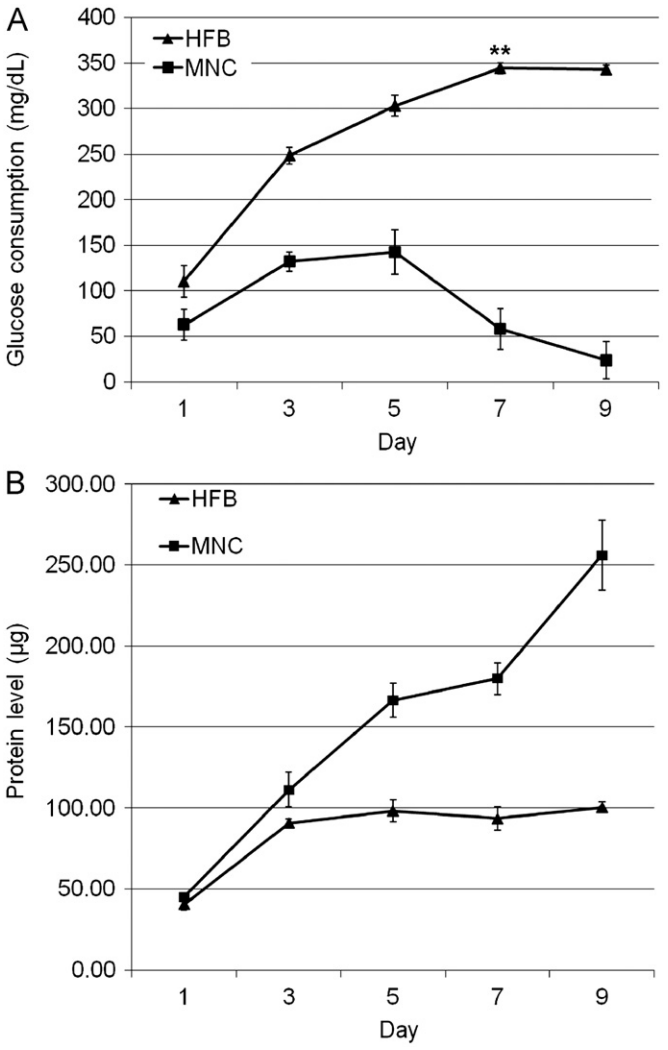

Fig. 1. (A) Comparison of glucose consumption in conditioned media (CM) from hollow fiber bioreactor (HFB) and monolayer culture (MNC) systems at days 1–9. (B) Total protein extracted from hollow fiber bioreactor (HFB) and monolayer culture (MNC) system at days 1–9.

constant level after day 3. However, since glucose consumption levels in MNC system suggest that cell number should decrease at day 5 (Fig. 1A), the significant increase in protein concentration obtained from MNC may be due to cellular protein contamination or cell lysis, rather than accumulation of secreted proteins [38,39]. On the other hand, the protein concentrations obtained from HFB were maintained at a constant level after day 3, implying absence of cellular protein contamination.

3.3. Cell leakage in both HFB and MNC systems was compared and the secreted proteins were verified by western blotting

Interference of cytoplasmic proteins is a major problem in secretome analysis, since this masks the truly secreted proteins [40]. Therefore, viability assays have been performed in parallel with secretome analysis to monitor the release of cytoplasmic contaminations from dying cells [6]. However, as mentioned above, direct cell viability assay cannot be performed with the HFB culture system. So leakage of cytosolic protein was followed by enzymatic assay of LDH leakage and Western blot analysis of major cytosolic proteins [36].

