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Original Article

Association of macrophage migration inhibitory factor (MIF) with metastasis of hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is well known for poor prognosis and short survival due to metastasis and recurrence even after curative treatment. This study aims to identify novel biomarkers to predict early-stage metastasis of HCC. The subcellular proteome of two HCC cell lines with differential metastasis ability was identified using one dimensional electrophoresis followed by liquid chromatography combined with tandem mass spectrometry. The candidate biomarker was further validated using conventional methods. Proteomic profiling analysis revealed a group of proteins upregulated in high metastasis cell line HCCLM9 compared with low metastasis cell line MHCC97L. Special attention was focused on macrophage migration inhibitory factor (MIF), which was confirmed *in vitro* and *in vivo*. RT-PCR showed that MIF mRNA expression was elevated 2.4 fold in HCCLM9 cells compared with MHCC97L cells. This translated into high protein levels as assessed by western blot of total cell lysates and by ELISA of MIF in the supernatant. In an *in vivo* xenograft model system, abundant MIF expression was observed in liver tumors, lung metastasis and serum of HCCLM9-nude mice compared with MHCC97L-nude mice. In conclusion, MIF expression was upregulated in highly metastatic HCCLM9 cells compared to lowly metastatic MHCC97L cells, which indicated that MIF might be a candidate biomarker for HCC metastasis prediction.

Keywords: Hepatocellular carcinoma, proteomics, macrophage migration inhibitory factor, metastasis

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide [1]. Most cases occur in Asia and sub-Saharan Africa due to high hepatitis B virus (HBV) infection [2]. Recently, the incidence of HCC is also rising in America possibly because of increased population infected with hepatitis C virus (HCV) [2]. Over the past years, impressive progress has been made in HCC treatment. However, conventional chemotherapeutic regimen is ineffective against HCC with a response rate between 5%-10%, and no single drug or "cocktail" could prolong the patient's survival [3]. Although curative therapies (surgical resection and liver transplantation) are possible if the lesion remains early and localized, almost 60% of resected cases recur within 5 years [4]. Subclinical preoperative metastasis may partly explain this phenomenon because metastasis is the major cause of recurrence. Identification of molecular markers could provide supplemental and useful information for predicting clinical outcome in patients with a given stage of disease and improve the selection of patients for adjuvant therapies after resection, [5] but no such molecular has been clinically applicable for HCC.

Two HCC cell lines, MHCC97L and MHCC97H, isolated from the same parental cell line MHCC97 were characterized with different metastatic potentials [6]. In order to obtain cells with increasing metastatic ability, MHCC97H cells were inoculated into BALB/c nude mice and the spontaneous pulmonary metastatic lesions were harvested and re-implanted into nude mice for the next round of *in vivo* selection [7]. The same procedure was repeated until a new cell line HCCLM9 was established from the ninth round. This model system has been

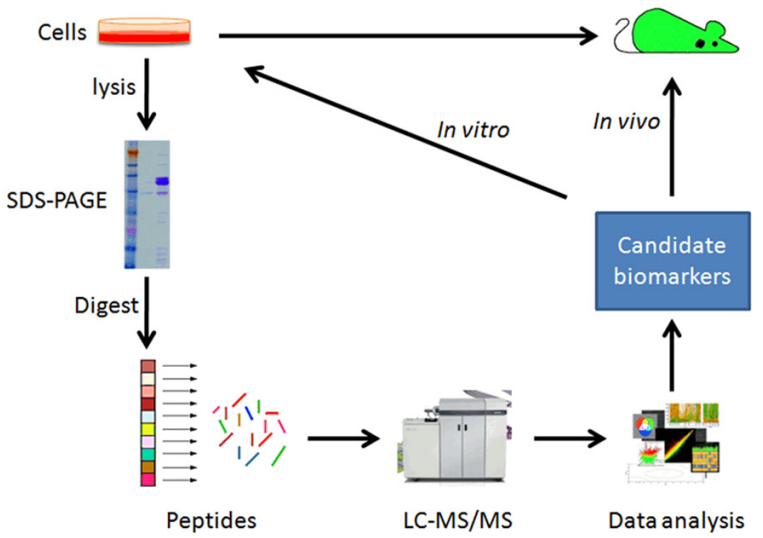

Figure 1. The flow chart of the experiment on identification and validation of biomarkers for HCC metastasis. SDS-PAGE, sodium sulfate polyacrylamide gel electrophoresis; LC-MS/MS, reverse liquid chromatography coupled with tandem mass spectrometry.

shown to be ideal for comparative study on the molecular events correlated with HCC metastasis in recent years [8-10].

Identification of cell proteome and comparison of their expression between cells with different phenotypic characteristics is crucial to the discovery of novel cancer biomarkers and drug targets as well as elucidating the basic biologic processes of cancer [11]. Our previous work has applied two-dimensional gel electrophoresis (2-DE) followed by matrix-assisted laser desorption/time of flight mass spectrometry to obtain the differential proteome of MHCC97L and MHCC97H cell lines, and identified cytokeratin 19 could be a useful marker for predicting tumor metastasis [12, 13]. However, some important information might be lost due to the limitation of the 2-DE strategy. Novel proteomic approaches in combination with subcellular fractionation procedures have recently made it possible to study the cellular proteome in more detail.

