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FLNA and PGK1 are Two Potential Markers for Progression in Hepatocellular Carcinoma

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Key Words

FLNA • PGK1 • Hepatocellular Carcinoma • ESI-Q-TOF MS/MS • Biomarkers

Abstract

Background/Aims: Hepatocellular carcinoma (HCC) is one of the most deadly diseases; metastasis and recurrence are the most important factors that affect the therapy of HCC chronically. Until now, the prognosis for the metastasis of HCC had not improved. Recently, several proteins that are related to metastasis and invasion of HCC were identified, but the effective markers still remain to be elucidated. Methods: In this study, comparative proteomics was used to study the differentially expressed proteins in two HCC cell lines MHCC97L and HCCLM9, which have low and high metastatic potentials, respectively. Results: Our findings indicated that filamin A (FLNA) and phosphoglycerate kinase 1 (PGK1) were two significantly differentially expressed proteins, with high expression in HCCLM9 cells, and may influence the metastasis of HCC cells. Conclusion: Taken together with the confirmation of expression on the mRNA level, we propose the use of FLNA and PGK1 as potential markers for the progression of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is currently the third most common cause of cancer-related mortality worldwide, but it is the second most common in China [1, 2]. Each year there are nearly 600,000 deaths worldwide as a result of HCC, and China accounts for over 50% of the world's cases [3, 4]. Although most HCC cases (>80%) occur in Eastern Asia and in sub-Saharan Africa [3, 5, 6], the incidence in the West is expected to double over the next 10 to 20 years [7]. Although surgery remains the primary treatment of HCC, the recurrence rate after resection is as high as 70% within 5 years [8]. Therefore, metastatic recurrence is believed to be the main barrier to the improvement of treatment efficacy [1]. Recently, many proteins related to metastatic recurrence of HCC were identified, but the molecular mechanisms that promote invasiveness and the biomarkers that can be widely used in clinical settings remain largely unknown.

For improved insight into the molecular mechanisms of HCC metastasis, we utilized two human HCC cell lines, HCCLM9 and MHCC97L, with high and low metastatic potentials, respectively. These cell lines were derived from two similar genetic backgrounds, although significant differences exist in their metastatic behaviors [9-11]. None-

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theless they provide a perfect model to learn the molecular mechanisms of HCC metastasis *in vitro* [1, 12, 13].

A proteomics approach provides a powerful tool to simultaneously analyze and identify many proteins exhibiting differential expression among different samples, including cell line and tissue samples. This technique enables the identification of cancer-related proteins for therapeutic intervention and the establishment of biomarkers for early diagnosis of metastatic recurrence [14, 15].

FLNA, a 280-kDa actin-binding protein encoded by an X-linked gene, has been demonstrated to interact with more than 70 diverse cellular proteins, including signaling molecules and membrane receptors. It thus provides essential scaffolding functions and crucial links from receptor-mediated signal transduction to the actin cytoskeleton [16-18]. It has been reported that FLNA is required for the migration of cortical neurons and melanoma cells [18-20] and plays a key role in the metastatic progression of prostate cancer and ovarian cancer [21, 22]. Hence, a role for FLNA in HCC metastasis and migration is to be expected.

PGK1 is an ATP-generating enzyme that is involved in the glycolytic pathway and is regulated by hypoxia-inducible factor-1α (HIF-1α) [23, 24]. Several proteins associated with PGK1 signaling have been identified already, including β-catenin (CTNNB1), chemokine receptor 4 (CXCR4), and hypoxia-inducible factor 1 (HIF1). All of them were also shown to promote invasion and metastasis in many carcinomas. At present, PGK1 is known to be overexpressed in several carcinomas, including breast, ovarian, pancreatic, and gastric carcinoma, and plays an important role in their malignancies [25-28]. Its role in HCC metastasis, however, remains to be further explored.

In the present study, proteins extracted from MHCC97L and HCCLM9 cells were analyzed by SDS-PAGE coupled with mass spectra (MS) technology. FLNA and PGK1 were identified for the first time and were overexpressed in HCCLM9 cells (lung metastasis rate 100%) as compared with in MHCC97L cells (lung metastasis rate 40%) [11-13]. In addition, the overexpression of FLNA and PGK1 at the mRNA level was further confirmed by RT-PCR in HepG2 cells. These findings suggested that FLNA and PGK1 might be two potential biomarkers for predicting the possibility of HCC metastasis progression and additionally could be targeted therapeutically for the treatment of HCC patients with metastases.

Materials and Methods

Cell culture

The two cloned cell lines, MHCC97L and HCCLM9, were received as gifts from Professor Yan Li (Wuhan University, Wuhan, China). These lines were derived from the same host cell line MHCC97 by cloning culture and 9 successive pulmonary metastases selection *in vivo*, as described previously [9-11]. And the other two cell lines, SMMC-7721 and HepG2, were obtained from cancer center of State Key Laboratory of Biotherapy in Sichuan University. These cells were respectively cultured in RPMI 1640 and DMEM (GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO, USA) and 100 units/ml streptomycin/penicillin at 37°C in a humidified atmosphere of 5% CO_2.

Migration assay

A wound-healing assay was used to investigate the cell migration in the cell lines as described previously [29]. The cells were seeded in six-well culture plates at a density of 10^5 cells per well. After one day of culture, a 10 μl pipette tip was used to scrape a line across the cell monolayer. The status of the wound line was recorded by microscopy at 0, 24, and 48 h respectively.

