Role of exosomes in hepatocellular carcinoma cell mobility alteration

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Abstract. Exosomes have gained increased research focus due to their key roles as messengers. The components of exosomes include proteins and RNAs that may be horizontally transferred between adjacent or distant cells. Hepatocellular carcinoma (HCC) is among the most malignant types of cancer worldwide, with exosomes implicated to play a crucial role in its regulation; however, the possible function of exosomes in modulating the motile ability of tumor cells and key molecules in HCC remain largely unknown. To investigate the regulatory effect of exosomes on the motile ability of HCC cells, exosomes from the culture medium of different HCC origins (high metastatic MHCC97-H and low metastatic MHCC97-L cells) were isolated for *in vitro* migration and invasion assays. The results indicated that the motile ability of MHCC97-L cells was significantly increased by pretreatment with MHCC97-H-derived exosomes when compared with MHCC97-L-exosome pretreatment (P<0.05). To further characterize the function of exosomes at the molecular level, protein profiling of exosomes from different cell origins was performed, which identified 129 proteins. Among these, adenylyl cyclase-associated protein 1, a protein implicated in HCC metastasis, was significantly enriched in exosomes from cells with high motile ability (P<0.05). The results of the present study validated the regulatory effect of exosomes on the motile ability of HCC cells. Furthermore, systematic analysis of the protein profiles of exosomes from different origins identified potential factors correlated with HCC metastasis, which may provide a basis for future functional analysis of exosomes regarding their involvement in cancer metastasis and recurrence.

Introduction

Exosomes are small endosome-derived vesicles that range between 30 and 100 nm in size, and are actively secreted through the exocytosis pathway (1). The major roles of exosomes are intercellular cross talk and receptor discharge (1-3), and they are typically released from high viability cells, including cancer cells (1). Previous studies have indicated that exosomes are capable of modulating intercellular communication and tumor progression through the transfer of proteins and RNA to adjacent and distant cells (1,4,5). Additionally, the functions of exosomes may vary depending on cell type and intracellular contents (2). In cancer, exosomes are considered to serve essential roles in tumor metastasis by regulating complex interactions between tumor cells and their microenvironment (6,7). However, the regulatory mechanisms of exosomes in tumor metastasis remain to be elucidated. As exosomes are carriers of multiple proteins and RNA molecules, the molecules contained within exosomes may themselves serve key roles in cell-cell communication (1). Thus, proteomics profiling and sequencing are promising platforms for systematically studying exosome components, which may ultimately improve understanding of exosome function.

At present, hepatocellular carcinoma (HCC) is a fatal primary malignancy of hepatocytes (8). Emerging diagnostic tools and novel therapeutic strategies for HCC have substantially improved the clinical outcomes; however, the long-term survival of patients with HCC remains relatively poor due to the high possibility of metastasis and/or recurrence (9). Whether a tumor is likely to undergo local or distant metastasis is principally determined by the metastatic potential of tumor cells and the corresponding microenvironment (10,11). As a major component of the cellular microenvironment, exosomes secreted by different tumor cell types are capable of inducing apoptosis of activated T cells by promoting the

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expression of cell death ligands (12-14), inhibiting natural killer cell functions (15,16), and promoting the generation of suppressor cells derived from myeloid precursors (13). Additionally, various signaling pathways and genes are involved in the communication between tumor cells and their microenvironment (17). For example, a previous study demonstrated that tumor-activated hepatocytes were capable of altering the expression profiles of colon cancer cells in order to support hepatic metastasis (18). However, despite progress in research regarding the role of exosomes in cell communication, the mechanism by which exosomes alter the metastatic potentials of different cell types, particularly liver cancer cells, still requires further investigation.

To investigate whether exosomes may alter the metastatic potential of cancer cells, the present study used two HCC cell lines with high and low metastatic potential, MHCC97-H and MHCC97-L, for exosome isolation and characterization. To evaluate the regulatory effect of exosomes on the mobility of HCC cells, exosomes from the culture medium of different HCC origins were isolated for *in vitro* migration and invasion assays. Additionally, protein profiling was performed on the exosomes from different origins to systematically characterize the content of the exosomes, in order to investigate the regulatory role of exosomes at the molecular level.

Materials and methods

Cell lines and cell culture. The in-house preserved MHCC97-H and MHCC97-L cell lines were provided by The Second Military Medical University of China (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle medium (DMEM; cat no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% FBS (cat no. 10100-147-FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml strepto-mycin at 37°C in a 5% CO_2 incubator for 24 h.

Exosome purification. Exosomes were isolated using a total exosome isolation kit (cat no. 4478359; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In brief, $5x10^6$ cells were seeded in a volume of 15 ml culture medium at 37°C for 24 h, prior to harvesting the cell culture medium. The culture medium was centrifuged at 2,000 x g at 4°C for 30 min to remove cells and debris. Subsequently, the supernatant containing the cell-free culture medium was transferred to a new tube, and then 15 ml of cell-free culture medium was mixed with 7.5 ml of total exosome isolation reagent. The culture medium/reagent was mixed by vortexing until homogenous, and the samples were incubated at 4°C overnight. Following incubation, the samples were centrifuged at 10,000 x g for 1 h at 4°C. The supernatant was aspirated and discarded, and the pelleted exosomes were resuspended in 1X phosphate-buffered saline (PBS). The exosomes were then washed with 1X PBS, ultra-filtrated with a molecular weight cut-off (MWCO) of 100,000 Da, and finally dissolved in 1X PBS.