When release of LDH into conditioned medium was measured to estimate cell leakage, there was still little LDH release with

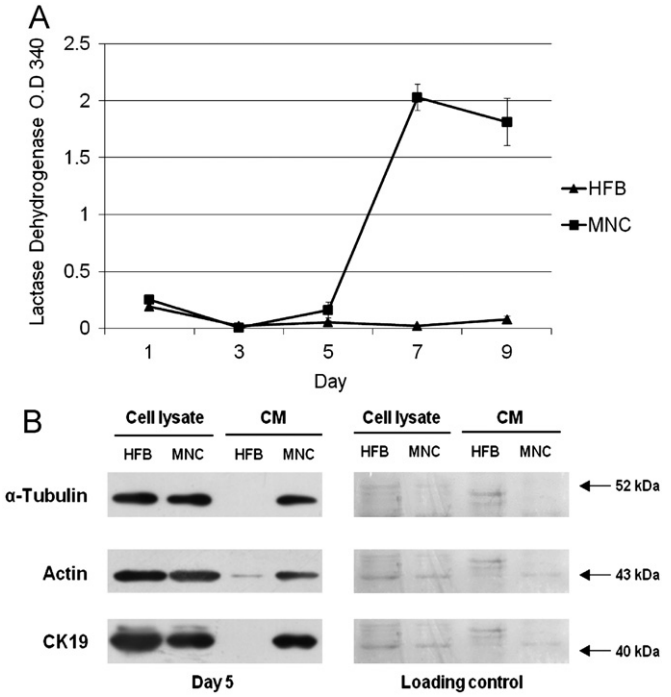

Fig. 2. (A) Measurement of LDH in conditioned media of HFB and MNC as an indicator of cellular protein leakage. (B) Western blots on selected cellular proteins α-tubulin, actin and cytokeratin-19 were performed to detect cellular protein leakage into condition media from day 5. The loading control images were obtained by staining of membrane with deep purple dye after the Western blotting.

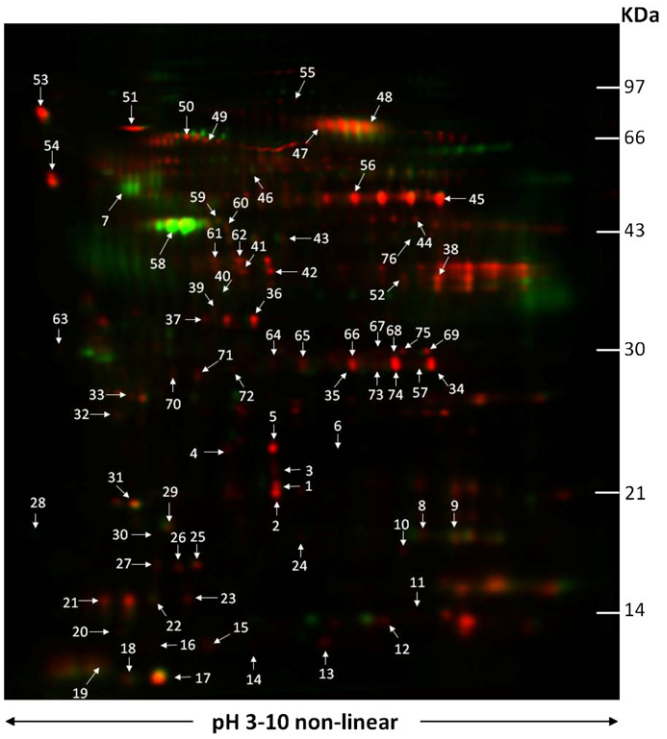

Fig. 3. 2D-DIGE of enriched CCA conditioned media (red) compared to cell lysate fraction (green) obtained from HFB culture system. Yellow indicates equivalent expression from both fractions.

both HFB and MNC systems for days 1–3 (Fig. 2A). However, while LDH release into conditioned medium was low and fairly constant for HFB, LDH release increased substantially in MNC after day 5 (Fig. 2A). In addition, Western blotting of two major cytosolic proteins (β-actin and α-tubulin) and the CCA marker cytosolic protein (cytokeratin-19) was also performed to study cell leakage into conditioned media from day 5. The results showed significant amounts of cytosolic proteins β-actin, α-tubulin and cytokeratin-19 (CK19) in conditioned media of MNC, but absence or near absence of these proteins in conditioned media of HFB (Fig. 2B). Since all three proteins were found in the cell lysate, these results provide further evidence to demonstrate lack of cytosolic protein leakage in the HFB system, unlike that found in the MNC system.