Protein extraction

Cells (10^7) were harvested following digestion with trypsin, collected by centrifugation at 1500g for 10 minutes (min) and washed three times with sufficient phosphate buffered saline (PBS). Cells were then counted using a hemocytometer. Five volumes (or 60-100 μl/1×10^6 cells) of lysis buffer was added, and samples were mixed. Cell lysates were then freeze-thawed three times in liquid nitrogen and sonicated for 60 seconds total, 1 s on, 1 s off, at 22% amplitude, rested at room temperature for 30 min. Finally, lysates were centrifuged at 40000 rpm for 1 hour (h) at room temperature, and the supernatants were collected and stored at -70°C.

Bradford assay

The staining solution was diluted 1:5 with water, and bovine serum albumin (BSA) was diluted to a concentration gradient of 0, 0.28, 0.56, 0.84, 1.12, and 1.4 mg/ml. Next, 100 μl of diluted standard protein and samples were independently added to 5 ml diluted staining solution. Samples were then mixed and incubated at room temperature for 5 min, and then absorbance at 595 nm was detected by the Multiskan Spectrum. After making a standard curve of the proteins, the precise concentration of samples can be calculated according to the formula of the standard curve.

SDS-PAGE analysis

First, 5X and 1X protein loading buffer (Takara, Japan) was added to protein extracted from MHCC97L and HCCLM9 cells. The mixture was then boiled in a microwave for 10 min and immediately placed on ice for 5 min. Samples were loaded onto a 10% SDS-PAGE gel, run at 60 V for 10 min and then 100 V for 1.5 h to separate the proteins. The gel was stained with

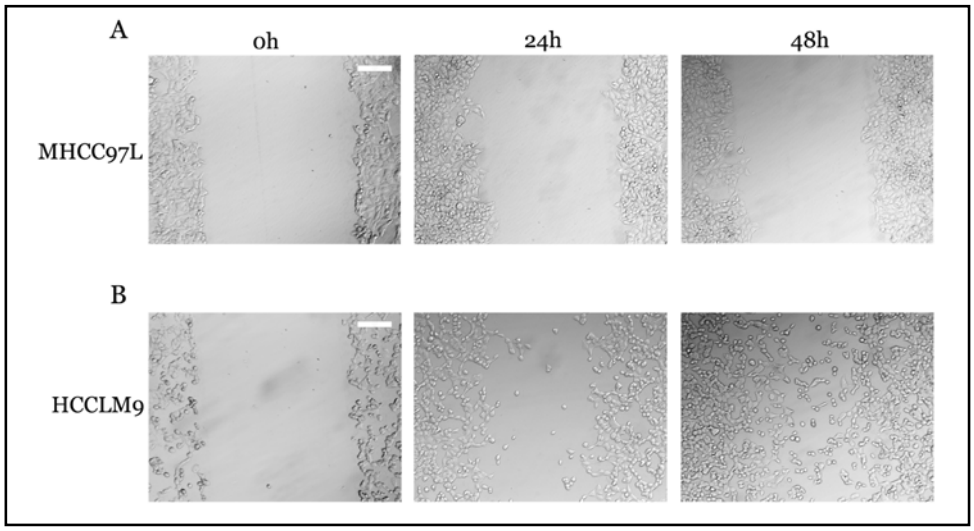

Fig. 1. Migration of MHCC97L and HCCLM9 cells. (A) The real-time images of migration status of MHCC97L cells; from 0 to 48 h, a faint migration occurs in these cells. (B) The real-time images of migration status of HCCLM9 cells; the cells showed strong ability to migrate and the wound appeared healed by 48 h.

coomassie brilliant blue (CBB) for 2 h and then destained to remove excess color by destaining solution (10% acetic acid and 5% ethanol).

Tryptic in-gel digestion

The differential protein bands were excised from the stained gel and cut into cubes of about 1-2 mm^3. The gel cubes were then immersed in 100 µl destaining solution and shaken for 20 min, discarding the solution completely. This procedure was repeated 1-2 times to clear remaining color, and then gels were dehydrated with 100 µl acetonitrile (ACN) for 5 min. Next, 50 µl of DTT solution was then added, and then samples were incubated at 56°C for 30 min. The solution was again discarded, and dehydration with ACN was repeated. Alkylation of the proteins was achieved by adding 50 µl iodoacetamide (IOA) and incubating the samples in the dark for 30 min. Dehydration of the gel with ACN was then repeated again. The gels were then incubated with 15-20 µl trypsin (0.01 µg/µl) at 4°C for 30 min, and then 15-20 µl trypsin buffer (25 mM NH_4HCO_3) was added while the trypsin was absorbed completely. Finally, the samples were incubated at 37°C overnight. The gels were then treated with extracting solution I (5% TFA) for 1 h at 40°C and sonicated per half an hour. The peptides were extracted again with solution II (50% ACN and 2.5% TFA) for 1 h at 40°C and sonicated per half an hour. At last, the samples were combined and evaporated by Speedvac (Savant).

ESI-Q-TOF

A Q-TOF mass spectrometer (Micromass, Manchester, UK) fitted with an ESI source (Waters, Massachusetts, USA) was used to obtain the mass spectra. The dry peptides were dissolved in 10 µl of 50% ACN. MS/MS was performed in a data-dependent mode in which the top 10 most abundant ions for each MS scan were selected for MS/MS analysis. The MS/MS data were acquired and processed using MassLynx software (Micromass, Manchester, UK), and MASCOT was used to search the database. Database searches were performed with using the following parameters: database, Swiss-Prot; taxonomy, Homo sapiens; enzyme, trypsin; mass tolerance, ± 0.1 Dalton (Da); MS/MS tolerance, ±0.05 Da, an allowance of one missed cleavage, and data format, Micromass (.pkl). In addition, variable modifications of methionine oxidation and fixed modifications of cysteine carbamidomethylation were allowed. Proteins with MOWSE scores exceeding their threshold ($p<0.05$) were considered to be positively identified, and the proteins with highest MASCOT scores belonging to H. sapiens were selected for further study [30].