Transmission electron microscopy (TEM). Exosomes isolated from MHCC97-H and MHCC97-L cells were identified for morphology by transmission electron microscopy (TEM) as previously described (19). In brief, exosomes were transferred to a copper grid coated with 0.125% Formvar in chloroform immediately after isolation. Then the grids were stained with 1% (v/v) uranyl acetate in double-distilled water right before examination. A Hitachi 7100 transmission electron micro-scope was applied for imaging.

Evaluation of HCC cell motile ability following exosome incubation. MHCC97-H and MHCC97-L cells were freshly cultured in DMEM supplemented with 10% FBS and incu-bated with 5% CO_2 in air at 37°C for 24 h. Exosomes were isolated from high metastatic MHCC97-H and low metastatic MHCC97-L cells as described above. A total of 10 μg pelleted exosomes from each of the MHCC97-H and MHCC97-L cell lines were individually resuspended in 1 ml culture medium. The MHCC97-L cells were mixed with the MHCC97-H- or MHCC97-L-derived exosomes, and the cells were cultured with 5% CO_2 in air at 37°C for 6 h prior to migration and invasion assays.

Migration and invasion assay. Cell migration was evaluated with a Transwell migration assay, while the invasion assays were performed using the Transwell units (Corning Incorporated, Corning, NY, USA) coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocols. A total of $1x10^5$ MHCC97-H and MHCC97-L cells, and MHCC97-L cells pretreated with exosomes, were seeded onto the upper chamber of the insert in serum-free DMEM (cat no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc.). After 6 h of incubation at 37°C, the membrane of the insert was fixed in 100% methanol at room temperature for 20 min and stained with crystal violet at room temperature for 20 min. After washing twice with PBS, tumor cells on the upper surface of the filters were removed by wiping with cotton swabs. The number of migrated or invaded cells that had passed through the filter to the lower surface were counted under an inverted microscope (Axiovert A1; Zeiss GmbH, Jena, Germany) in ~30 fields of view at x200 magnification. Mean values were determined from three independent experiments run in duplicate.

Statistical analysis of in vitro data. Data are presented as the mean ± standard deviation. Student's t test and analysis of variance (ANOVA) were used to determine whether the MHCC97-L and MHCC94-H groups were statistically signifi-cantly different in migratory and invasive ability. Following ANOVA results, Dunnett's test was used as a post hoc test. All data were analyzed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and P<0.05 was considered to indicate a statistically significant difference.

Protein preparation and isobaric tags for relative and absolute quantitation (iTRAQ). For each sample, proteins were precipitated by ice-cold acetone, and subsequently centrifuged at 10,000 x g at 4°C for 15 min. A total of 200 μl lysis buffer containing 8 M urea, 2% SDS and 1X protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) was added to resuspend the precipitate. The protein concen-tration of the samples was determined with a bicinchoninic acid assay (Beijing Transgen Biotech Co., Ltd., Beijing,

China) following the manufacturer's protocol. A total of 5 μl DTT (200 mM) was then added to the protein samples, and the samples were incubated at 55˚C for 1 h, after which 10 μl iodoacetamide (500 mM) was added to each sample for 30 min in the dark at room temperature to alkylate the proteins.

For each sample, the proteins were ultra-filtrated (MWCO, 10,000 Da) and dissolved in 100 μl triethylammonium bicarbonate (100 mM). The proteins were then digested with sequence-grade modified trypsin (Promega Corporation, Madison, WI, USA), and the resultant peptide mixture was labeled using an iTRAQ reagent kit (Shanghai AB SCIEX, Analytical Instrument Trading Co., Shanghai, China). The peptides were labeled with iTRAQ 8-plex reagent as follows: MHCC97-H and MHCC97-L were labeled with 113 and 114 isobaric tags, respectively; while the peptides from biological repetitions were labeled with 115 and 117, respectively (MHCC97-H), or 116 and 118, respectively (MHCC97-L). Equal amounts of labeled samples (100 μg) were then desalted with the Sep-Pak Vac C18 cartridges and dried in a vacuum centrifuge at 4˚C for 2 h.

High pH reverse-phase separation. A total of 400 μg peptide mixture was dissolved in solution A (5% acetonitrile and 0.1% formic acid in water; pH adjusted to 10.0 with ammonium hydroxide), and then fractionated by high pH separation using an Agilent 1260 Infinity System (Agilent Technologies GmbH, Waldbronn, Germany) connected to a reverse phase column (Durashell C18, 5 μm, 4.6x250 mm; Bonna-Agela Technologies, Inc., Tianjin, China). High pH separation was performed using a linear gradient of solution B [0.1% formic acid in 90% acetonitrile (ACN); pH adjusted to 10.0 with ammonium hydroxide] from 2 to 40% over 60 min. The column flow rate was maintained at 700 μl/min and the column temperature was maintained at 45˚C. Following separation, the column was re-equilibrated at the initial conditions for 15 min. A total of 40 fractions were collected, and any two fractions with the same time interval (including, 1 and 21, 2 and 22) were pooled to reduce the fraction numbers. In total, 20 fractions were obtained and dried in a vacuum concentrator at 4˚C for 2 h.