3.4. Secretomic analysis using 2D-DIGE with CyDye DIGE fluor saturation dye

The conditioned media from both HFB and MNC systems were lyophilized to reduce volume and then proteins were precipitated with TCA. Equal amounts (5 μg) of both HFB and MNC concentrated conditioned media were resolved by 2D-DIGE using CyDye DIGE Fluor saturation dye on 12.5% SDS-PAGE gel in triplicate. The protein patterns of HFB conditioned media differed remarkably from MNC conditioned medium as shown in Fig. 3. The Image Master software was used to compare the spots from HFB and MNC culture system. Only proteins whose expression levels differed by greater than 1.5 fold between the systems were considered significant and included in our analysis. Approximately 800 spot-features were visualized in each 2-DE gel. Of these, 427 spots (53.4%) were significantly increased in HFB culture, 218 spots (27.3%) were significantly increased in MNC culture, and 154 spots (19.3%) did not differ significantly between the two systems.

Of these, 75 spots showing differential expression in the two culture systems were characterized and identified by LC/MS/MS. On the basis of searching the mass spectrometry data against Swiss-Prot database, 106 proteins with significant hits proteins could be identified from the 75 spots (Supplement Table 1). These were categorized according to their functions into various categories, namely: chaperone/stress response, cytoskeleton/mobility, DNA replication/gene regulation, extracellular matrix, immunological response, oncogene/tumor suppressor genes, metabolism, protection and detoxification, protein synthesis and degradation, signal transduction, transport/binding proteins and unannotated/function inferred. Over half (approximately 56%) of the secreted proteins identified belong to three major categories, metabolism (22.6%), protein synthesis/degradation (17.9%) and cytoskeleton/mobility (15.1%) (Fig. 4). Our result is in agreement with the report of Gramer et al. [25] on the functions of the HepG2 secretomes, performed using the HFB culture system, where secreted proteins belonged mainly to protein metabolism (26%), and metabolism and energy pathways (23%).

Using 2D-DIGE in combination with LC/MS/MS to compare the differential protein expression of HFB and MNC culture system, two secreted proteins (C19orf10 and Cystatin-B) were found to be expressed only in conditioned medium from the HFB culture system. Among those highly expressed, 22 soluble-secreted proteins were enriched 5-fold higher in the HFB culture system compared to the MNC system. These proteins include: stathmin isoform a, nucleoside diphosphate kinase 3, thioredoxin peroxidase B, protein DJ-1, S100A11, S100A4, cystatin-A, annexin A3, serpin B5, isoprenyl-diphosphate delta-isomerase 2, phosphoserine phosphatase, triose phosphate isomerase. Only four proteins showed increased expression in the MNC system compared to the HFB culture system (alpha-tubulin, 39 S ribosomal protein L3, mutant beta-actin and gamma-actin). The remaining 78 proteins were expressed in the range of 1.5–5.0 folds (Supplement

