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Proteomics profiling of cholangiocarcinoma exosomes: A potential role of oncogenic protein transferring in cancer progression

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ABSTRACT

Cholangiocarcinoma (CCA), a common primary malignant tumor of bile duct epithelia, is highly prevalent in Asian countries and unresponsive to chemotherapeutic drugs. Thus, a newly recognized biological entity for early diagnosis and treatment is highly needed. Exosomes are small membrane bound vesicles found in body fluids and released by most cell types including cancer cells. The vesicles contain specific subset of proteins and nucleic acids corresponding to cell types and play essential roles in pathophysiological processes. The present study aimed to assess the protein profiles of CCA-derived exosomes and their potential roles. We have isolated exosomes from CCA cells namely KKU-M213 and KKU-100 derived from Thai patients and their roles were investigated by incubation with normal human cholangiocyte (H69) cells. Exosomes were internalized into H69 cells and had no effects on viability or proliferation of the host cells. Interestingly, the exosomes from KKU-M213 cells only induced migration and invasion of H69 cells. Proteomic analysis of the exosomes from KKU-M213 cells disclosed multiple cancer related proteins that are not present in H69 exosomes. Consistent with the protein profile, treatment with KKU-M213 exosomes induced β-catenin and reduced E-cadherin expressions in H69 cells. Collectively, our results suggest that a direct cell-to-cell transfer of oncogenic proteins via exosomal pathway may be a novel mechanism for CCA progression and metastasis.

1. Introduction

Cholangiocarcinoma (CCA), a severe neoplasm of biliary tract epithelia [1], is the second most common primary hepatic tumor globally [2]. CCA is often enigmatic, characterized by poor prognosis, and unresponsive to chemotherapeutic agents [3]. This type of cancer represents 10–25% of primary liver cancers worldwide and is highly prevalent in Asian countries including Thailand [4]. It responds poorly to the presently available therapies [5] and surgical resection is the only mean to extend lifespan for no more than 5 years [6]. It is, therefore, considered to be an incurable and rapidly lethal malignancy. In view of the lack of a valid early stage detection method and potential curative treatment, an effective early detection and/or an alternative therapeutic strategy with high sensitivity to specific targets is urgently needed.

Exosomes are small (40–100 nm in diameter) membrane bound vesicles that are initially formed within the endosomal compartment and secreted upon fusion of the limiting membrane of multi-vesicular bodies (MVBs) with plasma membrane [7]. These homogeneous vesicles are released by most of cell types including cancer cells and are considered as messengers of intercellular communications [8]. Biochemical and proteomic analysis of exosomes reveal that, besides a common set of membrane and cytosolic molecules, these vesicles contain cell and cell state specific cargos of proteins, mRNAs, and miRNAs that characterize their functional activities [9]. Of particular interest, tumor cells exhibit enhanced production of exosomes. The exact function and the need of such high amount of exosomes in malignant cells are not clearly understood. Recent investigations suggest the roles in cell-to-cell communication, tumor–stroma interaction, antigen presentation and establishment of tumor microenvironments that potentially uphold cancer progression at different steps [10]. Although exosomes from a variety of cell types have been isolated and characterized and their functions have been previously reported, the presence and the role of exosomes in CCA carcinogenesis are presently not known. We hypothesized that CCA cells may

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manufacture exosomes that reinforce CCA development. The present piece of work investigated whether CCA cells produce exosomes and what are their potential roles on malignant phenotypes. We found that KKU-M213 cells, an aggressive human CCA cell line, released exosomes in culture media with basic characteristics similar to those isolated from other sources [11]. A comparative proteomics approach between KKU-M213 and human cholangiocyte (H69) exosomes disclosed vast differences in their active protein contents. Furthermore, exosomes from KKU-M213 cells increased motility and altered cell adhesion related protein expressions in recipient H69 cells. Our results indicate that exosomes may play a role on CCA pathogenesis and identification of their cargo proteins may serve as a biomarker for early detection and thus might enlighten new treatment strategies.

2. Materials and methods

2.1. Reagents and antibodies

Ham's F12 nutrient mixture, DMEM, DMEM/F-12 media and antibiotic–antimycotic were purchased from Invitrogen (CA, USA). Fetal bovine serum (FBS), protease and phosphatase inhibitor, adenine, insulin, epinephrine, 3,3′,5 Triiodothyronine, apo-transferrin, epidermal growth factor and hydrocortisone were obtained from Sigma-Aldrich Co. (MO, USA). RIPA cell lysis buffer and SuperSignal West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Cramlington, UK). Anti-flotillin-1 and anti-E-cadherin primary antibodies were purchased from BD Biosciences (CA, USA). Anti-GSK3β and anti-phospho-GSK3β (Ser-9) were obtained from Cell Signal Technologies (MA, USA). Anti-β-catenin and anti-CD81 (B-11) antibodies were from Santa Cruz Biotechnology, Inc. (TX, USA). Anti-TSG101[4A10] and anti-CD63 were from abcam (Cambridge, UK). Anti-β-actin was form Sigma-Aldrich Co. (MO, USA). Trans-well chambers were obtained from Corning Life Sciences (MA, USA). All other reagents were of the highest analytical grades available and unless otherwise stated were purchased from Sigma-Aldrich (MO, USA).

2.2. Cell culture

KKU-100 and KKU-M213, the CCA cell lines established from primary tumors of *Opisthorchiasis*-associated Thai CCA patients [12], were kindly provided by Dr. Banchob Sripa. KKU-100 is poorly differentiated adenocarcinoma cell line with compact polygonal to spindle shape [12] whereas KKU-M213 is well-differentiated adenocarcinoma cell line [13,14]. These cells were cultured in Ham's F12 nutrient media, supplemented with 10% heat inactivated FBS and 1% antibiotic–antimycotic, at 37 °C in a humidified 5% CO_2 incubator. A human cholangiocyte cell line (H69) immortalized with the simian virus 40 (SV40) large T antigen was obtained from Dr. Gregory J. Gores, Mayo Clinic College of Medicine and cultured in DMEM/Ham-F12 media containing 10% FBS. Although immortal, this cell line is non-malignant; *in vitro*, it does not display anchorage independent growth and, *in vivo*, it does not produce tumors in nude mice [15].

2.3. Exosome isolation

CCA or H69 cells were plated in 75 cm^2 culture flasks. Cells were cultured in conditioned medium depleted of contaminating vesicles and protein aggregates by overnight centrifugation at 110,000 ×*g*. Culture supernatants were collected 48 h after changing the medium and exosomes were purified by differential centrifugation as described previously [16]. Briefly, culture supernatant was collected and first centrifuged 300 ×*g* at 4 °C for 10 min to remove lifted cells followed by centrifugation at 1,200 ×*g* for 10 min and 10,000 ×*g* at 4 °C for 30 min to remove cell debris. The resultant supernatant was subjected to filtration on 0.22 μm pore filters, followed by ultracentrifugation (Beckman Coulter Inc.; CA, USA) at 110,000 ×*g* for 70 min. The resulting pellets were re-suspended in chilled PBS, pooled, and again ultra-centrifuged at 110,000 ×*g* for 70 min at 4 °C. The final pellet of exosomes was re-suspended in 50–100 μl PBS, aliquoted and stored at −80 °C until use.

2.4. Electron microscopy

For electron microscopic (EM) observation of exosomes, pellets obtained after centrifugation at 110,000 ×*g* were fixed in 4% paraformaldehyde (PFA) (1:1 ratio) for 10 min and loaded on Formvar/carbon-coated EM grids by floating the grid over small drops of PBS containing fixed exosomes. The exosomes were then post fixed in 1% glutaraldehyde for 5 min and contrasted successively in freshly prepared 2% uranyl acetate, pH 7, and 2% methylcellulose/0.4% uranyl acetate, pH 4. Observations were made with a FEI Technai G^2 electron microscope (The Netherlands) equipped with a thermionic tungsten filament and operated at an acceleration voltage of 120 kV. Images were taken with a cooled slow-scan CCD camera at a magnification of 50,000×.

2.5. Migration and invasion assay

Cell migration and invasion assays were performed using trans-well chambers with polycarbonate inserts containing 8 μm pores, as described elsewhere [17]. Briefly, H69 cells were detached from culture plates by gentle pipetting. Cells (1×10^5 cells) were mixed with PBS or 200 μg/ml exosomes from either H69 cells or CCA cells, placed inside the upper chamber, and maintained in serum- and growth factor-freed medium for 48 h. For an invasion assay, a thin layer of matrigel was layered on top of the insert in the upper chambers. Cells, incubated at 37 °C in a humidified 5% CO_2 incubator, were allowed to migrate or invade for 48 h to the lower chamber containing 750 μl culture medium supplemented with 20% exosome-freed FBS as chemo-attractant. After incubation, non-migrated and non-invaded cells in the upper chamber were completely removed using cotton swabs. Cells that had migrated or invaded across the porous membrane were fixed in 25% methanol for 30 min, stained with DAPI (1 μg/ml) for 5 min in darkness or 0.5% crystal violet and washed 3 times. The membranes were carefully removed and mounted on slides. The nuclei of migrated and invaded cells were observed under a confocal fluorescence microscope and photographed. Cells were automatically counted using image J software. The background was subtracted using rolling ball feature, jointed cells were separated by pixel mapping and parameter of the software was set to exclude particles of smaller or bigger than the average nuclear size to minimize erroneous counting.