Low pH nano-liquid chromatography-mass spectrometry (nano-LC-MS)/MS analysis. The fractions were resuspended with 80 μl solution C (0.1% formic acid in water), separated by nano-LC and analyzed by electrospray tandem mass spectrometry. The experiments were performed on a Nano LC1000 system (Thermo Fisher Scientific, Inc.) connected to a quadrupole-Orbitrap mass spectrometer (Q-Exactive Plus; Thermo Fisher Scientific, Inc.), equipped with an online nano-electrospray ion source. A total of 2 μl peptide sample was loaded onto the trap column (Thermo Fisher Scientific Inc., Acclaim PepMap C18, 100 μm x 2 cm) with a flow rate of 10 μl/min, and subsequently separated on the analytical column (Acclaim PepMap C18, 75 μm x 15 cm; Thermo Fisher Scientific, Inc.), with a linear gradient of solution D (0.1% formic acid in ACN) between 3 and 35%. The column flow rate was maintained at 300 nl/min, the column temperature was maintained at 40˚C, the nebulizer pressure of ~15 MPa and an electrospray voltage of 2.8 kV at the inlet

of the mass spectrometer was used. Following the nano-LC separation, the column was re-equilibrated at the initial conditions for 15 min.

The Q-Exactive Plus mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (*m/z* 300-1,500) were acquired with a mass resolution of 70 K, followed by 10 sequential high-energy collisional dissociation MS/MS scans with a resolution of 17.5 K. In all cases, one microscan was recorded using a dynamic exclusion of 30 sec.

Mass spectrometry data analysis. The raw files from the Q-Exactive instrument were searched against the human database provided by the Universal Protein Resource (http://www.uniprot.org/uniprot, released on 10 April 2014, with 20,264 entries) using Proteome Discoverer (PD) 1.4 (Thermo Fisher Scientific, Inc.). The enzyme specificity of trypsin and a maximum of two missed cleavages were selected for protease digestion. PD was used with a parent ion tolerance of 10 parts per million and a fragment ion mass tolerance of 0.05 Da. Carbamidomethylation of cysteine, as well as iTRAQ modification of the peptide N-terminus and lysine residues, were set as a fixed modification; oxidation of methionine and iTRAQ 8-plex labeling of tyrosine were specified as variable modifications.

A decoy database search strategy was adopted to estimate the false discovery rate (FDR) for peptide identification. Scaffold (version 4.3.2, Proteome Software, Inc., Portland, OR, USA) was used to validate the MS/MS based peptide and protein identifications. The proteins were assembled using the parsimony method and accepted if the peptide FDR was <1% and the protein probability was >99.0%. Proteins containing similar peptides that could not be distinguished based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Differentially expressed protein filtering and gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway analyses. Proteins with expression fold change >2 and Student's t-tests, P<0.05 were filtered as differentially expressed proteins between exosomes isolated from MHCC97-H and MHCC97-L cells. GO and KEGG pathway analyses were conducted using the R packages GO.db (version 3.4.1), KEGG.db (version 3.23) and KEGGREST (version 1.16.1). The P-value threshold was set at 0.01 to filter significantly enriched biological processes and KEGG pathways.

Identification of significantly altered subnetworks. Subnetwork identification was conducted with a heat-diffusion model based on the HotNet2 algorithm. The expression profile change was used as a heat signal input. The networks used for this analysis were obtained from the Human Protein Reference Database (HPRD) (20), iRefIndex (21) and Multinet (22) as recommended by the algorithm authors. A subnetwork identification result with P<0.05 and minimum edge weight threshold δ≥0.0003 were selected for subsequent consensus subnetwork construction. The consensus subnetworks were derived by the following steps: Initially, a complete weighted graph combining all the subnetworks identified from each interaction network was generated. In the weighted graph,

proteins served as vertices and the edge between any pair of proteins were weighted by the number of networks, in which HotNet2 reports them in the same subnetwork. Then, the consensus subnetworks were identified by i) initializing the consensus subnetworks with connected components with edge weights ≥2 (connected components were defined as core genes of the consensus subnetworks), and ii) extending the subnetworks by adding similar genes to a given subnetwork until all weight one edges ended in the consensus subnetworks. The construction of the consensus networks was performed using customized Python scripts (version 2.7.13, Python Software Foundation, Wilmington, DE, USA).

Results

Electron microscopy of isolated exosomes. Exosomes were isolated from the culture media of MHCC97-H and MHCC97-L cells using a total exosome isolation kit. To verify that the isolated structures were exosomes, the isolates were examined by electron microscopy (Fig. 1). The electron images depicted rounded structures with a size range of 50-100 nm in diameter and a cup-shaped morphology, which confirmed the successful isolation of exosomes according to previously described exosome characteristics (5,23,24).

Exosomes from different origins significantly alter the migratory and invasive abilities of cancer cells. The HCC cell lines, MHCC97-H and MHCC97-L, were used in the present study to assess the potential tumor regulatory role of exosomes. MHCC97-H cells exhibited a higher motile ability compared with that of MHCC97-L cells when analyzed by *in vitro* migration and invasion assays (Fig. 2A). Statistical analyses confirmed that, the migratory and invasive capacity of MHCC97-H cells was ~1.5 times higher than that of the MHCC97-L cells (Fig. 2B; P<0.0001 and P<0.005), respectively, which is in accordance with a previous study (25).