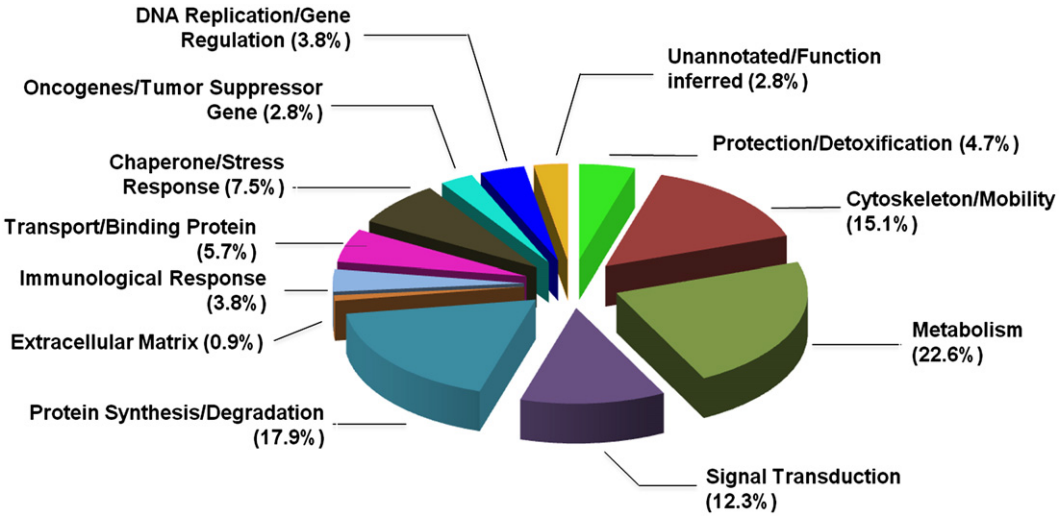

Fig. 4. Biological functions of secreted proteins in hollow fiber bioreactor were shown distributed using a pie chart.

Table 1), such as superoxide dismutase [Cu–Zn], nuclear transport factor 2, coactosin-like protein, annexin A2, proprotein convertase subtilisin kexin type 9 (PCSK9), heat shock cognate 70 kDa protein, enolase (alpha, beta and gamma), E-cadherin, lamin A/C, isoform CRA_a, peroxiredoxin-6, disintegrin and metalloproteinase domain-containing protein 21, cyclophilin A, S100A6 and espin.

3.5. *Validation of the enrichment of CCA secretomes in the HFB culture system*

The present study shows that secreted proteins from CCA were considerably enriched with the HFB culture system compared to the conventional MNC system. The two secreted proteins, C19orf10 and Cystatin-B, expressed only in HFB culture system are of much interest. C19orf10 is a secretory protein, which was reported to be overexpressed in AFP-positive HCC cases. C19orf10 is believed to regulate the Akt/MAPK and cell cycle pathways, and was suggested to be a potential tumor marker for HCC treatment [41]. Cystatin-B is a cathepsin protease inhibitor that was shown to be increased in many types of cancer including ovarian, lung, laryngeal, bladder, colorectal and hepatocellular carcinoma [42].

In this study, some proteins were found to show increases in expression as high as 9.5-fold, differentially expressed proteins were categorized into two groups: 1.5 to 5-fold change and more than 5-fold change. Some proteins showing fold changes as low as 1.5-fold were included, because they may be potential biomarkers and warrant further study. For example, four types of HSP70 (spots 55–58), heat shock proteins (HSPs) related to stress in cells, have been found to be expressed when cells are under lethal conditions. HSPs help cells to survive and prevent cellular apoptosis [43]. Potential biliary markers of HSP70 for cholangiocarcinoma detection have been reported, where HSP27 and HSP70 levels seemed to be increased significantly in the bile of cholangiocarcinoma patients, compared to lithiasis patients [44].

We further used immunodetection to verify some distinct secreted proteins that may be useful in cancer biomarker study including C19orf10, Cystatin B, PCSK9, HSC-70, serpin B5, annexin A2, DJ-1, stathmin, S100A6 and S100A11. Samples studied include fractions from conditioned media of HFB and MNC of HuCCA-1 cells. Our results confirmed significant increases in these proteins in HFB conditioned medium compared to MNC conditioned medium (Fig. 5). C19orf10 and Cystatin B were confirmed to be expressed only in the HFB system. Some proteins in S100 family showed increased expression in the HFB system compared to the