2.6. Cell viability and cell proliferation assay

Cell viability was measured by MTT assay. H69 cells were plated in 96-well plates for 24 h. The supernatants were withdrawn from sub-confluent cultures, the cells were washed with serum-freed medium and incubated with PBS or 200 μg/ml of exosomes from either H69 or CCA cells, in serum- and growth factor-freed medium for 12, 24 or 48 h. Culture supernatants were then carefully removed and MTT (0.5 mg/ml) solutions were added and incubated at 37 °C, 5% CO_2 incubator for 4 h. Then, MTT solutions were removed and 100 μl DMSO was added to dissolve the formazan crystals before measurement at an absorbance of 590 nm by Multiskan micro plate reader (Thermo Scientific; Cramlington, UK). The result was calculated as % of cell viability. Cell proliferation upon exosomes treatment was further analyzed by staining Ki67 protein in growing cells. H69 cells were cultured on cover slips in 24-well culture plates. After 24 h, supernatants were withdrawn from sub-confluent cultures, the cells were washed with serum-freed medium and incubated with PBS or 200 μg/ml of exosomes from either H69 or CCA cells, in serum- and growth factor-freed medium. After culture, supernatants were carefully removed, cells were washed twice in PBS and fixed in 4% PFA. Cells were then blocked in 10% BSA for 1 h, washed in PBS and incubated with cocktail antibody solution in 1% BSA containing anti-ki67 and anti-actin antibodies for 2 h at room

temperature. Then, the cells were washed thrice and incubated with fluorescent tagged secondary antibodies for another 1 h at room temperature in darkness. Cells were then washed and nuclei were counter stained with DAPI (0.5 mg/ml) for 5 min, mounted on glass slides and visualized under a confocal microscope (Fluoview fv10i, Olympus).

2.7. SDS-PAGE and nano LC/MS/MS

Exosome samples (30 μg) from H69 and KKU-M213 cells were re-suspended in NuPAGE SDS sample buffer and separated under reducing conditions on NuPAGE 3–8% gradient precast tris-acetate SDS–polyacrylamide gel (Life Technologies; NY, USA). Gels were stained with Coomassie brilliant blue g-250 gel staining solution (Bio-Rad; CA, USA), and de-stained in ultrapure LC/MS grade water (Fisher Scientific; Loughborough, UK). Each gel lane was trimmed in pieces and de-stained in 50% acetonitrile (in 25 mM NH_4HCO_3) until colorless. Freshly prepared 10 mM dithiothreitol (in 25 mM NH_4HCO_3) was added to reduce the proteins for 15 min at 60 °C. The gel pieces were cooled down to room temperature and freshly prepared 55 mM iodoacetamide (in 25 mM NH_4HCO_3) were added to alkylate the proteins for 30 min at room temperature in the dark. Thereafter, solutions were removed and acetonitrile was added to dehydrate gel pieces. The gel pieces were allowed to dry

Table 1
Protein identification from H69 and KKU-M213-derived exosomes.

No	Accession no.	Protein	emPAI	
			H69	KKU-M213
1	CLH1_HUMAN	Clathrin heavy chain 1	0.49 ± 0.03	0.41 ± 0.04
2	FINC_HUMAN	Fibronectin	0.2 ± 0.05	0.1 ± 0.03
3	HSP7C_HUMAN	Heat shock cognate 71 kDa protein	0.75 ± 0.05	0.17 ± 0.1
4	PDC6I_HUMAN	Programmed cell death 6-interacting protein	0.52 ± 0.17	0.7 ± 0.11
5	UBIQ_HUMAN	Ubiquitin	6.06 ± 1.24	3.77 ± 1.52
6	EF1A1_HUMAN	Elongation factor 1-alpha 1	0.43 ± 0.01	NI
7	HS90B_HUMAN	Heat shock protein HSP 90-beta	0.19 ± 0.28	NI
8	ACTB_HUMAN	Actin, cytoplasmic 1	0.29 ± 0.00	0.29 ± 0.00
9	TERA_HUMAN	Transitional endoplasmic reticulum ATPase	0.18 ± 0.00	0.18 ± 0.00
10	HSP76_HUMAN	Heat shock 70 kDa protein 6	0.17 ± 0.03	NI
11	ANXA2_HUMAN	Annexin A2	0.32 ± 0.00	0.32 ± 0.00
12	ACTBL_HUMAN	Beta-actin-like protein 2	0.19 ± 0.00	0.19 ± 0.00
13	ALBU_HUMAN	Serum albumin	0.17 ± 0.00	0.3 ± 0.00
14	TS101_HUMAN	Tumor susceptibility gene 101 protein	0.28 ± 0.07	NI
15	LRRK2_HUMAN	Leucine-rich repeat serine/threonine-protein kinase 2	NQ	NI
16	ATD3B_HUMAN	ATPase family AAA domain-containing protein 3B	NQ	NI
17	RRBP1_HUMAN	Ribosome-binding protein 1	NQ	NI
18	LG3BP_HUMAN	Galectin-3-binding protein	NI	1.56 ± 0.23
19	FPRP_HUMAN	Prostaglandin F2 receptor negative regulator	NI	0.68 ± 0.07
20	4F2_HUMAN	4F2 cell-surface antigen heavy chain	NI	0.89 ± 0.04
21	MVP_HUMAN	Major vault protein	NI	0.44 ± 0.06
22	ITB1_HUMAN	Integrin beta-1	NI	0.39 ± 0.1
23	AT1A1_HUMAN	Sodium/potassium-transporting ATPase subunit alpha-1	NI	0.21 ± 0.13
24	TSP1_HUMAN	Thrombospondin-1	NI	0.18 ± 0.02
25	K1199_HUMAN	Protein KIAA1199	NI	0.15 ± 0.04
26	TACD2_HUMAN	Tumor-associated calcium signal transducer 2	NI	0.49 ± 0.09
27	EF2_HUMAN	Elongation factor 2	NI	0.16 ± 0.01
28	1A01_HUMAN	HLA class I histocompatibility antigen, A-1 alpha chain	NI	0.55 ± 0.07
29	G3P_HUMAN	Glyceraldehyde-3-phosphate dehydrogenase	NI	0.35 ± 0.09
30	ITB4_HUMAN	Integrin beta-4	NI	0.07 ± 0.00
31	LAMC1_HUMAN	Laminin subunit gamma-1	NI	0.06 ± 0.00
32	1B55_HUMAN	HLA class I histocompatibility antigen, B-55 alpha chain	NI	0.3 ± 0.07
33	TPP2_HUMAN	Tripeptidyl-peptidase 2	NI	0.14 ± 0.04
34	TINAL_HUMAN	Tubulointerstitial nephritis antigen-like	NI	0.23 ± 0.01
35	HSP71_HUMAN	Heat shock 70 kDa protein 1A/1B	NI	0.11 ± 0.00
36	ANXA1_HUMAN	Annexin A1	NI	0.2 ± 0.09
37	HS90A_HUMAN	Heat shock protein HSP 90-alpha	NI	0.14 ± 0.06
38	BASI_HUMAN	Basigin	NI	0.29 ± 0.08
39	UBA1_HUMAN	Ubiquitin-like modifier-activating enzyme 1	NI	0.1 ± 0.00
40	IMB1_HUMAN	Importin subunit beta-1	NI	0.08 ± 0.00
41	IGSF8_HUMAN	Immunoglobulin superfamily member 8	NI	0.18 ± 0.00
42	ITA3_HUMAN	Integrin alpha-3	NI	0.1 ± 0.00
43	ITA2_HUMAN	Integrin alpha-2	NI	0.09 ± 0.02
44	TBB2C_HUMAN	Tubulin beta-2C chain	NI	0.16 ± 0.11
45	APOB_HUMAN	Apolipoprotein B-100	NI	0.01 ± 0.00
46	VTNC_HUMAN	Vitronectin	NI	0.14 ± 0.06
47	MFGM_HUMAN	Lactadherin	NI	0.09 ± 0.02
48	ITA6_HUMAN	Integrin alpha-6	NI	0.06 ± 0.00
49	EPCAM_HUMAN	Epithelial cell adhesion molecule	NI	0.23 ± 0.1
50	KPYM_HUMAN	Pyruvate kinase isozymes M1/M2	NI	0.13 ± 0.07
51	TBA1B_HUMAN	Tubulin alpha-1B chain	NI	0.15 ± 0.08
52	PLAK_HUMAN	Junction plakoglobin	NI	0.09 ± 0.00
53	ARRD1_HUMAN	Arrestin domain-containing protein 1	NI	0.17 ± 0.00
54	AAAT_HUMAN	Neutral amino acid transporter B(0)	NI	0.14±
55	PGBM_HUMAN	Basement membrane-specific heparan sulfate proteoglycan core protein	NI	0.01±
56	PB1_HUMAN	Protein polybromo-1	NI	NQ
57	CENPE_HUMAN	Centromere-associated protein E	NI	NQ