To investigate the impact of exosomes on the motile ability of HCC cells, exosomes were individually isolated from MHCC97-H and MHCC97-L cells and incubated with MHCC97-L cells for 6 h. Following incubation, further migration and invasion assays were performed to detect changes in the motile abilities of the MHCC97-L cell groups. As depicted in Fig. 3, according to the migration and invasion assays, MHCC97-L cells incubated with MHCC97-H-derived exosomes exhibited higher motile ability compared with that of MHCC97-L cells incubated with MHCC97-L-derived exosomes. MHCC97-L cells incubated with MHCC97-H-derived exosomes demonstrated significantly increased migratory ability (~2-fold; P<0.05) than those incubated with MHCC97-L-derived exosomes. For the invasion assay, MHCC97L cells incubated with MHCC97-H-derived exosomes displayed higher invasive ability compared with those incubated with MHCC97-L-derived exosomes (~1.5-fold; P<0.05). Taken together, these results indicate the ability of exosomes to regulate the motile capacity of HCC cell lines. It is established that exosomes serve an important role in cell-cell communication by transferring molecules between cells (5). Furthermore, a number of proteins and RNAs have been reported to be enriched in exosomes, which may be responsible for the regulatory role of exosomes (1,26).
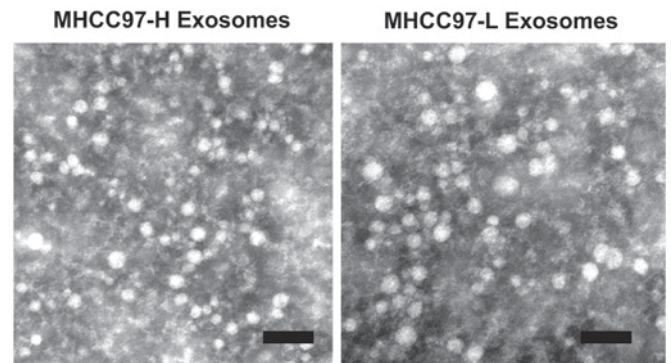

Figure 1. Transmission electron microscopy images of exosomes released from MHCC97-H and MHCC97-L cells. Scale bar, 100 nm.

Therefore, the change in motile ability induced by exosomes in the present study was the basis for subsequent analysis of the molecular content of exosomes.

Mass spectrum analysis of exosomal proteins from different origins. Using a quantitative MS-based discovery strategy, the overall proteomes of exosomes extracted from MHCC97-H and MHCC97-L cells were investigated to characterize the molecular mechanism by which exosomes regulate the motile ability of cells. Exosomes were extracted from the supernatant of the HCC cell lines, and eluted proteins were digested, separated by high pH reversed-phase LC and analyzed by iTRAQ 2D LC-MS/MS. In total, three biological repeats from MHCC97-H and MHCC97-L exosomes were labeled and processed for quantitative analysis. The representative MS/MS result is depicted in Fig. 4. Reporter ion intensities of representative peptides derived from MHCC97-H and MHCC97-L exosomes are depicted in the inset. Peptides from the MHCC97-H exosomes were labeled with 113, 115 and 117 isobaric reagents, respectively, while peptides from the MHCC97-L exosomes were labeled with 114, 116 and 118 isobaric reagents, respectively. The Scaffold software quantified a total of 129 proteins in the exosome samples isolated from MHCC97-H and MHCC97-L cells.