Semi-quantitative RT-PCR

To check the mRNA levels of differentially expressed proteins observed in the CBB stained SDS-PAGE gel, semi-quantitative RT-PCR was performed in triplicate. Total RNA was extracted with Trizol Reagent (Invitrogen), first strand cDNA was synthesized with EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech), and RT-PCR was carried out using a PCR kit from Fermentas. Semi-quantitative RT-PCR was performed according to the manufacturer's instructions using the primers as shown in Table 2. GAPDH was used for normalization. The PCR amplification profile whouras as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 7 min.

Statistical analysis

Data are presented as means ± the standard deviation (SD) or means ± the standard error of the mean (SEM). Statistical significance for comparisons between groups was determined using a Student's paired t-test in Prism 4.0, and $p < 0.05$ was the level of significance.

Results

Migration ability of HCCLM9 cells is stronger than MHCC97L cells

To investigate the difference in migration ability between MHCC97L and HCCLM9 cells, a wound-healing assay was employed. The real-time images of cell migration were obtained after the monolayer of cells was wounded by a 10-µl pipette tip. At 0, 24, and 48 h, we observed the status as the wound healed. As shown in

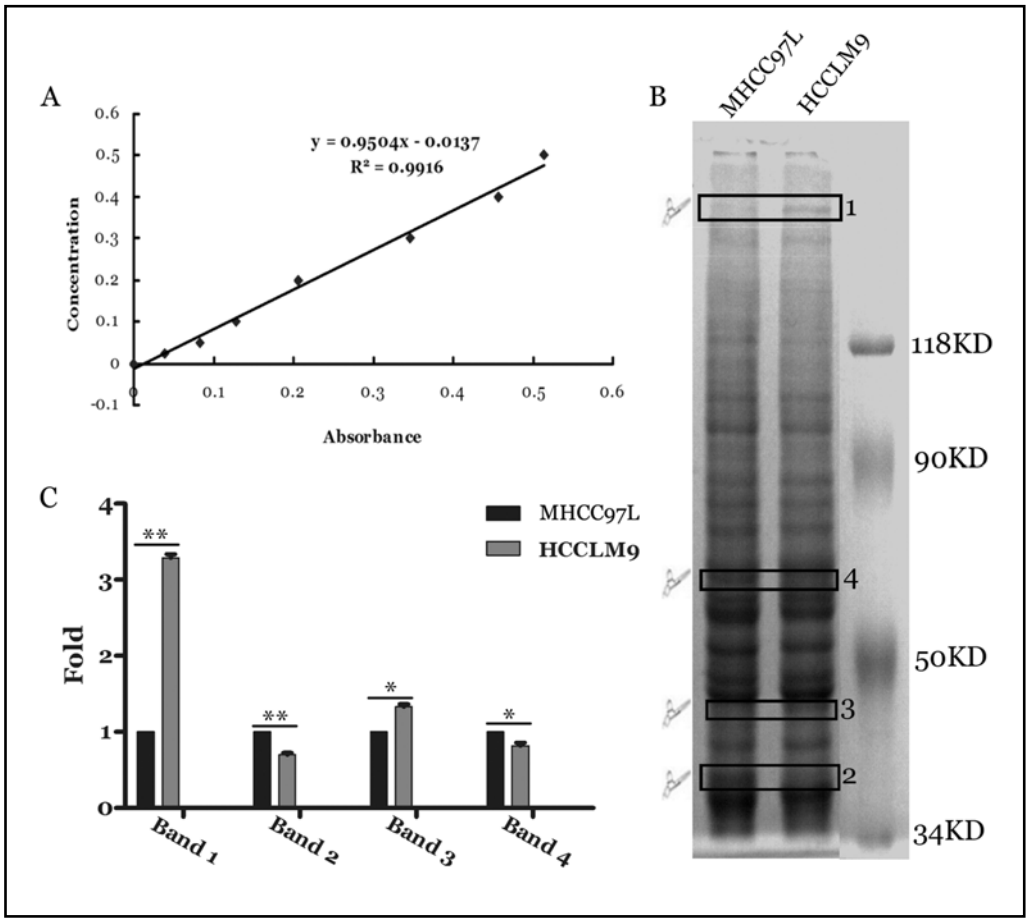

Fig. 2. Separation of total proteins by SDS-PAGE. (A) The concentration of total proteins was measured by the Bradford assay, and the values of the gradient concentration of proteins and gradient absorbance were used to make the standard curve. (B) Total proteins were separated by SDS-PAGE and stained with coomassie brilliant blue (CBB). The gel was scanned and analyzed by ImageScan, and four differential bands were found. (C) The four differential bands were quantified by gray scale. Bands 1 and 3 are brighter in HCCLM9 than in MHCC97L; in contrast, bands 2 and 4 are brighter in MHCC97L than in HCCLM9, *p < 0.05; **p < 0.01; ***p < 0.001.

Fig. 1A, MHCC97L cells did not clearly present the ability of migration at all time points; in contrast, HCCLM9 cells primarily showed aspects of migration at 24 h, and the wound was almost healed at 48 h (Fig. 1B). These findings suggest the migration ability of HCCLM9 cells is markedly greater than that of MHCC97L cells, and this phenomenon is in agreement with previous reports [6].