NI: No identificationand NQ: no quantification by emPAI.

completely in a fume hood. Trypsin solution (0.01 mg/ml) was added to digest the gel pieces and incubated at 37 °C overnight. The peptides were extracted by adding acetonitrile (10 mg/ml in 50% HPLC grade acetonitrile, 0.1% TFA) and subsequently concentrated using a concentrator. The samples were stored at −20 °C prior to mass spectrometric analysis. Each tryptic digested fractions were re-suspended in 0.1% formic acid containing 2% acetonitrile and analyzed by MicroToF Q II mass spectrometer (Bruker; Bremen, Germany). The front end of the mass spectrometer was coupled with an Ultimate 3000 nano-LC system (Dionex; Surrey, UK). The separation was done at a flow rate of 300 nl/min. 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) were used to establish 45 min gradient. The gradient started with 10 min 2–10% B, followed by 33 min 10–40% B, ramped rapidly (1 min) 40–95% B and maintained at 95% B for 1 min. The eluent was sprayed and ionized in the nano-electrospray source of the mass spectrometer. Data were acquired using Hystar software. The MS and MS/MS spectra were acquired in mass range of m/z 400–2000 m/z 50–1500, respectively.

2.8. Analysis of LC/MS/MS data

The mass spectrometric data was smoothed, centroided and converted into a mascot generic file (.mgf) using data analysis software version 4.0. Mascot v.2.3.0 (Matrix Science, London, UK) was used to search data against Swissprot_57.15 (total sequences is 515203) human protein database using trypsin with one possible missed cleavage allowed at 1. The mass tolerances for precursor and fragment ions were set to 1.2 Da and 0.6 Da, respectively. The peptide charge was selected as 2^+, 3^+ and 4^+. Oxidations of methionine and carbamidomethylation of cysteine were set as variable modifications. To reduce false positive identification, only peptides scored above 20 were reported in this study and each identified protein contained at least 2 peptides. Due to the same amount of proteins loaded into SDS-PAGE,

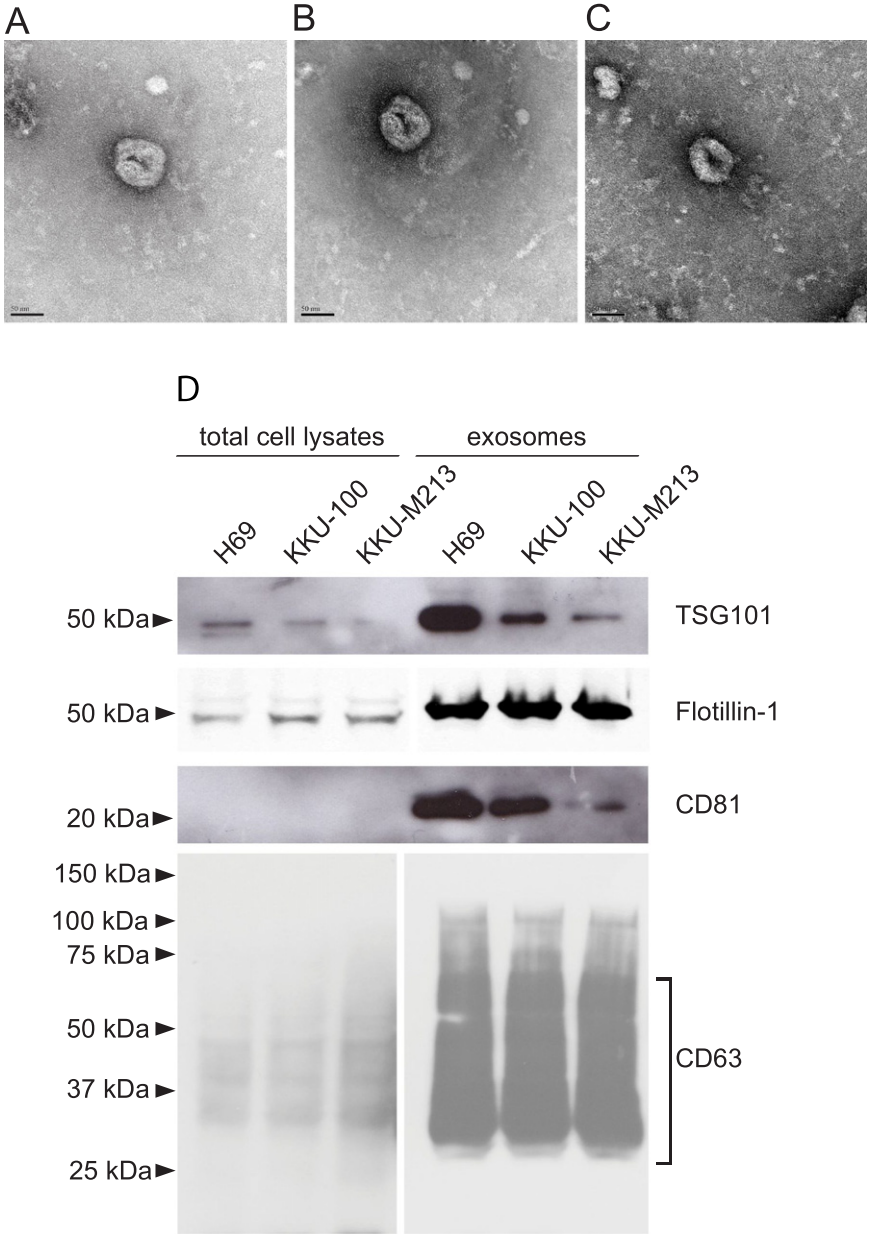

Fig. 1. Characterization of exosomes purified from CCA cell lines. Transmission electron microscopic images of exosomes isolated from (A) H69, (B) KKU-100, and (C) KKU-M213 cell lines. (D) Western blot analysis of exosome marker proteins. Total cell lysates and isolated exosome fractions (50 μg for CD63, 25 μg for flotillin-1, 1 μg for TSG101 and CD81) from three different cell lines were separated by 10% SDS-PAGE followed by Western blotting with specific antibodies. Bars = 50 nm.

quantification information of H69 and KKU-M213 was performed using exponentially modified protein abundance index (emPAI) provided by the Mascot. The emPAI value is based on an equation shown below:

$$\text{emPAI} = 10^{\frac{N_{observed}}{N_{observable}}} - 1$$

where $N_{observed}$ is the number of experimentally observed peptides and $N_{observable}$ is the calculated number of observable peptides for each protein [18]. The success of this label freed quantitative approach has been shown in many studies. Indeed, secretomes of *Aspergillus fumigatus* were profiled at different temperatures [19]. Moreover, the mannose-binding proteins (MBPs) from the normal donor and hepatocellular carcinoma (HCC) patient sera were also quantified by emPAI [20]. All emPAI values in Table 1 are means of three MS analysis. Protein class and pathway categorization were carried out using Panther classification system available at http://www.pantherdb.org/. The functional annotation of identified proteins was mainly retrieved from the Swissprot database (http://www.uniprot.org/).

2.9. Western blot analysis

H69 cells were cultured on 6-well culture plates. After treatment with exosomes in serum- and growth factor-freed medium for the desired period, cells were washed thrice with chilled PBS. Cells were then harvested, lysed with radio-immuno-precipitation assay (RIPA) cell lysis buffer (50 mM Tris–HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF) containing protease and phosphatase inhibitors. Lysates were cleared by centrifugation at 14,000 ×*g* for 20 min at 4 °C. The supernatant fractions were used for Western blotting. The protein concentrations were measured using BCA (Bicinchoninic acid) protein assay kit (Thermo Fisher; MA, USA). Equal amount of protein extracts from different treatment sets were mixed with Laemmli sample buffer and heated at 95 °C for 5 min. The proteins were resolved by 10% SDS-PAGE and transferred onto PVDF membrane at a constant voltage of 100 V at 4 °C for 2 h. Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and probed with indicated antibodies overnight at 4 °C. Membranes were then washed thrice and incubated with HRP conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature and signals were detected using enhanced SuperSignal West Pico Chemiluminescent (Thermo Scientific; MA, USA).