GO and KEGG enrichment analyses of differentially expressed exosomal proteins in MHCC97-H and MHCC97-L cells. Among the identified genes, adenylyl cyclase-associated protein 1 (CAP1) had a significantly altered expression pattern in exosomes isolated from MHCC97-H cells compared with those from MHCC97-L cells (fold change ≥2; P≤0.05). Other differentially expressed genes in the MHCC97-H-derived exosomes included peptidylprolyl isomerase A (PPIA), keratin, type I cytoskeletal 20 (KRT20) and inter-α-trypsin inhibitor heavy chain family member 4 (ITIH4; fold-change ≥1.5; P≤0.05). The differentially expressed genes information is presented in Table I. As the aim was to elucidate the roles of exosomes in different biological processes and pathways, GO and KEGG enrichment analyses were performed for all the proteins identified by MS-based discovery. The 129 identified proteins were over-represented in 316 biological processes and 6 KEGG pathways. The top 10 filtered biological processes were 'response to stimulus' (GO:0050896), 'response to stress' (GO:0006950), 'wound healing' (GO:0042060), 'response to
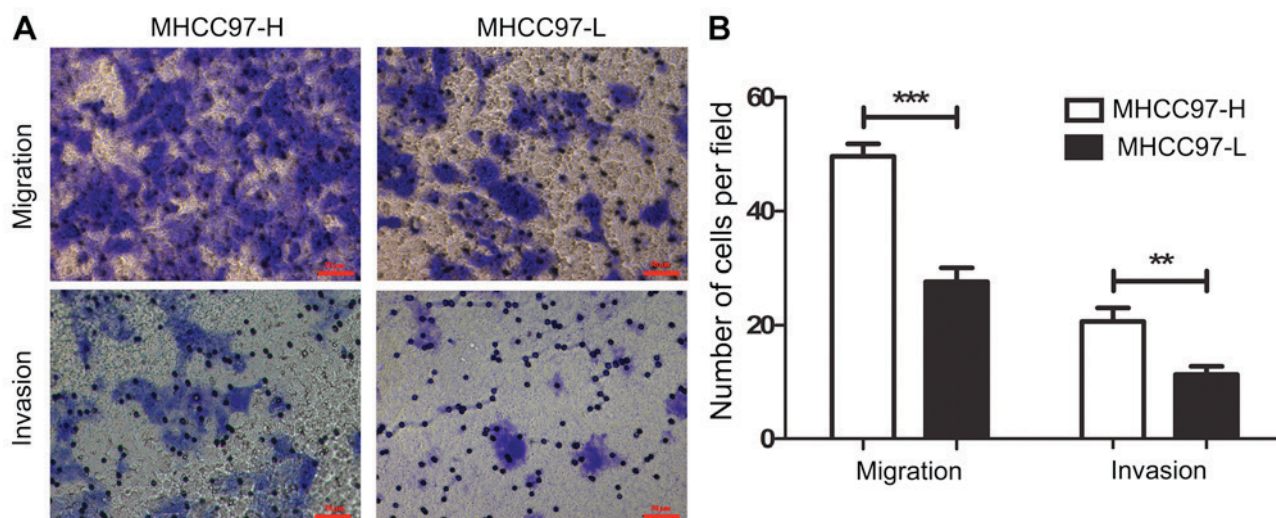
Figure 2. *In vitro* migration and invasion assays of MHCC97-H and MHCC97-L hepatocellular carcinoma cells. (A) Representative images and (B) quantification of migration and invasion assays, at magnification, x200; scale bar, 50 μm. **P<0.01, ***P<0.001.
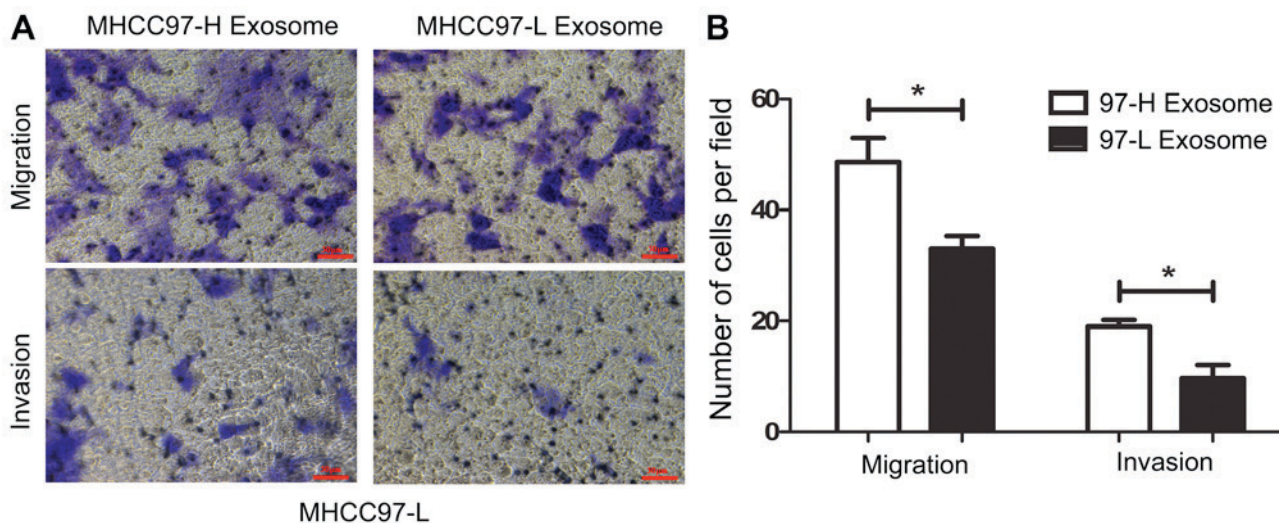

Figure 3. Impacts of exosomes from different origins. *In vitro* migration and invasion assays were performed to assess the altered motile ability of MHCC97-L cells following incubation with exosomes of different origins. (A) Representative images and (B) quantification of migration and invasion assays, at magnification, x200; scale bar, 50 μm. *P<0.05.

wounding' (GO:0009611), 'regulation of body fluid levels' (GO:0050878), 'blood coagulation' (GO:0007596), 'coagulation' (GO:0050817), 'hemostasis' (GO:0007599), 'platelet activation' (GO:0030168) and 'platelet degranulation' (GO:0002576), as depicted in Fig. 5. Significantly enriched KEGG pathways were glycolysis/gluconeogenesis, focal adhesion, extracellular matrix-receptor interaction, and complement and coagulation cascades (Table II; P<0.001). These results indicated enrichments in proteins involved in cell microenvironment construction and cell responses to environmental stress, thus suggesting the regulatory role of exosomes within the cell microenvironment.

Subnetwork identification based on a heat-diffusion-like model. To elucidate the potential influences of differentially expressed proteins, a heat-diffusion-like model was constructed based on the HotNet2 algorithm (27). This model