Separation of total proteins by SDS-PAGE electrophoresis

After confirming the differential migration ability of MHCC97L and HCCLM9 cells, efforts were made to identify the proteins that actually affect the migration between the two cells. Proteins were extracted from the two cell types, and the protein concentration was determined using the Bradford assay (Fig. 2A). Next, 50 μg of protein was separated on a 10% SDS-PAGE gel and stained by CBB, and the protein bands were analyzed (Fig. 2B). The differentially stained bands were quantified by grayscale as shown in Fig. 2C. These data indicate significant differential expression between MHCC97L and HCCLM9 cells. Four differentially stained bands were extracted to be further identified by ESI-Q-TOF MS/MS as described previously [30].

Identification of differentially expressed proteins by ESI-Q-TOF MS/MS

To clearly identify the differentially expressed proteins, peptides of these proteins were freeze-dried and dissolved in 18 μl of 50% ACN. The top 10 most abundant ions for each MS scan were selected for MS/MS analysis with a data dependent mode when we performed the ESI-Q-TOF MS/MS. As shown in Fig. 3, each of the four samples presented showed excellent and dependable quality, thus the proteins identified were accurate. Peptides derived from trypsin and keratin were automatically excluded. The proteins identified by MS/MS are listed in Table 1, along with accession numbers, protein names, gene names, and MS/MS scores. In addition, as shown in Fig. 4A and 4B, proteins were classified into seven groups based on their biological function and divided into 3 parts according to their subcellular localization. Detailed information regarding proteomic identification of FLNA and PGK1 are presented in Fig. 4C and 4D respectively. Both FLNA and PGK1 had high MOWSE scores, and at least eight peptides matched to their protein sequences. These data

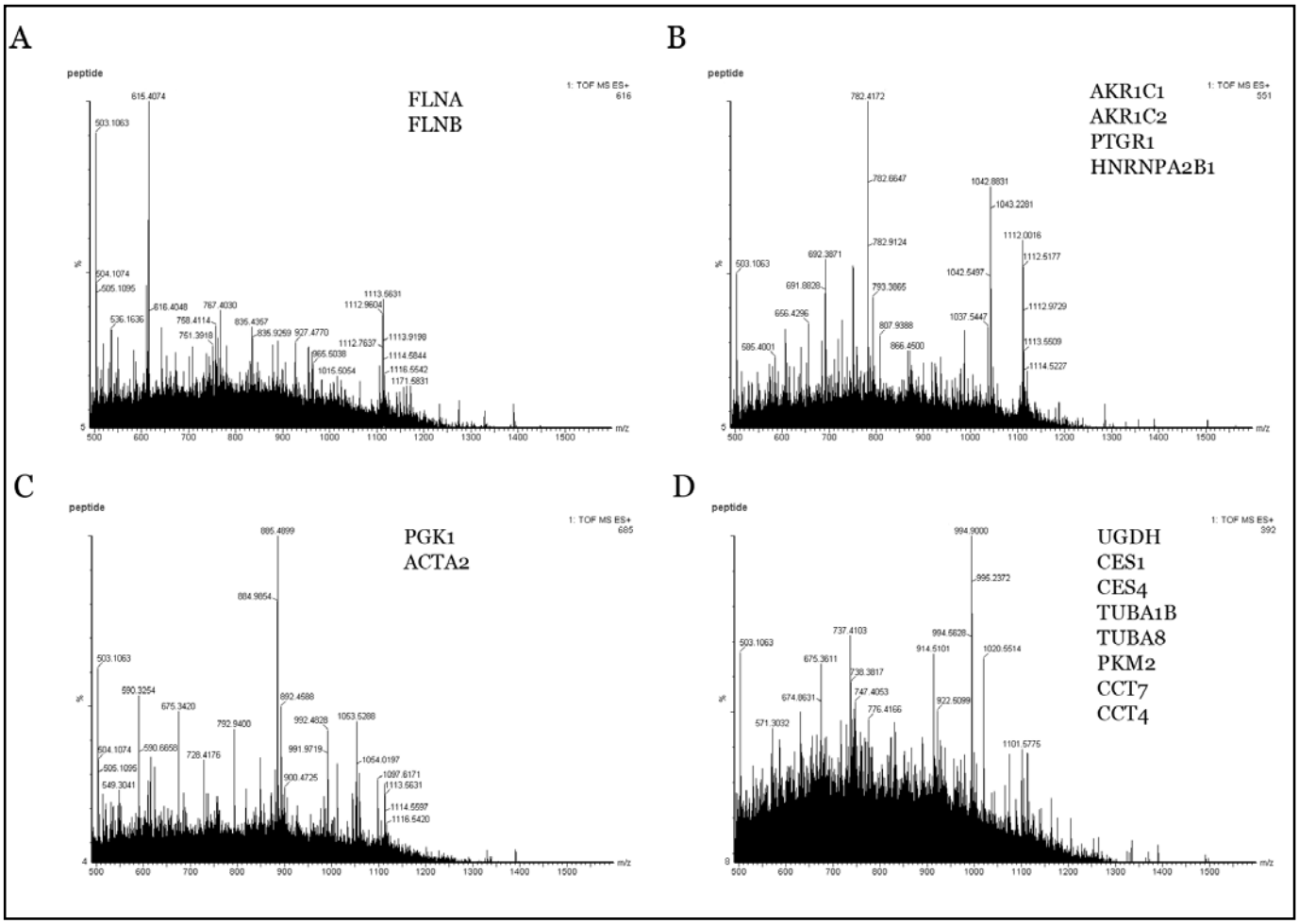

Fig. 3. MS/MS spectra image of identified proteins. (A) The MS/MS spectra of FLNA and FLNB. (B) The MS/MS spectra of AKR1C1, AKR1C2, PTGR1 and HNRNPA2B1. (C) The MS/MS spectra of PGK1 and ACTA2. (D) The MS/MS spectra of UGDH, CES1, CES4, TUBA1B, TUBA8, PKM2, CCT7 and CCT4.