2.10. Confocal fluorescence microscopy

H69 cells were plated on cover slips in 12-well plates for 24 h. Subconfluent cell mono-layers were washed with PBS and treated with PBS or exosomes from either H69 or CCA cells in serum- and growth factor-freed, and exosome-depleted medium for the indicated time. Cells were then washed thrice with cold PBS, fixed with 4% PFA and completely washed with PBS-Tween 20 (0.02% v/v). After blocking with 1% (w/v) BSA at room temperature for 1 h, cells were treated with anti-mouse

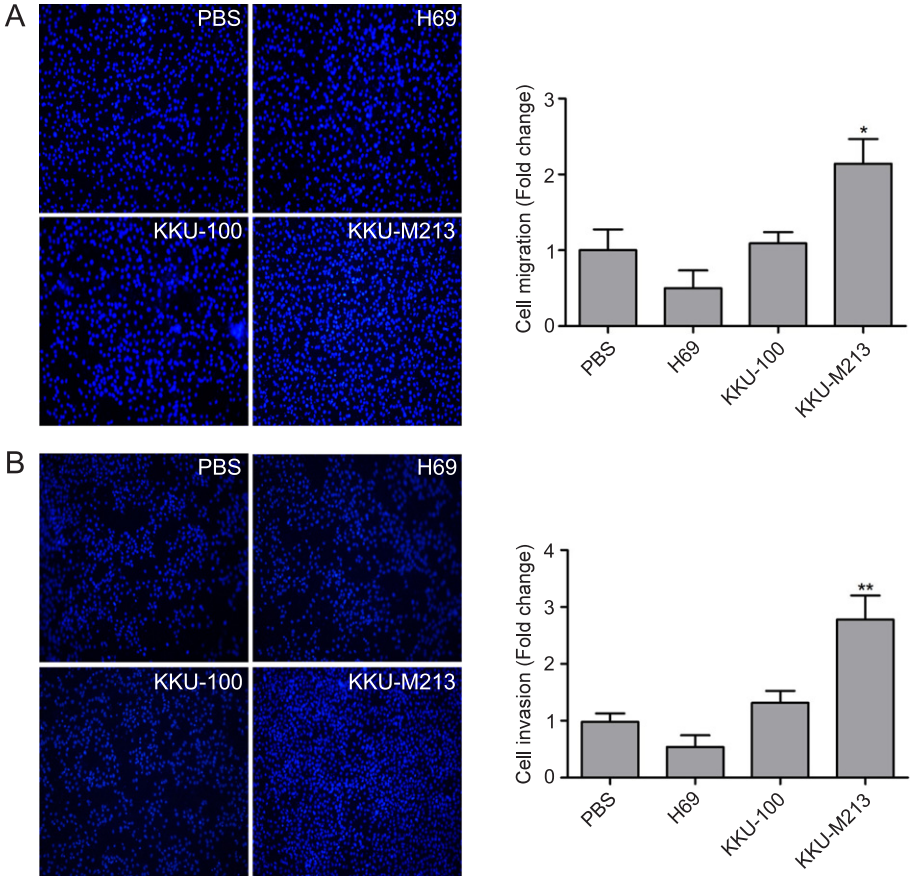

Fig. 2. Effects of H69- and CCA-derived exosomes on H69 cell migration and invasion. (A) KKU-M213-derived exosomes induced H69 cell migration. H69 cells were cultured in the upper chamber of trans-well in exosome-depleted and growth factor-freed medium. Cells were then incubated with PBS or 200 μg/ml of exosomes isolated from H69, KKU-100, and KKU-M213 cell lines for 48 h. Non-migrated cells were removed by cotton swab. Migrated cells in the lower chamber of trans-well were stained with DAPI and observed under a confocal fluorescence microscope. (B) KKU-M213-derived exosomes induced H69 cell invasion. The invasion assay was performed as in the migration assay except a thin layer of matrigel was placed on top of the insert in the upper chamber of trans-well. Non-invaded cells were removed by cotton swab and invaded cells in the lower chamber of trans-well were stained with DAPI and observed under a confocal fluorescence microscope. Bar graphs are quantitative analyses of the number of migrated and invaded cells. Data represent means ± S.E.M. (n = 5) and presented as fold change. *$p < 0.01$; and **$p < 0.001$ compared to PBS control (ANOVA).

E-cadherin and anti-rabbit β-catenin antibodies cocktail overnight at 4 °C. Cells were washed with PBS three times for 5 min, and treated with goat anti-mouse IgG (H + L) Alexa 488 and anti-rabbit IgG Alexa 568 (Invitrogen; CA, USA) in the blocking buffer at room temperature for 1 h. The cells were subsequently washed with excess volumes of PBS four times and stained with DAPI. Finally, each slide was mounted and examined by a confocal fluorescence microscopy (Fluoview fv10i, Olympus). Representative images were chosen and digitally recorded at the same sensitivity and magnification.

2.11. Statistical analysis

The statistically significant differences among groups were compared using one-way analysis of variance (ANOVA) followed by Tukey–Kramer post hoc. Data were analyzed by using the statistical software package, GraphPad Prism version 5.0.

3. Results

3.1. Characterization of exosomes released from CCA and human cholangiocyte cells

To investigate the potential role of CCA cells derived exosomes in tumor progression, the exosomes isolated from normal human cholangiocyte cells (H69) and two human CCA cell lines (KKU-M213 and KKU-100) were first analyzed by electron microscopy. As shown in Fig. 1A–C, electron microscopy of negatively stained exosome preparation showed a characteristic saucer-like structure with a diameter ranged from 40 to 100 nm and crescent shaped membrane invagination that is limited by a lipid bilayer. SDS gel followed by Western blotting of whole cell lysates and exosome fractions revealed the well-known multi-vesicular body markers flotillin-1, TSG101, tetraspanin CD81 and CD63 proteins, which were abundant in the exosomes of both H69 and CCA cells (Fig. 1D). In addition, we have determined the viability of H69, KKU-100 and KKU-M213 cell lines at the time of culture mediums were collected for exosome isolation by trypan blue staining assay. We found that more than 97% of cells were viable, indicating that exosomes are released from viable CCA and cholangiocytes cells (data not shown). The results suggest that, in CCA patients, these micro-vesicles are synthesized in cholangiocarcinoma cells and may be secreted into the circulation.

3.2. KKU-M213 exosomes promote H69 cell migration and invasion

Increases in cell migration, invasion, and proliferation are hallmarks of cancer progression. The effects of CCA cells derived exosomes on H69 cell migration, invasion, and proliferation were, therefore, examined. We cultured H69 cells on top of trans-well for cell migration assay. For invasion assay a thin layer of matrigel was layered on top of trans-well chambers. As shown in Fig. 2, exosomes derived from KKU-100 cells did not induce H69 cell migration (Fig. 2A) or invasion (Fig. 2B) compared to PBS treated set. In contrast, exosomes isolated from KKU-M213 cells not only increased the migration (Fig. 2A) but also markedly increased the invasion of H69 cells (Fig. 2B). Of note, exosomes from H69 cells failed to alter both H69 cell migration and invasion. This was not due to the effect of differential exosome uptake by H69 cells. Indeed, the exosomes isolated from all cell lines were able to either be internalized or attached onto normal human cholangiocytes (data not shown). Increases in cell migration and invasion may occur as a result of an increase in cell proliferation. We next examined whether adding exosomes purified from CCA cell lines to normal cholangiocyte cells in culture would promote cell proliferation. As shown in Fig. 3A, H69 cell proliferation was not affected upon exosome treatments up to 48 h as depicted by MTT assay. The effect of different set of exosomes on H69 cell proliferation was further confirmed by Ki67 staining, a marker protein for cell proliferation. Consistent with