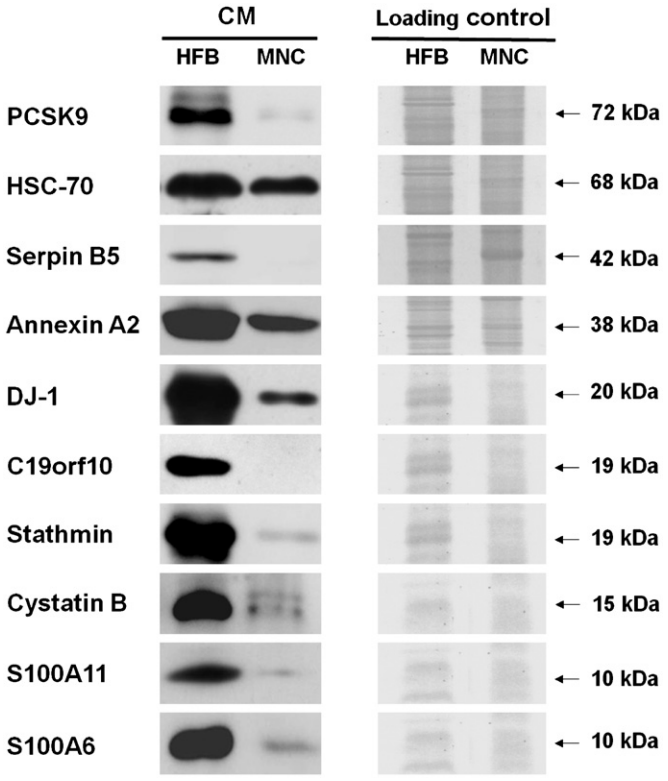

Fig. 5. Western blot of conditioned media (CM) from hollow fiber bioreactor (HFB) and monolayer culture (MNC). Results show enrichment of C19orf10, cystatin B, HSC70, serpin B5, annexin A2, DJ-1, Stathmin, S100A6 and S100A11 in the HFB secretome fraction. The loading control images were obtained by staining subsequently with deep purple dye of membrane after the Western blotting.

MNC system including S100A4, S100A6 and S100A11, with fold changes of 6.98-fold, 2.10-fold and 6.98-fold respectively. Western blot results also confirmed increased levels of S100A6 and S100A11 (Fig. 5). S100 calcium binding proteins are acidic low molecular weight proteins that normally exist as symmetric homodimers stabilized by noncovalent interactions [45]. S100 proteins have been reported to perform many roles, such as cell growth, cell movement, oxidative cell damage protection, and cell cycle progression [46]. Even though S100 proteins lack the classical signaling sequence for secretion, previous studies have likewise demonstrated an alternate secretory pathway,

independent of the classical ER–Golgi route [47]. Among S100 protein family, S100A4 has also been reported to show a distinct expression in CCA [48].

Our previous study [16] showed that annexin A1, annexin A2, annexin IV variant, and annexin A5 were expressed in this cholangiocarcinoma cell line. In the present study, annexin A1, annexin A2 and annexin A3 were significantly increased by 1.97-fold, 2.2-fold and 9.1-fold respectively in the HFB culture system compared to the MNC system. Annexins fall into 12 subfamilies (A1–A11 and A13) in humans, and play both intra- and extracellular roles in cell signaling, apoptosis, cell division and ion transport. Extracellular occurrence has been shown consistently for several annexins and different means of unconventional secretion have been reported. Annexins A1, A2, and A5 interact with cytoskeletal proteins [49]. Annexin A1 is considered to be a mediator of glucocorticoid actions in anti-inflammatory scenarios [50], while annexin A2 is present on the external surface of endothelial cells as a co-receptor for tissue plasminogen activator, which mediates the conversion of plasminogen to plasmin [51]. Annexin A2 and annexin A4 appeared to be potential markers of interest for diagnosis of colorectal cancer [52]. Most interesting is Annexin A3, which was also reported to be associated with lung [53], colorectal [54] and prostate cancer [55] and should be studied further.

Our results showed high expression of DJ-1 (9.41 folds), which was further confirmed by immunodetection. DJ-1, a secreted oncoprotein, is known as Parkinson's disease associated protein and was found to be involved in cancer. It is overexpressed in many types of cancer including cervical cancer [56], breast cancer [57] and lung cancer [58].