used the different expression profiles between exosomes from MHCC97-H and MHCC97-L cells as stimulated directed heat signals radiated from the corresponding protein to its interacting partner. Subsequently, diffusion was evaluated in the genome-scale interaction networks to identify significantly altered gene subnetworks, which revealed a combination of proteins across different pathways and complexes. The three interaction networks were extracted from the HPRD (20), iRefIndex (21) and Multinet (22). A single subnetwork was significantly altered in the exosomes of MHCC97-H cells when compared with MHCC97-L cells (P=0.0001), as depicted in Fig. 6. This subnetwork contained 38 proteins (Presented in Table III), of which the core components principally belonged to three different groups according to their interactions. The first group contained apolipoprotein E (APOE), low density lipoprotein receptor-related protein 1 (LRP1), talin 1 (TLN1), filamin A (FLNA), lamin A, β-actin
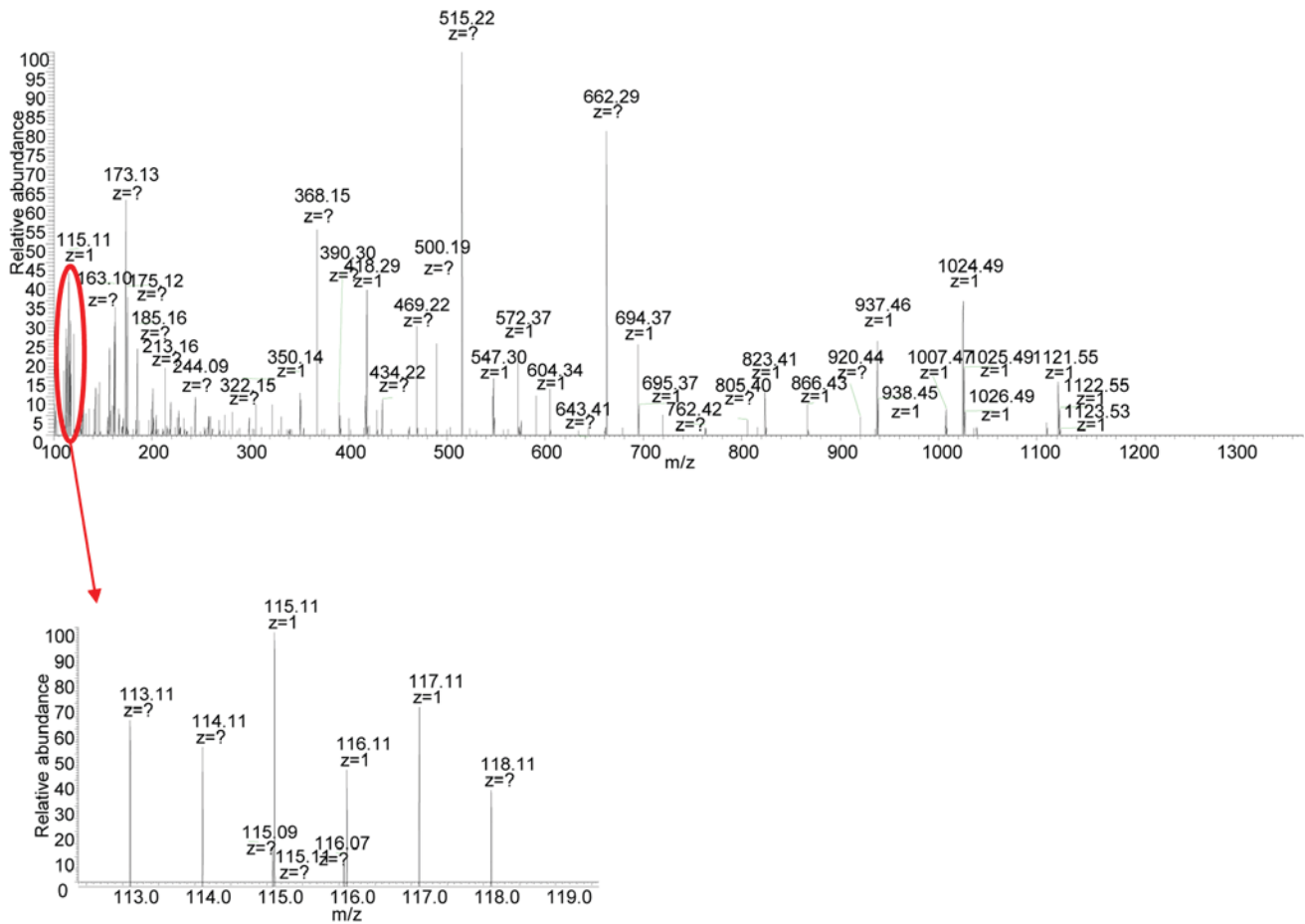
Figure 4. Representative tandem mass spectrometry spectrum of differentially expressed proteins. The inset depicts the isobaric tags for relative and absolute quantitation reporter ion intensities for representative peptides in exosomes from MHCC97-H and MHCC97-L cells. MHCC97-H exosome peptides were labeled with 113, 115 and 117 isobaric tags and MHCC97-L exosome peptides were labeled with 114, 116 and 118 isobaric tags.

(ACTB) and CAP1. These core components were indicated to directly interact and were thus associated. Furthermore, it has been reported that these proteins are associated with cell-cell interactions in cell migration indicating their relevance to cancer metastasis (28). Proteins with close interactions to the core components of the subnetwork (PYGL, G6PD, LUM and TKT) were associated with glucose metabolization, which likely provides the energy required for cell transformation and migration. CAP1, the expression of which was significantly altered between MHCC97-H and MHCC97-L exosomes, was a core component and placed centrally in the subnetwork, implicating it as a key linker gene. The other two highly interactive groups contained laminin subunit γ1, nidogen 1 and fibulin 1 (FBLN1), and actinin α1 (ACTN1) and glycogen phosphorylase B, respectively.

All the core components were classified based on function, as the majority were either associated with lipoprotein activity (APOB, APOE and LRP1, depicted as blue nodes in Fig. 6), or with the cell microenvironment and extracellular matrix formation (including FLNA, TLN1, FBLN1, ACTN1/4 and ACTB, depicted as green nodes in Fig. 6). The other core components that did not belong to an interactive group were MET and ras-related nuclear protein (RAN; depicted as red nodes in Fig. 6), indicating that these genes exert a distinct influence in the subnetwork while remaining

indirectly associated with the CAP1-centered core. MET and RAN are established cancer-associated genes, particularly regarding cancer development and migration (29,30). Thus, taken together these results indicated an altered fraction of genes that may serve crucial functions in cancer cell-derived exosomes.