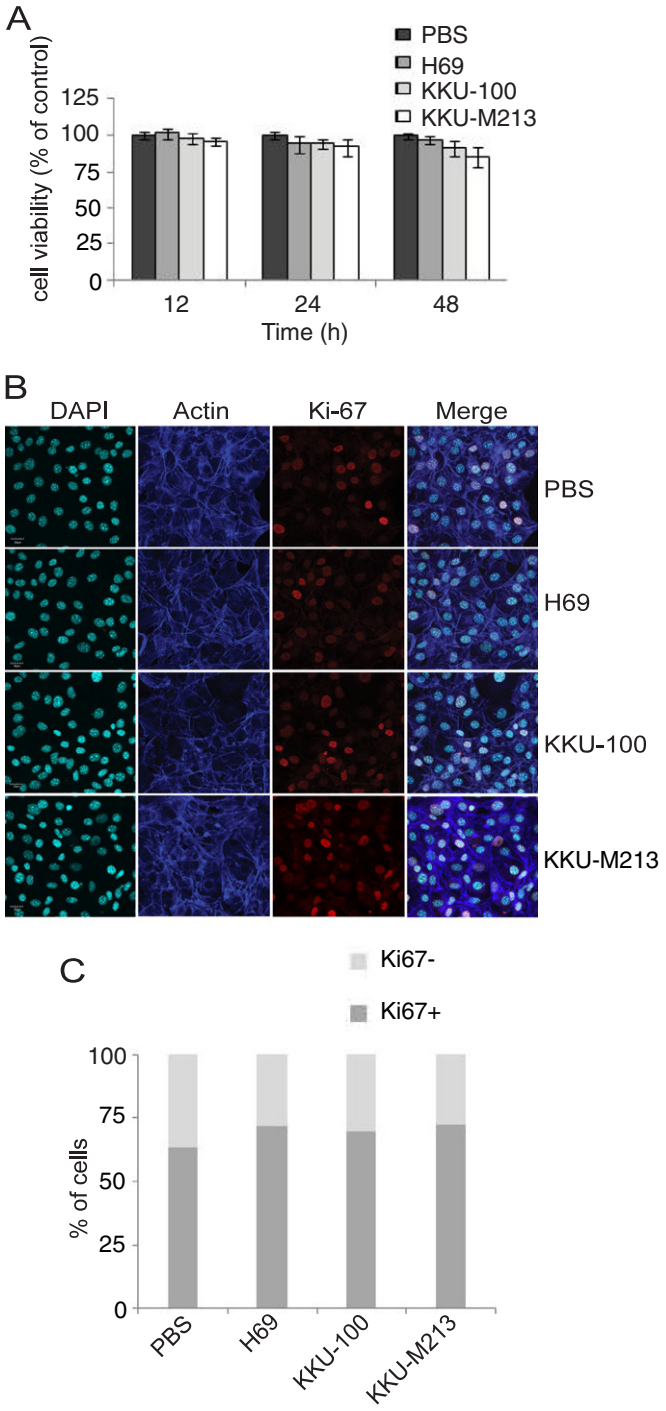

Fig. 3. Effects of exosomes on H69 cell viability and proliferation. (A) Exosomes isolated from CCA cell lines have no effect on cell viability. H69 cells were cultured in 96-well plates in complete medium. Cells were washed twice with PBS prior to incubation with PBS or 200 μg/ml of exosomes isolated from H69, KKU-100, and KKU-M213 cell lines in exosome-depleted and growth factor-freed medium for 12, 24, and 48 h. After incubation, MTT dye was added and incubated for 4 h. DMSO was added to dissolve the formazan crystals and colorimetric measurement was made at OD 590 nm. Data are means ± S.E.M. (n = 3) and presented as % of control (PBS). (B) Effect of exosomes on Ki-67 protein expression in H69 cells. H69 cells were routinely cultured on cover slips in 24-well plates for 24 h, washed twice in PBS and incubated with either PBS or 200 μg/ml exosomes isolated from H69, KKU-100, and KKU-M213 in exosome-depleted and growth factor-freed medium for 48 h. Cells were fixed by 4% PFA and stained with anti-Ki-67 antibody. Cell nuclei were stained with DAPI and visualized under a confocal microscope. (C) Bar graphs represent percentages of Ki-67 positive cells among total number of cells in randomly observed fields (n = 3).

the MTT data, the expression pattern of Ki67 protein was almost similar in all conditions upon treatment with exosomes isolated from CCA cells (Fig. 3B and 3C), suggesting that CCA-derived exosomes do not enhance or suppress normal cell proliferation.

3.3. Proteomic analysis of exosomes derived from human cholangiocyte and CCA cells

Exosomes isolated from KKU-M213 cells, but not from other set of cells tested, promote H69 cell migration and invasion, suggesting the different sets of proteins are carried by exosomes in normal and CCA cell lines. We further investigated the protein composition in exosomes isolated from KKU-M213 and H69 cells by proteomic approaches. First, we separated the total exosomal proteins (30 μg) from KKU-M213 and H69 cells by 3-8% gradient SDS-PAGE and stained with Coomassie blue. The protein bands were distinctly different between two sets of exosomes (data not shown). To identify the protein profile of CCA and H69 exosomes, individual slice of gels were subjected to in-gel trypsin digestion followed by mass spectrometry. The data analysis using Mascot database has identified proteins and their score for both H69 and KKU-M213 derived exosomes (Tables 1, S1 and S2). As shown in Table 1, a number of proteins were exclusively found in the exosomes purified from normal human cholangiocyte cells, not in KKU-M213 cells. Among the identified proteins, 10 proteins were found to be expressed in both exosome samples. Interestingly, the exosomes isolated from KKU-M213 cells accommodated 38 distinct proteins, compared to those of H69 cells, including cancer related proteins such as galectin-3-binding protein, prostaglandin F2 receptor negative regulator, 4F2heavy chain, integrin-β1, major vault protein and integrin β1 (Table 1). Our proteomic results suggest that CCA may promote cell invasion and migration via oncogenic molecules carried by exosomes.

Proteomics of shed vesicles, like exosomes, substantially contribute to the understanding of biological functions of exosomes. Ontology enrichment is the most ubiquitous type of functional analysis, which evaluates relative representation of biological functions, or ontology terms, such as pathways and cell processes, for the proteomic profile of interest. In our study, the Gene Ontology (GO) analysis by open source panther database was employed for prediction of any specific proteins or pathways that may affect H69 cells motility upon treatment with KKU-M213 exosomes. As shown in Fig. 4A and B, different classes of proteins with a variety of functions were identified in two different sets of exosomes purified from cholangiocyte and CCA cell lines. H69 exosomes contain higher chaperone and cytoskeletal proteins (Fig. 4A). While, cell adhesion molecules, extracellular matrix protein, oxidoreductase, receptor, transporter and protease were identified only in KKU-M213 exosomes (Fig. 4B), indicating that CCA derived exosomes are packed with enormous functional ability and/or potential to manipulate recipient cells in a wider aspect and that different to those of normal cholangiocyte cells. Interestingly, pathway classification revealed that only the exosomal proteins from KKU-M213 cells are related to metabolism and cancer-related signaling pathway such as glycolysis, pyruvate metabolism, gonadotropin releasing hormone receptor pathway, ubiquitin proteasome pathway and p53 pathway (Fig. 5A and B). The number of proteins related to integrin signaling pathway which plays an important role in cancer progression was increased in exosomal proteins from KKU-M213 cells compared to

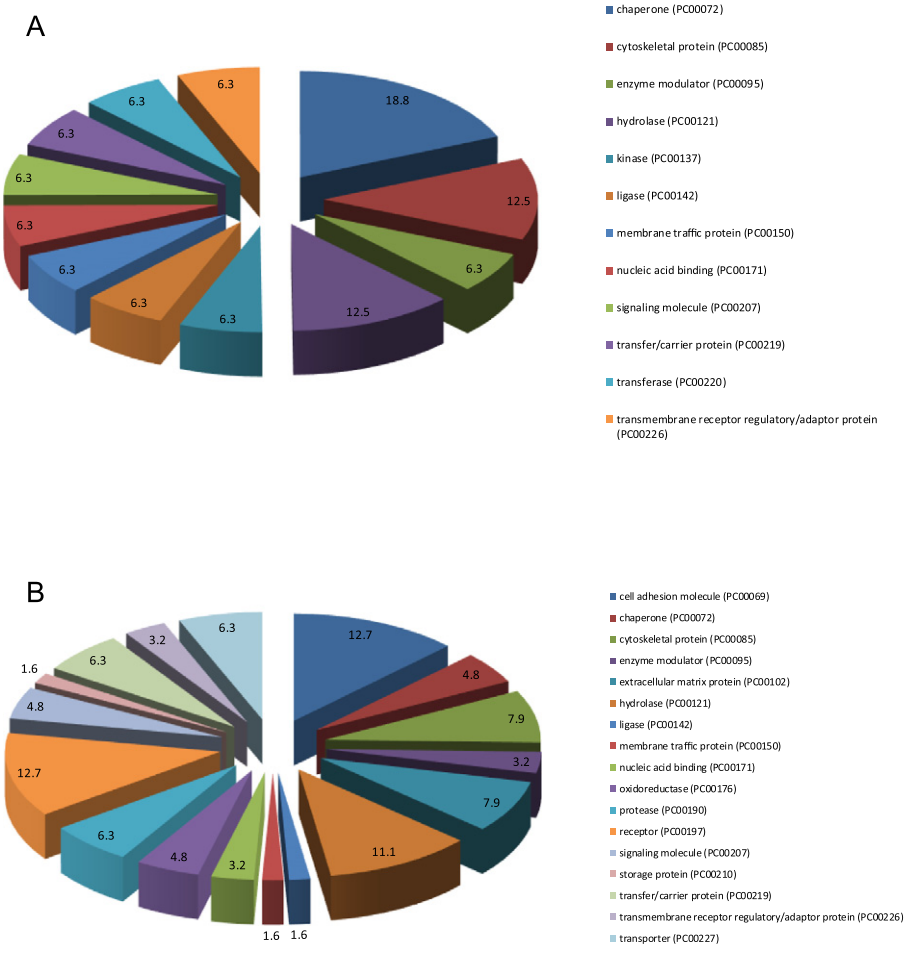

Fig. 4. Proteomic analysis of purified exosomes from H69 and KKU-M213 cell lines. Classification of proteins identified from H69 (A) and KKU-M213 (B) derived exosomes according to their protein classes using PANTHER Classification System.