Band NO.	Accession NO [a]	Protein name	Gene name	Theoretical molecular mass [b]	No. of peptides	Coverage (%)	MS/MS Score [c]	Location [b]
1	P21333	Filamin-A	FLNA	283301	8	4	205	Cytoplasm
	O75369	Filamin-B	FLNB	280157	6	2	89	Cytoplasm
2	Q04828	Aldo-keto reductase family 1 member C1	AKR1C1	37221	18	51	287	Cytoplasm
	P52895	Aldo-keto reductase family 1 member C 2	AKR1C2	37111	14	39	165	Cytoplasm
	Q14914	Prostaglandin reductase 1	PTGR1	36075	3	11	31	Cytoplasm
	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNP A2B1	37464	1	4	27	Nucleus/Cytoplasm
3	P00558	Phosphoglycerate kinase 1	PGK1	44985	23	31	319	Cytoplasm
	P62736	Actin	ACTA2	42381	2	4	68	Cytoplasm
4	O60701	UDP-glucose 6-dehydrogenase	UGDH	55674	14	30	136	cytoplasm
	P23141	Liver carboxylesterase 1	CES1	62766	12	17	134	Endoplasmic reticulum lumen
	O9UKY3	Putative inactive carboxylesterase 4	CES4	30773	4	9	118	secreted
	P68363	Tubulin alpha-1B chain	TUBA1B	50804	3	6	61	Microtubule
	O9NY65	Tubulin alpha-8 chain	TUBA8	50746	3	6	61	Microtubule
	P14618	Pyruvate kinase isozymes M1/M2	PKM2	58470	2	3	37	Cytoplasm/Nucleus
	O99832	T-complex protein 1 subunit eta	CCT7	59842	1	2	19	Cytoplasm
	P50991	T-complex protein 1 subunit delta	CCT4	58401	2	4	19	Cytoplasm

Table 1. Proteins identified by ESI-Q-TOF. All protein spots were identified by ESI-Q-TOF MS/MS. [a]Accession numbers were derived from the ExPASy database. [b]Theoretical molecular mass (Da) and location from the ExPASy database. [c]Probability-based MOWSE scores.

suggest that the proteins identified by MS/MS are accurate, and it is worthwhile to analyze the levels of mRNA expression from which these proteins are encoded.