Discussion

The interactions between cancer cells and their microenvironment are important in tumor development (16). In recent studies on HCC, it has been demonstrated that exosomes secreted by cancer cells serve as messengers in cell-cell communication by conveying molecular information between tumor and adjacent cells (31,32). However, while exosomes are a key component of the cellular microenvironment, whether they regulate the migration and invasion of HCC cells remains unresolved. Additionally, the molecular contents of exosomes derived from HCC cells remain largely unclear, and the regulatory role of exosomes regarding metastatic potential requires further elucidation. The present study has indicated that horizontal transfer of exosomes between cells of different origin may lead to changes in the receiver cell metastatic potential, which thus provides insight into how exosomes may be involved in cancer development. This alteration in

Table I. Differentially expressed exosomal proteins in MHCC97-H and MHCC97-L cells.

Protein name	Fold-change	P-value
CAP1	4.047908066	0.035
PPIA	1.827357238	0.028
KRT20	1.583989312	0.041
ITIH4	1.944871172	0.048

Table II. Significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways associated with proteins identified by mass spectrum-based discovery.

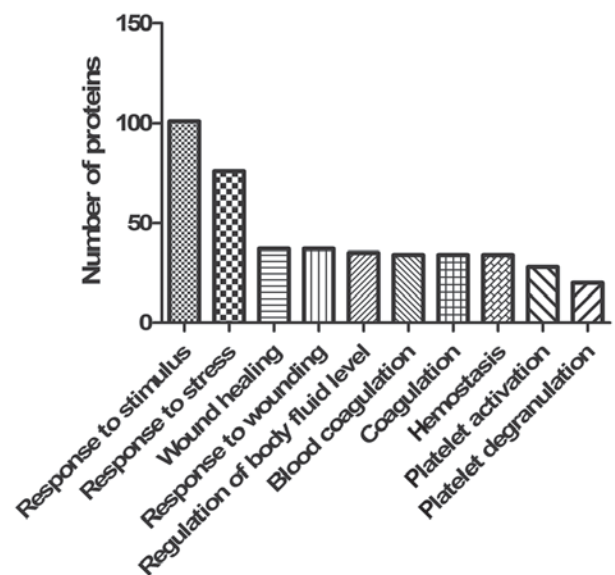| ID	Description	Count	P-value
hsa00010	Glycolysis/gluconeogenesis	10	4.16×10^{-8}
hsa04510	Focal adhesion	16	4.69×10^{-8}
hsa04512	ECM[a]-receptor interaction	11	5.38×10^{-8}
hsa04610	Complement and coagulation	10	7.51×10^{-8}

[a]ECM, extracellular matrix.

Figure 5. GO analysis of the involved biological processes of the differentially expressed proteins between MHCC97-H and MHCC97-L exosomes. The analysis was performed using the Database for Annotation, Visualization and Integrated Discovery and GO annotations. GO, Gene Ontology.
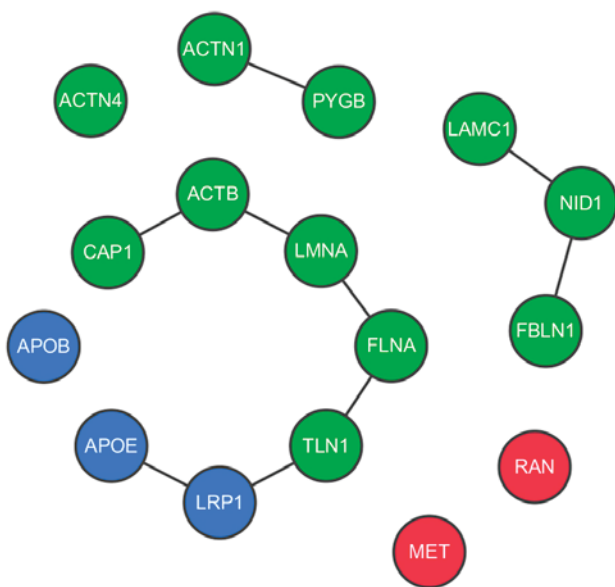

Figure 6. Interactions between core proteins in the subnetwork. Interactions between proteins in the subnetwork determined from three different databases. Each type of protein is depicted as a different color node (blue represents lipoprotein-associated proteins; green represents cell microenvironment and extracellular matrix formation-associated proteins; red represents oncogene encoded proteins). CAP1, adenylyl cyclase-associated protein 1; ACTN1/4, actinin α1/4; PYGB, glycogen phosphorylase B; ACTB, β-actin; LMNA, lamin A; FLNA, filamin A; TLN1, talin 1; LRP1, low density lipoprotein receptor-related protein 1; APOB, apolipoprotein B; APOE, apolipoprotein E; LAMC, laminin subunit γ1; NID1, nidogen 1; FBLN1, fibulin 1; RAN, ras-related nuclear protein.

metastatic ability is unlikely to result from a change in the direct expression of several individual proteins, but rather from changes in complex mechanisms consisting of multiple biological pathways (33).