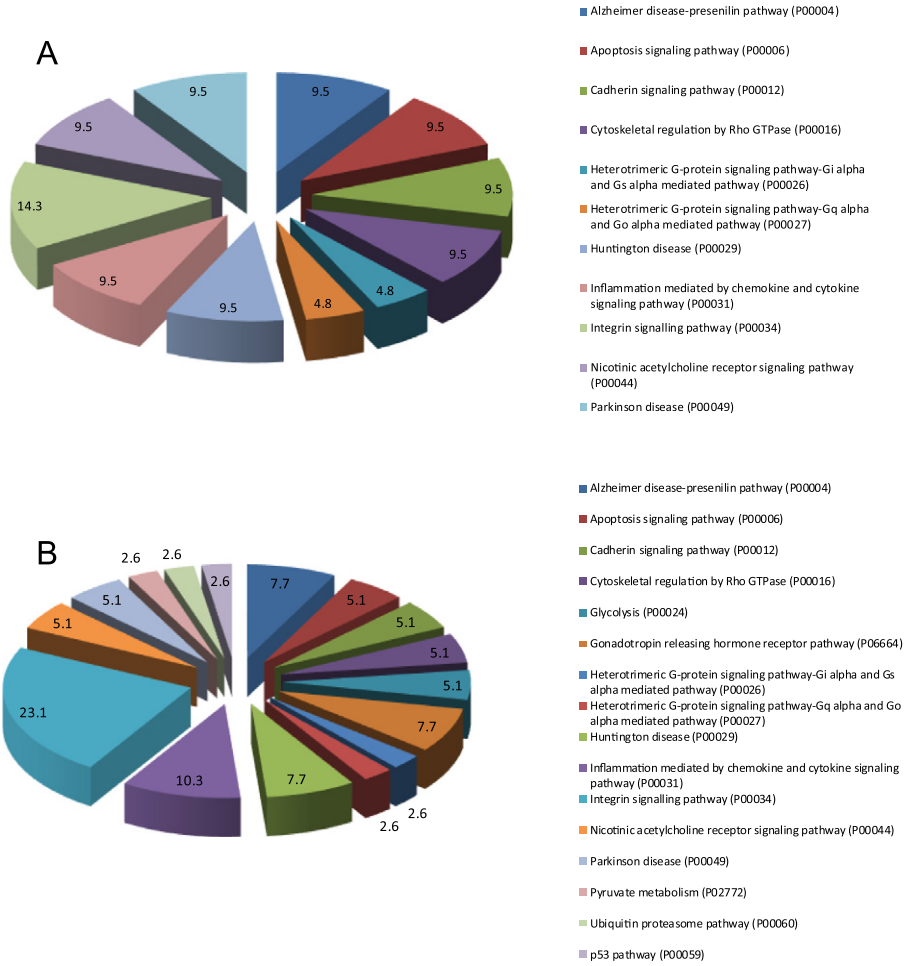

Fig. 5. Proteomic analysis of purified exosomes from H69 and KKU-M213 cell lines. Classification of proteins identified from H69 (A) and KKU-M213 (B) derived exosomes according to their related pathways using PANTHER Classification System.

normal cholangiocyte cells. Moreover, the number of proteins related to apoptosis pathway and other normal cellular activity was reduced. Taken together, the results show that proteins identified in H69 exosomes are mostly meant for maintenance of normal cellular activity compared to exosomes from KKU-M213 cells that are packed with set of proteins having significant potential to vast range of normal biological functions and disease development such as cancer.

3.4. CCA exosomes alters E-cadherin and β-catenin expression in H69 cells

Since treatment with KKU-M213 exosomes increased invasion and migration of H69 cells, we then assessed the cancer and cell motility related proteins, β-catenin and E-cadherin, by fluorescence microscopy. As shown in Fig. 6A, KKU-M213 exosomes increased β-catenin and reduced E-cadherin expression in H69 cells whereas the expression patterns remain unchanged after treatments with H69 or KKU-100 exosomes. Western blot analysis also confirmed the increase (1.7 folds) in expression of β-catenin (Fig. 6B and C). However, the phosphorylation of GSK-3β, a well-known β-catenin regulator, was not affected, suggesting that the increase in β-catenin expression in H69 cells was GSK-3β-independent. Collectively, these results indicate that exosomes from CCA cells carry cancer-specific proteins that may modulate normal cells toward tumor characteristics.

4. Discussion

Exosomes play a critical role in various pathological conditions [21]. Their biogenesis, intra-vesicular content, and transfer to recipient cells are of enormous biological interest. Having a unique molecular cargo, exosomes can reprogram the recipient cells. A number of recent studies have demonstrated that tumor derived exosomes can modulate functional geometry of recipient cells. It may help establish oncogenic niche systematically via delivery of proteins, mRNAs or miRNAs, which, in turn, promote cancer cell progression and metastasis [22]. Despite the potential importance of exosomes in the context of cancer progression, the presence of exosomes in human CCA cell line and their possible role in CCA pathogenesis have not yet been reported. Herein we demonstrated for the first time that two CCA cell lines derived from Thai cholangiocarcinoma patients and SV40-transformed human cholangiocyte H69 cells released exosomes into the extracellular media. These exosomes share similar biophysical and biochemical properties such as shape, size and specific membrane protein content of exosomes from other sources [23]. Our study provides insights into the role of CCA-derived exosomes in cancer progression. We showed that exosomes released by CCA cells *in vitro* have a potential to modulate normal cholangiocyte cell motility. Thus, exosomes released from KKU-M213 were internalized by H69 cells, deliver functional biochemical constituents and induced cell migration and invasion, but not proliferation. By means of proteomic approach, proteins identified in H69 exosomes are mostly associated with normal cellular activity. In contrast, exosomes from KKU-M213 cells are packed with a set of proteins having significant potential to intervene in cancer progression. Further, our data demonstrate that CCA-derived exosomes carried cancer-specific proteins, highlighting a novel source of diagnostic biomarkers and therapeutic target for cholangiocarcinoma.