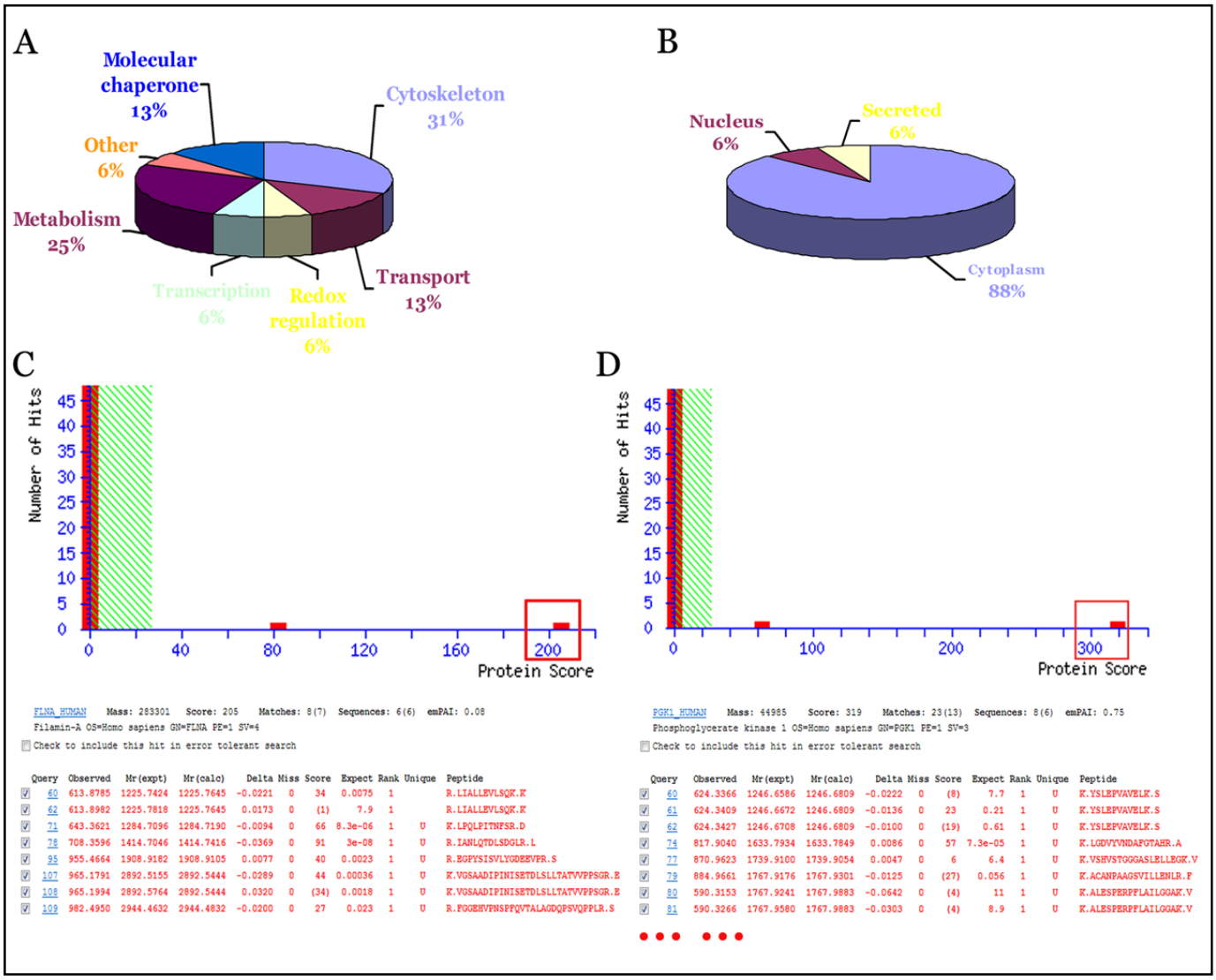

Fig. 4. Classification of identified proteins and proteomic identification of FLNA and PGK1. (A) First, 16 distinct proteins were classified into 7 groups based on their biological functions: cytoskeleton (31%), transport (13%), redox regulation (6%), transcription (6%), metabolism (25%), molecular chaperone (13%) and others (6%). (B) The identified proteins were categorized into groups according to their subcellular locations. Among them, 88% of the proteins were located in the cytoplasm, and the others were located in nucleus and secreted as indicated. (C) MS/MS identification of FLNA revealed 8 matched-peptides, with a MOWSE score of 205. (D) MS/MS identification of PGK1 revealed 23 matched-peptides, with a MOWSE score of 319.

Detection of mRNA levels of selected proteins and PGK1 signaling by RT-PCR in MHCC97L and HCCLM9 cells

To confirm the differential expression of identified proteins at the mRNA level, semi-quantitative RT-PCR was performed using the primers for each gene as listed in Table 2 and using *GAPDH* as an internal control. As shown in Fig. 5A and 5B, expression of *FLNA*, *PGK1*, and *ACTA2* was significantly increased in HCCLM9 cells, but *AKR1C1*, *UGDH*, and *CES1* were dramatically downregulated in MHCC97L cells. Furthermore, proteins related to the PGK1 signaling pathway were also detected by RT-PCR. β-catenin (*CTNNB1*), chemokine receptor 4 (*CXCR4*), and hypoxia-inducible factor 1 (*HIF1*) were overexpressed in HCCLM9 cells (Fig. 5C and 5D). These findings indicate that FLNA and PGK1 can be two biomarker candidates for progression of HCC.

Confirmation of differential expression of FLNA and PGK1 by RT-PCR in SMMC-7721 and HepG2 cells

To confirm the differential expression of *FLNA* and *PGK1* in cell lines own different metastatic abilities, we

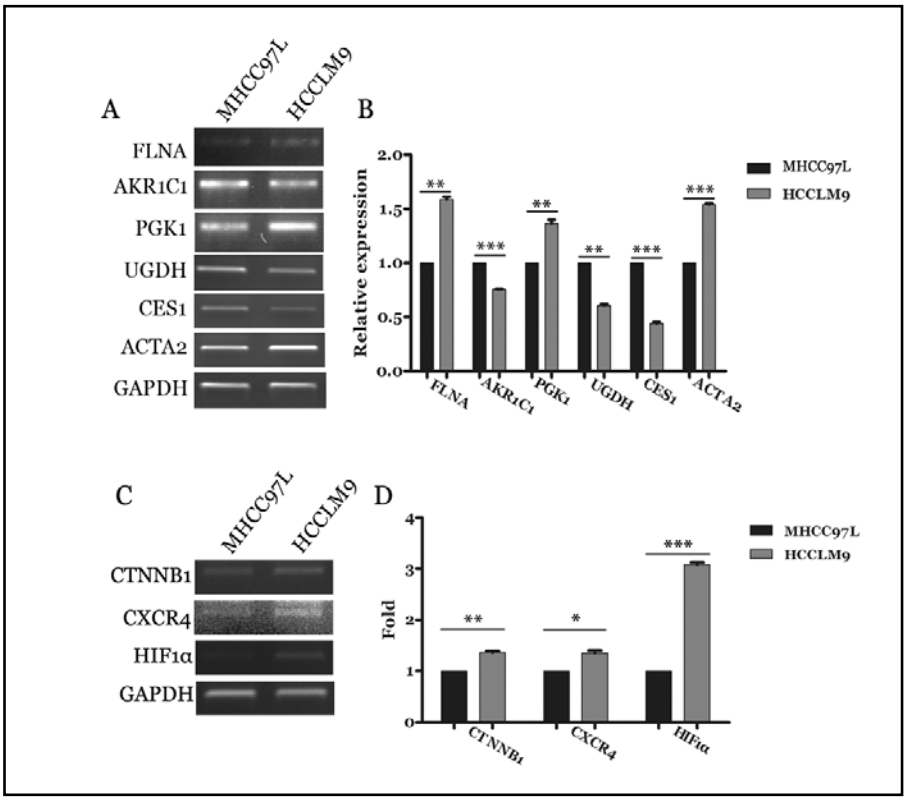

Fig. 5. Semi-quantitative RT-PCR confirmation of identified proteins. (A) Among the 16 identified proteins, the transcripts that encode 6 of the proteins were further assayed by RT-PCR. Total mRNA was normalized by GAPDH. (B) The electrophoresis bands were analyzed by Quantity-One software, and the average intensity of each band was obtained by triplicate experiments. (C) PGK1-related proteins were tested by RT-PCR. Three proteins including CTNNB1, HIF1, and CXCR4 were upregulated in HCCLM9 cells. (D) The quantification and statistical analysis of the electrophoresis bands presented in (C), *p < 0.05; **p < 0.01; ***p < 0.001.

further selected two other hepatoma carcinoma cells, SMMC-7721 and HepG2 cells, which own differential ability of migration. As shown in Fig. 6A and 6B, the ability of migration of HepG2 cells is stronger than SMMC-7721 cells significantly. Next, total RNA was extracted from those two cell lines, the differential expression of *FLNA* and *PGK1* was also detected by RT-PCR. Compared with SMMC-7721, the expression of *FLNA* and *PGK1* were markedly increased in HepG2 cells (Fig. 6C and 6D), *GAPDH* as an internal control. These findings confirmed the differential expression of *FLNA* and *PGK1* in HCC cells with different ability of migration.