A number of proteins are enriched in exosomes, including membrane trafficking proteins (Rab proteins and Annexins), adhesion molecules (lactadherin) and signal transduction proteins (protein kinases) (1,26,34). Following validation of the regulatory role of exosomes regarding cell mobility, the present study conducted protein profiling of exosomes from different origins to systematically investigate the differentially expressed proteins within the exosomes. Among the differentially expressed proteins, CAP1 was revealed to be significantly upregulated within exosomes from high metastatic HCC cells. CAP1 has been demonstrated to be significantly overexpressed in human HCCs and correlated with HCC metastasis, and thus was suggested to be a potential independent prognostic factor in patients with liver cancer (35). Loss of CAP1 expression may lead to cell polarity defects and altered distributions of actin filaments and mRNA determinants during development (36). Meanwhile, a previous study demonstrated that CAP1 was overexpressed in pancreatic cancer, and indicated an involvement of CAP1 in aggressive pancreatic cancer cell behavior (37).

The present study identified that CAP1 expression was relatively higher in the exosomes of MHCC97-H cells compared with those of MHCC97-L cells, which indicates a regulatory role of CAP1 in cell mobility alterations associated with secreted exosomes. However, a limitation of the present study is that RNA-sequencing screening was not performed to compare the genetic components within exosomes of different origins. It has previously been indicated that the exchange of genetic materials, including mRNA and microRNA, is an alternative way of exosome-mediated intercellular communication that may reprogram the recipient cells (5,38). Thus, future studies should focus on the potential regulatory functions of genetic materials within exosomes. Additionally, the level of CAP1 within exosomes and its association with the

Table III. Consensus subnetworks identified by HotNet2 algorithm.

Group	Component	Size
1	APOB, APOE, FLNA, LRP1, PYGL, TLN1, UBA1, FBLN1, LAMC1, NID1, ACTN1, ACTN4, PYGB, LMNA, ACTB, ANXA2, CAP1, WDR1, G6PD, LUM, PGK1, PPIA, TKT, MET, RAN	25
2	C1S, ITIH4, ITIH3, ALDOA, LDHB, MYH9, NRP2, NAMPT, PSMD3, UGDH, SDC4, MYL6, PLEC	13

regulation of the motile ability of cancer cells requires further characterization.

By constructing a heat-diffusion-like model, a fraction of genes were identified that was significantly altered between exosomes isolated from MHCC97-H and MHCC97-L cells. Genes within this group were divided into several classes. A major part of this subnetwork consisted of lipoprotein genes, which is consistent with the origin of exosomes, as cell-derived vesicles with membranous structure. These lipoproteins are frequently present in different classes of extracellular vesicles, including microvesicles (39), microparticles (40,41) and exosomes (42,43).

Lipoproteins are key components of exosome structure and participate in exosome synthesis, transport and interactions with cells. It has also been reported that APOE and APOB were associated with hepatocyte-derived exosomes (44), while other proteins of this group (including CLIC1 and NRP2) have been associated with the cell microenvironment and extracellular structure (45,46), thus indicating that these proteins may serve key roles in the exosomal regulation of cell metastatic ability. Additionally, it has been reported that tumor cells may modulate the surrounding microenvironment to enhance their metastatic potential through the secretion of exosomes (47). In the present study, the altered expression of exosome lipoproteins may have resulted from differences in the status of the origin cell, as the cell lines possessed different motile abilities. Indeed, several of these proteins (including APOB and APOE) have previously been identified to be associated with cancer (48,49).

FLNA, which crosslinks actin into dynamic extracellular networks and interacts with multiple binding proteins with different biological functions, has been associated with human cancer proliferation, migration and invasion (50), and thus is correlated with cancer development (51,52). Furthermore, TLN1, which encodes a cytoskeletal protein important in the assembly of actin filaments, is considered as a diagnostic and prognostic marker in human HCC (53,54). FBLN1, another component of the fibrillary extracellular matrix, has also been associated with cancer (55). As these cell microenvironment-associated proteins have been associated with cancer migration and metastasis, afforded by their biological functions, alterations in the expression of these proteins in exosomes may impact on the target cell micro environment, and ultimately cause a change in the motile ability of HCC cells, as observed in the present study. Indeed, the results of the present study were consistent with previous observations, indicating that exosomes may not only affect cells but also the surrounding environment (56).

Also noteworthy was the identification of two established oncogenes (MET and RAN) in the differentially expressed subnetwork. The subnetwork structure indicated that these oncogenes were indirectly associated with the extracellular microenvironment and membrane structure, since they are highly interactive with each other (57). This result suggests that oncogenes may affect HCC cell status and migration by changing the extracellular environment, thus leading to a change in the motile ability of cells. MET, which is frequently identified as a key gene in the process of cancer cell migration, serves a role in epithelial-to-mesenchymal transition and contributes to the tumor microenvironment (58). Meanwhile, RAN is associated with the formation and organization of the microtubule network, and previous results have demonstrated that microtubule perturbation may regulate remodeling of the tumor microenvironment to alter cell migratory potential (57). The tumor cell secretome has also been implicated in this mechanism (59). The identification of MET and RAN oncogenes within HCC exosomes indicates that these genes may not only influence the origin cells, but also have an impact on adjacent cells through exosome transportation. Thus, the identification of these genes provides insight into the complex mechanisms of exosome-associated processes and pathways, particularly regarding cancer development and migration. Further studies of these genes may provide more novel perspectives on the association between exosomes and cancer.

In conclusion, the results from the present study indicated that exosomes may alter the metastatic potential of cancer cells. To the best of our knowledge, the present study was the first to conduct protein profiling of exosomes from different HCC cell origins, which identified protein candidates associated with metastasis and recurrence. Collectively, these data may provide the foundation for further studies into the regulatory role of exosomes in cell-cell communication in HCC and other cancers.

Acknowledgements

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