Stathmin, oncoprotein 18, a microtubule-destabilizing protein, has an influence on cell proliferation, differentiation and in cell movement. High levels of stathmin have been reported in many cancers including leukemia, prostate cancer, breast cancer and sarcoma [59]. In liver, high expression of stathmin was correlated with hepatocarcinogenesis and tumor progression [60]. Stathmin has also been reported to play a role in cancer cell migration and as a prognostic marker in colorectal cancer [61]. Our results showed 5.42 fold increase of stathmin in HFB compared with MNC, and this was again confirmed by immunodetection (Fig. 5). It would be interesting to compare the expression of stathmin in HuCCA-1 and HCC liver cancer cell lines.

Proprotein convertase subtilisin kexin type 9 or PCSK9 is believed to be involved in lipid disorders and atherosclerosis [37], and was reported to have three isoforms. All three isoforms were found in the present study, with two high molecular weight isoforms (spot no. 47 and spot no. 48), and one low molecular weight form (spot no. 31). Our studies suggest that spot 47 is the intact PCSK9, while spot 31 is the propeptide part and spot 48 is PCSK9 lacking the propeptide. The expression of PCSK9 is high in liver, with an N-terminal prodomain, a catalytic domain, and a carboxyl-terminal domain. The prodomain is cleaved by an autocatalytic reaction, which is required for the secretion of PCSK9 [62].

Serpin B5 or maspin is an apoptosis inducer, angiogenesis inhibitor and tumor growth suppressor. There is suggestion that the combination of the expression of serpin5 and Bax proteins may be involved in delaying the progression of intrahepatic cholangiocarcinoma [63].

4. Conclusion

The secretome has many functional components, such as enzymes, cell–cell signals, immune defense etc., and is a useful source for identifying circulating biomarkers in blood secreted from cancer cells. The main challenges in secretome analysis are the complexity of sample preparation, serum protein and contamination by cell lysis. Most secretome studies tend to use 1-DE for analysis [34,38,64,65]. Our previous study on the CCA secretome in the monolayer culture system was performed by using 1-D SDS gel electrophoresis and LC/MS/MS, because only small amounts of sample were available, and this showed that only 83 proteins can be detected. To obtain additional information, the HFB culture system was used here since it can be used to culture large numbers of cells [14,15], with the bioreactor cartridge being designed to house 10^6–10^9 cells without sacrificing nutrient or oxygen deprivation. In addition, the HFB culture system has a number of advantages in providing greater cell attachment area, good delivery of nutrients to cells, and continuous removal of waste, which allows cells to continue growth for longer periods, as found here. In addition, the system offers a means for collection and enrichment of secreted proteins.

Thus, in the present study with the HFB culture system, secreted proteins from CCA were considerably enriched compared to the conventional MNC system, allowing isolation of sufficient amounts for 2-DE, LC/MS/MS characterization and other studies. The present studies show that approximately 107 proteins could be identified in HFB secretomes, which is much greater than the number previously identified in the MNC secretomes. This was not due to cell lysis, since the LDH release with the HFB system was much lower than in the MNC system, as was the level of actin and α-tubulin (Fig. 2). This indicates that the HFB culture system not only provided an enrichment of secreted proteins, but decreased the level of contamination by intracellular proteins.

Comparisons of the protein patterns of HFB and MNC conditioned media, showed that 75 spots were significantly increased in HFB conditioned media compared to that from MNC. Nine proteins were verified by Western blotting, namely C19orf10, Cystatin B, HSC-70, serpin B5, Annexin A2, protein DJ-1, stathmin, S100A6 and S100A11, which were all found to be increased in HFB conditioned media compared to MNC conditioned media. The two novel secreted proteins (C19orf10 and Cystatin-B) were only detected in HFB and could be the potential biomarkers for cholangiocarcinoma.

Thus, HFB culture system provides enrichment of secreted proteins, which may allow the identification of other novel secreted proteins in CCA, improving the prospects for further development of diagnostic markers and/or therapeutic strategies for CCA as well.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2012.05.054.

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