Target protein	Primer sequence (sense (+) and antisense (-))	Annealing temperature	Product length
FLNA	(+)5' -CCGCAATGACAATGACACC-3' (-)5'-TGGAGATACTGCCACTGAGA-3'	48 °C	421bp
ACTA2	(+)5' -ACGTGACATCCGCAAAGAC-3' (-)5'-AAAGGGTGTAACGCAACTAA-3'	48 °C	302 bp
AKR1C1	(+)5' -CTGGGATTTGGCACCTAT -3' (-)5'-TTGGGATCACTTCCTCACC-3'	48 °C	337 bp
PGK1	(+)5' -CGGTAGTCCTTATGAGCC -3' (-)5'-CATGAAAGCGGAGGTTCT-3'	48 °C	207 bp
UGDH	(+)5' -TGGGCTCCACAATGAACT-3' (-)5'-GAGAATGGCGTGAACCTG-3'	48 °C	353 bp
CES1	(+)5' -CTCCGTGCCTTTATCCTG-3' (-)5'-TGTTCTCCTTTCGGTTTGT-3'	48 °C	316 bp
CTNNB1	(+)5' -ACCTCCCAAGTCCTGTAT-3' (-)5'-CCTGGTCCTCGTCATTTA-3'	45 °C	322 bp
CXCR4	(+)5' -AAAATCTTCCTGCCCACC-3' (-)5'-ATCCAGACGCCAACATAG-3'	50 °C	374 bp
HIF1A	(+)5' -GCTTGGTGCTGATTTGTG -3' (-)5'-TGTTTGTTGAAGGGAGAA-3'	45 °C	416 bp
GAPDH	(+)5' -GGAGTCCACTGGCGTCTT-3' (-)5'-CATCATATTTGGCAGGTTTT -3'	48 °C	482 bp

Table 2. Information of RT-PCR for selected genes.

Discussion

Hepatocellular carcinoma (HCC) is currently the third leading cause of cancer mortality worldwide [2], and the incidence is expected to increase over the next few decades due to increased rates of HBV and HCV infection [3, 7]. We thus have to develop systems for the diagnosis, prognosis, and treatment of HCC. Several proteins including alpha-fetoprotein (AFP) and fibroleukin were identified as potential makers for early HCC diagnosis, and AFP is used most widely to test for HCC [31, 32]. Even after curative therapies while HCC remains early and localized, the incidence of metastatic recurrence for HCC is still high [6]. Many efforts have been made to identify more effective molecules related to the progression and metastasis of HCC, including transforming growth factor-b1 (TGFb) [33], insulin-like growth factor-II (IGF-II) [34], and human cervical cancer oncogene (HCCR) [35]. However, these currently available biomarkers have inadequate specificity and sensitivity. Hence, the specific prognostic biomarkers for metastatic