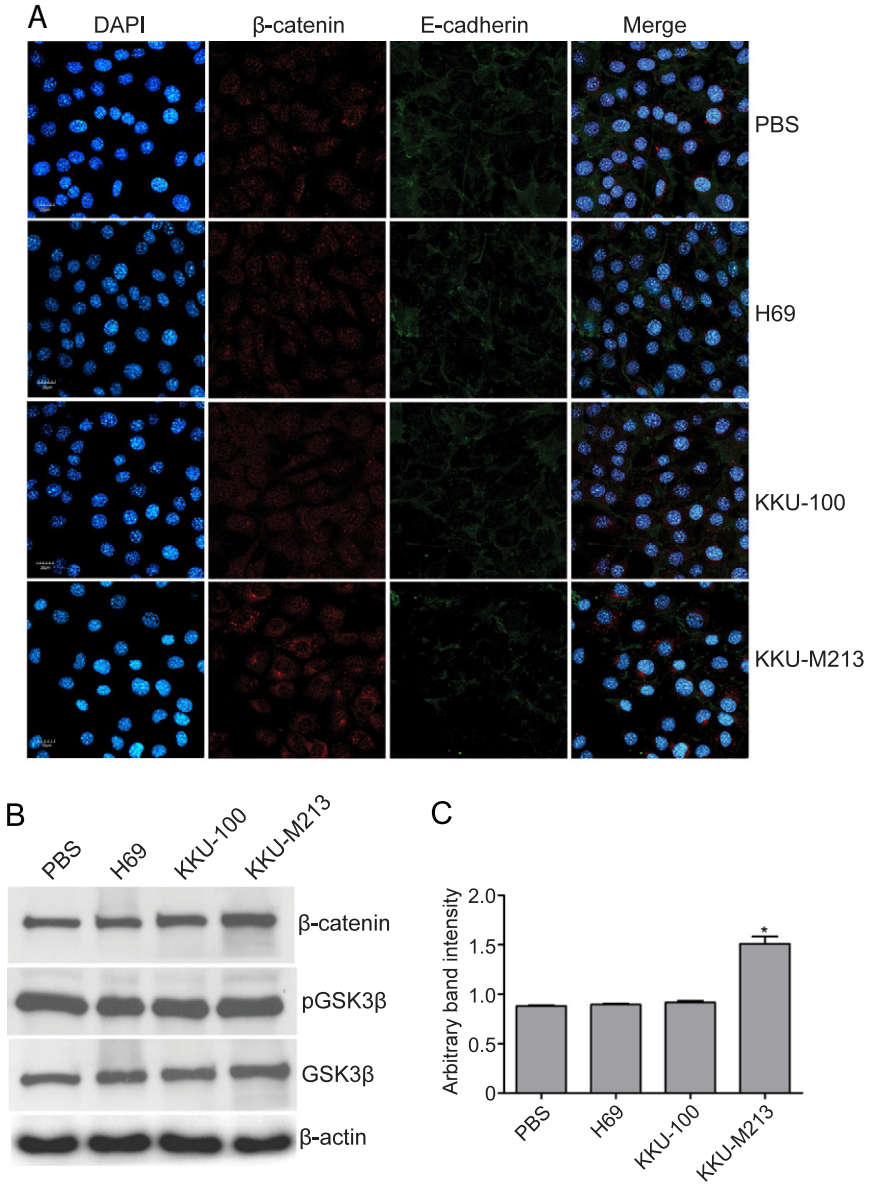

Fig. 6. KKU-M213 exosomes reduce E-cadherin and induce β-catenin expression in H69 cells. (A) Fluorescence microscopic analysis of E-cadherin and β-catenin. After treatment of H69 cells with PBS or 200 μg/ml exosomes for 24 h, cells were fixed and stained for E-cadherin and β-catenin using anti-E-cadherin (green) and anti-β-catenin antibody (red). DAPI was used as a nuclear marker (blue). Samples were visualized with a confocal laser microscope. (B) Western blot analysis of β-catenin in H69 after treatment with exosomes. H69 cells were treated with PBS or 200 μg/ml exosomes isolated from different cell lines for 24 h. Cells were then lysed and 25 μg proteins were separated by 10% SDS-PAGE and immunoblotted with respective antibodies. (C) Bar graphs representing the quantitative analysis of Western blotting of β-catenin from 3 independent experiments. Data are means ± S.E.M., $^{*}p < 0.0001$ compared to PBS control (ANOVA).

MS-based proteomics continues to contribute enormously to our understanding of the molecular composition and functions of exosomes. The concept suggests that digging out the composition and the abundance of each protein in exosomes from different sources may be useful for the identification of novel biomarker candidates, if the proteomic profiling is planned in a comparative approach [24]. The subset of proteins packed into exosomes can be functional in new environment after being transferred to other cells, which is an exhilarating new development to untie exosome saga. It has been reported that exosomes released by different cell types are different with regard to their specific cellular components [25]. Our proteomic study revealed different proteins in two sets of exosomes (Table 1). Exploring these protein profiles revealed that exosomes from H69 and KKU-M213 cells contained several proteins which are consistent with exosome biosynthesis such as transitional endoplasmic reticulum ATPase, molecular motors such as tumor susceptibility gene 101 and ubiquitin. Through our proteomic approach, we have identified exosomal proteins that are known to be associated with cancer cell adhesion, migration, and invasion. A number of these proteins have previously been identified by other researchers in similar studies. The categorization revealed some interesting facts that KKU-M213 exosomes contain broad spectrum of functional proteins involved in cancer progression more than H69 exosomes. We detected integrin α and β, lactadherin, and vitronectin in KKU-M213 exosomes. Recent studies demonstrated that integrins involved in cell growth and migration through interaction with vitronectin [26], and exogenous lactadherin directly activated AKT-dependent pathway through interaction with integrins that subsequently lead to neo-vascularization, supporting their functional roles in cancer progression.

Although the functions of exosomal proteins and lipid are well characterized, data concerning glycoprotein composition are scarce [27]. In our proteomic study, we found that the exosomes from KKU-M213

cells were enriched with a sialoglycoprotein which was identified by peptide mass fingerprinting as the galectin-3-binding protein (LGALS3BP or LG3BP). Several lines of evidence indicate the essential contribution of galectin-binding glycoproteins in different events associated with tumor growth and metastasis [28]. The role of LGALS3BP in neoplastic progression is strongly supported by numerous studies. The expression levels of this protein in sera and neoplastic tissue from cancer patients tightly correlate with poor prognosis and the occurrence of metastasis [29]. In addition, Escrevente et al. speculated that exosomal proteins may involve in exosome/target cell interactions and the LG3ALSBP protein may serve as an exosome biomarker for ovarian carcinoma [27]. Since LGALS3BP is known to bind several proteins on the cell surface such as collagens, fibronectin, galectin-3 and integrin beta1, it is possible that exosomal LGALS3BP interacts with target cells through binding with extracellular matrix proteins, which, in turn, triggers the cellular signaling pathways associated with cancer progression. In agreement with this notion, Kim et al. demonstrated that suppression of LGALS3BP decreased cell motility compared to the parental cells whereas high levels of LGALS3BP led to increased cell migration [30].

Our MS analysis also identified several other proteins in KKU-M213 exosomes that have been established to play critical roles in cancer progression. Up-regulations of LAT1 and 4F2hc that fasten formation of solid tumors were detected in several cancer cell lines including CCA cells [31,32]. Tumor associated calcium signal transducer 2 protein stimulated several human cancer growth and up-regulation of this protein was associated with poor prognosis particularly in invasive cancers [33]. High levels of vault protein expression are correlated with a poor prognosis in certain cancers with some multi-drug resistant (MDR) cancer cell lines [34]. Silencing a novel endoplasmic reticulum protein KIAA1199 discovered earlier to induce cell migration in invasive cancers caused a reduction of 75% in cell migration and 80% in cell invasion [35]. Another KKU-M213 exosome protein, CD147, was reported to play important roles in hepatocellular carcinoma invasion and metastasis [36]. Epithelial cell adhesion molecule observed in KKU-M213 exosome, is a transmembrane glycoprotein that has oncogenic potential due to its ability to aid cell migration, invasion, and metastasis [37]. An aberrant over-expression of pyruvate kinase isozymes M1/M2 (KPYM) associated with aggressive tumor features has been proposed to serve as a novel biomarker and a potential treatment target for thyroid cancer [38]. Although, KKU-100 and KKU-M213 cell lines were derived from CCA patients, however only exosomes from KKU-M213 cells induced H69 cell invasion and migration. These results might be due to the differences in their malignancy characteristics. Consistent with our finding, KKU-M213 cells have previously been reported to exhibit higher motility and invasive abilities compared to KKU-100 cells [39]. Roles of exosomes in cancer cell motility, metastasis and growth have recently received wide attention. For example, Luga et al., have recently proposed that fibroblast exosomes promoted breast cancer cell protrusive activity, motility and metastasis but not tumor growth through induction of autocrine Wnt-PCP signaling mechanism [40]. Although, some of similar cancer related molecules were also present in our proteomic study, however, Wnt signaling and PCP molecules were not detected. Therefore, at present, the roles of these exosomal proteins in CCA carcinogenesis remain unclear and required further investigation. It is now well established that accumulation of β-catenin is frequently observed in many invasive cancers. In addition, Hayashida et al. postulated that a loss of E-cadherin liberates β-catenin protein from the cadherin/catenin complexes that elicits an alternative β-catenin-mediated pathway to make cancer cells more motile and invasive [41]. Consistent with this fact, we observed an acute loss of E-cadherin expression in H69 cells upon treatment with KKU-M213 exosomes. Furthermore, an increase in β-catenin expression was observed in the same treatment indicating that KKU-M213 exosome somehow negatively influenced the cadherin/catenin complex and subsequently facilitated an increase in H69 cell motility.

5. Conclusion

In conclusion, our results suggest that the exosomes from KKU-M213, an aggressive CCA cell line, might induce human cholangiocyte cell migration and invasion by a direct cell-to-cell transfer of oncogenic proteins that influence specific intracellular mechanisms related to CCA carcinogenesis. The exosomal oncogenic proteins may serve as diagnostic/prognostic and therapeutic marker candidates which will have more immediate medical applications for CCA treatment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.06.024.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Conflict of interest

The authors have no conflicts of interest to disclose

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References

[1] P.C. de Groen, G.J. Gores, N.F. LaRusso, L.L. Gunderson, D.M. Nagorney, N. Engl. J. Med. 341 (1999) 1368–1378.
[2] T. Patel, Nat. Rev. Gastroenterol. Hepatol. 8 (2011) 189–200.
[3] A. Saborowski, M. Saborowski, M.A. Davare, B.J. Druker, D.S. Klimstra, S.W. Lowe, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 19513–19518.
[4] G.L. Tyson, H.B. El-Serag, Hepatology 54 (2011) 173–184.
[5] J. Meza-Junco, A.J. Montano-Loza, M. Ma, W. Wong, M.B. Sawyer, V.G. Bain, Can. J. Gastroenterol. 24 (2010) 52–57.
[6] I. Endo, M. Gonen, A.C. Yopp, K.M. Dalal, Q. Zhou, D. Klimstra, M. D'Angelica, R.P. DeMatteo, Y. Fong, L. Schwartz, N. Kemeny, E. O'Reilly, G.K. Abou-Alfa, H. Shimada, L.H. Blumgart, W.R. Jarnagin, Ann. Surg. 248 (2008) 84–96.
[7] J.S. Schorey, S. Bhatnagar, Traffic 9 (2008) 871–881.
[8] S. Taverna, A. Flugy, L. Saieva, E.C. Kohn, A. Santoro, S. Meraviglia, G. De Leo, R. Alessandro, Int. J. Cancer 130 (2012) 2033–2043.
[9] S. Mathivanan, H. Ji, R.J. Simpson, J. Proteome 73 (2010) 1907–1920.
[10] M. Iero, R. Valenti, V. Huber, P. Filipazzi, G. Parmiani, S. Fais, L. Rivoltini, Cell Death Differ. 15 (2008) 80–88.
[11] U. Putz, J. Howitt, A. Doan, C.P. Goh, L.H. Low, J. Silke, S.S. Tan, Sci. Signal. 5 (243) (2012) ra70.
[12] B. Sripa, S. Leungwattanawanit, T. Nitta, C. Wongkham, V. Bhudhisawasdi, A. Puapairoj, C. Sripa, M. Miwa, World J. Gastroenterol. 11 (2005) 3392–3397.
[13] N. Kunkeaw, S.H. Jeon, K. Lee, B.H. Johnson, S. Tanasanvimon, M. Javle, C. Pairojkul, Y. Chamgramol, W. Wongfieng, B. Gong, C. Leelayuwat, Y.S. Lee, Oncogene 32 (2013) 3722–3731.
[14] P. Yonglitthipagon, C. Pairojkul, Y. Chamgramol, J. Mulvenna, B. Sripa, Int. J. Parasitol. 40 (2010) 1203–1212.
[15] D.M. Harnois, F.G. Que, A. Celli, N.F. LaRusso, G.J. Gores, Hepatology 26 (1997) 884–890.
[16] C. Thery, A. Regnault, J. Garin, J. Wolfers, L. Zitvogel, P. Ricciardi-Castagnoli, S. Amigorena, J. Cell Biol. 147 (1999) 599–610.
[17] Y.S. Kim, S.Y. Hwang, H.Y. Kang, H. Sohn, S. Oh, J.Y. Kim, J.S. Yoo, Y.H. Kim, C.H. Kim, J.H. Jeon, J.M. Lee, H.A. Kang, E. Miyoshi, N. Taniguchi, H.S. Yoo, J.H. Ko, Mol. Cell. Proteomics 7 (2008) 1–14.
[18] Y. Ishihama, Y. Oda, T. Tabata, T. Sato, T. Nagasu, J. Rappsilber, M. Mann, Mol. Cell. Proteomics 4 (2005) 1265–1272.
[19] S.S. Adav, S.K. Sze, J. Proteome Res. 12 (2013) 2715–2731.

[20] G. Yang, W. Chu, H. Zhang, X. Sun, T. Cai, L. Dang, Q. Wang, H. Yu, Y. Zhong, Z. Chen, F. Yang, Z. Li, Proteomics 13 (2013) 878–892.
[21] S. Pant, H. Hilton, M.E. Burczynski, Biochem. Pharmacol. 83 (2012) 1484–1494.
[22] H. Peinado, M. Aleckovic, S. Lavotshkin, I. Matei, B. Costa-Silva, G. Moreno-Bueno, M. Hergueta-Redondo, C. Williams, G. Garcia-Santos, C. Ghajar, A. Nitadori-Hoshino, C. Hoffman, K. Badal, B.A. Garcia, M.K. Callahan, J. Yuan, V.R. Martins, J. Skog, R.N. Kaplan, M.S. Brady, J.D. Wolchok, P.B. Chapman, Y. Kang, J. Bromberg, D. Lyden, Nat. Med. 18 (2012) 883–891.
[23] C. Thery, S. Amigorena, G. Raposo, A. Clayton, Current Protocols in Cell Biology/Editorial Board, Juan S. Bonifacino … [et al.], 2006. (Chapter 3:Unit 3 22).
[24] F. Raimondo, L. Morosi, C. Chinello, F. Magni, M. Pitto, Proteomics 11 (2011) 709–720.
[25] M. Simons, G. Raposo, Curr. Opin. Cell Biol. 21 (2009) 575–581.
[26] C.N. Landen, T.J. Kim, Y.G. Lin, W.M. Merritt, A.A. Kamat, L.Y. Han, W.A. Spannuth, A.M. Nick, N.B. Jennnings, M.S. Kinch, D. Tice, A.K. Sood, Neoplasia 10 (2008) 1259–1267.
[27] C. Escrevente, S. Keller, P. Altevogt, J. Costa, BMC Cancer 11 (2011) 108.
[28] F.T. Liu, G.A. Rabinovich, Nat. Rev. Cancer 5 (2005) 29–41.
[29] I. Iurisci, N. Tinari, C. Natoli, D. Angelucci, E. Cianchetti, S. Iacobelli, Clin. Cancer Res. 6 (2000) 1389–1393.
[30] Y.S. Kim, J.A. Jung, H.J. Kim, Y.H. Ahn, J.S. Yoo, S. Oh, C. Cho, H.S. Yoo, J.H. Ko, Biochem. Biophys. Res. Commun. 404 (2011) 96–102.
[31] O. Yanagida, Y. Kanai, A. Chairoungdua, D.K. Kim, H. Segawa, T. Nii, S.H. Cha, H. Matsuo, J. Fukushima, Y. Fukasawa, Y. Tani, Y. Taketani, H. Uchino, J.Y. Kim, J. Inatomi, I. Okayasu, K. Miyamoto, E. Takeda, T. Goya, H. Endou, Biochim. Biophys. Acta 1514 (2001) 291–302.
[32] K. Janpipatkul, K. Suksen, S. Borwornpinyo, N. Jearawiriyapaisarn, S. Hongeng, P. Piyachaturawat, A. Chairoungdua, Cell. Signal. 26 (2014) 1668–1679.
[33] H. Lin, J.F. Huang, J.R. Qiu, H.L. Zhang, X.J. Tang, H. Li, C.J. Wang, Z.C. Wang, Z.Q. Feng, J. Zhu, Exp. Mol. Pathol. 94 (2013) 73–78.
[34] G.L. Scheffer, A.B. Schroeijers, M.A. Izquierdo, E.A. Wiemer, R.J. Scheper, Curr. Opin. Oncol. 12 (2000) 550–556.
[35] N.A. Evensen, C. Kuscu, H.L. Nguyen, K. Zarrabi, A. Dufour, P. Kadam, Y.J. Hu, A. Pulkoski-Gross, W.F. Bahou, S. Zucker, J. Cao, J. Natl. Cancer Inst. 105 (2013) 1402–1416.
[36] J. Xu, H.Y. Xu, Q. Zhang, F. Song, J.L. Jiang, X.M. Yang, L. Mi, N. Wen, R. Tian, L. Wang, H. Yao, Q. Feng, Y. Zhang, J.L. Xing, P. Zhu, Z.N. Chen, Mol. Cancer Res. 5 (2007) 605–614.
[37] W.A. Osta, Y. Chen, K. Mikhitarian, M. Mitas, M. Salem, Y.A. Hannun, D.J. Cole, W.E. Gillanders, Cancer Res. 64 (2004) 5818–5824.
[38] C. Feng, Y. Gao, C. Wang, X. Yu, W. Zhang, H. Guan, Z. Shan, W. Teng, J. Clin. Endocrinol. Metab. 98 (2013) E1524–E1533.
[39] W. Treekitkarnmongkol, T. Suthiphongchai, World J. Gastroenterol. 16 (2010) 4047–4054.
[40] V. Luga, L. Zhang, A.M. Viloria-Petit, A.A. Ogunjimi, M.R. Inanlou, E. Chiu, M. Buchanan, A.N. Hosein, M. Basik, J.L. Wrana, Cell 151 (2012) 1542–1556.
[41] Y. Hayashida, K. Honda, M. Idogawa, Y. Ino, M. Ono, A. Tsuchida, T. Aoki, S. Hirohashi, T. Yamada, Cancer Res. 65 (2005) 8836–8845.