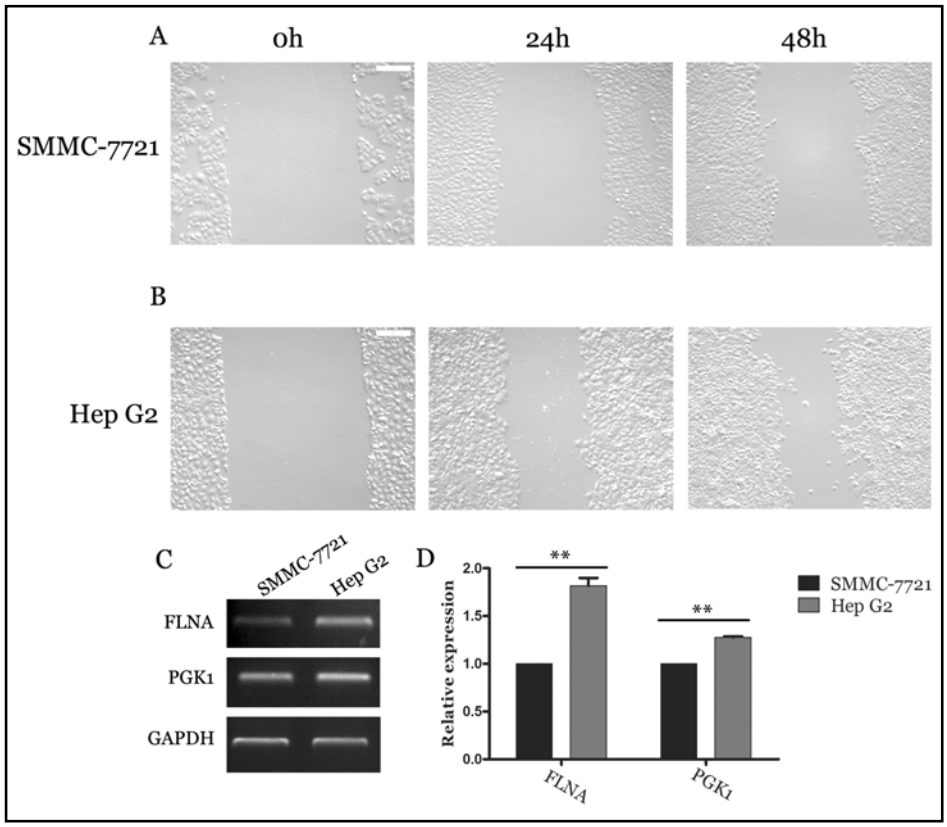

Fig. 6. Confirmation of the differential expression of FLNA and PGK1 in SMMC-7721 and HepG2 cells. (A) The real-time images of migration status of SMMC-7721 cells, from 0 to 48 h. (B) The real-time images of migration status of HepG2 cells, from 0 to 48h, these cells showed a stronger ability of migration than SMMC-7721 cells. (C) The differential expression of FLNA and PGK1 were further examined by RT-PCR at the levels of mRNA. Both of the proteins identified previously were overexpressed in HepG2 cells, which have higher ability of migration. (D) The quantification and statistical analysis of the electrophoresis bands presented in (C), *p < 0.05; **p < 0.01; ***p < 0.001.

progression of HCC are still lacking.

It is believed that the metastatic recurrence of HCC is a complex process with numerous molecular alterations [31]. The current biomarkers seem to be insufficient to monitor the metastasis of HCC. It is possible to improve the diagnosis and prognosis of HCC metastasis with global profiling of metastatic progression of HCC, thus providing more novel and effective biomarkers.

In the present study, we coupled SDS-PAGE with MS/MS technology to address the large-scale differences in protein expression between MHCC97L and HCCLM9 cells, a perfect cell model to study the metastasis of HCC[1, 12, 13] (Fig. 2). Sixteen proteins were identified in four bands (Table 1): four were identified in bands 1 and 3 and overexpressed in HCCLM9 cells, namely FLNA, FLNB, PGK1, and ACTA2. The other proteins identified from bands 2 and 4 were upregulated in MHCC97L cells. Two proteins that were overexpressed in HCCLM9 cells, FLNA and PGK1, were further selected, due to their high MOWSE score, to confirm their expression at the mRNA level and study their involvement with metastasis in HCC. Indeed, *FLNA* and *PGK1* were overexpressed in HCCLM9 cells, as confirmed by RT-PCR (Fig. 5).

FLNA is one of the members of the filamin family, and the other two members are FLNB and FLNC. These three proteins are highly conserved, showing 60-80% homology over their entire sequence to each other [20, 36, 37]. In this study, FLNB was identified meantime, and FLNA and FLNB were reported to form a heterodimer to compensate for the loss of FLNA [20]. So far, more than 70 proteins were validated to interact with FLNA, which works as a cellular scaffold as well as a linker between receptor signaling and the cytoskeleton [16-18]. In addition, FLNA may play an important role in vascular development and tumor cell metastasis [38].

Recently, R.G. Bedolla et al. [21] reported that the expression of FLNA in the cytoplasm (280 kDa) promotes cell invasion of prostate cancer cells, whereas the nuclear translocation (90 kDa) of the protein inhibits this process[21]. In our study, FLNA was overexpressed in HCCLM9 cells, which have a 100% lung metastasis rate and was localized in the cytoplasm of these cells, as indicated in Fig. 2B. We propose that cytoplasmic FLNA also plays a role in the metastasis of HCCLM9 cells. Cytoplasmic FLNA also serves as an actin-binding protein that further promotes cell motility [21]. Our data indicate that actin was indeed markedly upregulated in HCCLM9 cells compared with MHCC97L cells (Fig. 5).

Another potential biomarker for metastasis of HCC identified in this study is PGK1. PGK1 had been identified as a potential marker for peritoneal dissemination in gastric cancer [27] and is known to be involved in the

onset of several malignancies, such as multi-drug resistant ovarian cancer, prostate, breast, pancreatic cancer, and gastric cancer [24-28, 39, 40], but its role is ambiguous in HCC. Moreover, RT-PCR was employed to detect the expression of the PGK1 signaling pathway, including CTNNB1, CXCR4, and HIF1α; all were overexpressed in HCCLM9 cells. It was reported that CTNNB1 is involved in angiogenesis as well as tumor metastasis [41], and its overexpression in HCCLM9 cells suggests CTNNB1 may play a role in metastatic progression of HCC. In addition, CXCR4 plays an important role in the metastatic homing of malignant cells [42], and the tight relationship between CXCR4 and PGK1 was confirmed by D. Zieker et al. [27]. HIF1α is an upstream regulator of PGK1[23], so overexpression of PGK1 in HCCLM9 cells may partially be attributable to the upregulation of HIF1α. Overexpression of HIF1α resulted in the changes of niche that HCC cells lived, supplied enough energy to the tumor cells under the condition of oxygen limited, therefore, overexpression of HIF1α in HCCLM9 cells may promote the metastasis of HCC according to energy supply. Taken together, we postulate that the alterations of PGK1 and its targets comprehensively promote the metastasis of HCC, and these proteins may serve as prognostic biomarkers and/or therapeutic targets to prevent the metastatic recurrence of HCC.

Aside from the proteins described above, another group of proteins identified in this study was downregulated in MHCC97L cells. These proteins comprised of metabolism- and cytoskeleton-related proteins. These will soon be studied in detail.

For the first time, the current study demonstrated that both FLNA and PGK1 were overexpressed in HCCLM9 cells at the protein and mRNA level. Because of the high metastasis potential of HCCLM9 cells, it is convincing that FLNA and PGK1 are two potential biomarkers for the metastasis of HCC and may serve as possible therapeutic targets for the treatment of HCC patients with metastases.

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