In the present study, subcellular protein profiles of MHCC97L and HCCLM9 cell lines were compared with sodium sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by liquid chromatography plus tandem mass spectrometry (LC-MS/MS), in an effort to identify specific biomarker candidates and gain further insight into HCC metastasis biology. Special attention was focused on macrophage migration inhibitory factor (MIF), because it was considered to be a potential missing link between inflammatory activation and malignant progression [14, 15]. Furthermore, serum MIF levels in patients with hepatitis, cirrhosis and HCC were found to be gradually increased [16]. In addition, recombinant MIF enhanced the invasion and migration of HCC cells in an *in vitro* cell migration assay [17]. Therefore, we determined the expression levels of MIF *in vitro* and *in vivo* to evaluate if MIF could to be a biomarker for metastasis prediction of HCC.

Materials and methods

Cell lines and cell culture

Two cloned HCC cell lines, MHCC97L and HCCLM9, were derived from the same host cell line MHCC97 in a process of cloning culture and 9 successive *in vivo* selections as described previously [6, 7]. Cells were grown in RPMI 1640 medium (Mediatech, Manassas, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Carlsbad, USA). Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2 and passaged grown to 80% confluence.

Protein profiles identified by SDS-PAGE combined with LC-MS/MS

The protein profiles of MHCC97L and HCCLM9 were identified by SDS-PAGE combined with LC-MS/MS as described previously [18]. Briefly, the proteins were extracted using ProteoExtract® subcellular proteome extraction kit (Merck, Darmstadt, Germany) according to the manufacturer's instruction. The proteins contained in fraction 2 (membrane and organelle) were used for the subsequent studies. After protein concentration quantification with BCA assay (Pierce, Rockford, USA), equal amounts of proteins were loaded and separated on 12% SDS-PAGE. Each lane was cut into ten equal pieces and transferred into 1.5 ml Eppendorf tubes. In-gel digestion with MS grade trypsin (Sigma, St. Luois, USA) was performed. The peptides were collected, lyophilized, dissolved

in 0.1% acetic acid and separated by reverse-phase liquid chromatography. MS/MS analysis was conducted on a Q-TOP mass spectrometer (Bruker, Germany). Data-dependent MS/MS mode was used, and peptides were identified by searching a non-redundant protein sequence database (SWISS-Prot) using the proteinPilotTM 2.0.1 software (Applied Biosystems, Foster City, USA). The work flow chart was shown in **Figure 1**.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured HCC cells using TRIzol reagent and reverse transcribed using SuperScript® III First-Strand Synthesis system (Invitrogen, USA). PCR was carried out using Golden Fast PCR kit (TIANGEN, Beijing, China). The following primers were used to amplify a 185 base pair fragment of MIF: sense primer, 5'-GTT CCT CTC CGA GCT CAC CCA GCA GC-3'; antisense primer, 5'-GCA GCT TGT AGG AGC GGT TCT G-3'. Primers for the amplification of human β-actin mRNA were as follows: sense primer, 5'-ATG GAA TTC CCG TGG AAG AAC AAG AAT GAG ATC AG-3'; antisense primer, 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCA GC-3'. PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and analyzed by densitometry. Expression of MIF mRNA was measured as the intensity ratio of MIF over β-actin in the corresponding band.

Western blot

Western blot analyses were performed on cell lysates prepared from HCC cell lines. For preparation protein lysates, cells were first washed with PBS and lysed in 2× sodium dodecyl sulphate sample buffer (100 mM Tris-HCl pH6.8, 200 mM DTT, 4% SDS, 20% glycerol and 0.2% bromoplenol blue). Cell lysates were separated by 12% SDS-PAGE. Proteins were transferred to PVDF membranes (Immobilon 0.2 μm, Millipore, USA), which was then immersed in a blocking solution containing 5% non-fat milk and 0.1% tween-20 for 1 h. Afterwards, the membranes were washed and incubated with rabbit anti-MIF antibody (1:1000) or rabbit anti-β-actin antibody (1:1000) for 2 h and then with goat anti-rabbit secondary antibody (1:10000) for 1 h at room temperature. All the antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Enhanced chemiluminescence (Beyotime, Shanghai, China) was used to visualize the immuno-reactive bands.

Enzyme-linked immunosorbent assay

MIF levels were measured by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, USA) according to the manufacturer's protocols. A subset of samples was re-assayed five times in every ELISA plate for quality control.

Nude mice model of spontaneous pulmonary metastasis

Male athymic BALB/c nu/nu mice, 4-6 weeks old, were obtained from Experimental Animal Institute of Hubei Center for Disease Control and Prevention and housed in specific pathogen-free (SPF) condition at the Animal Experiment Center of Wuhan University. The facilities and the protocols of experiment were consistent with the regulations on animal use for biomedical experiments issued by the Ministry of Science and Technology of China, and approved by the Animal Care Committee of Wuhan University. Nude mice models with different metastatic potential were established as described previously [7]. Briefly, Both MHCC97L and HCCLM9 cells (5×10^6 cells each) in 0.1 ml phosphate buffered saline (PBS) were injected subcutaneously into the left upper flank region of 1 nude mouse, respectively. The subcutaneous tumors were removed when they reached 8 mm in diameter, and minced into pieces (1 mm^3) to perform orthotopic transplantation into livers of nude mice (n=8 each group). The behaviors and body weight of animals were monitored. All mice were sacrificed under deep anesthesia by peritoneal injection of 3% phentobarbital chloride at 6 weeks post inoculation. Tumor tissues and lungs were collected, fixed with paraformaldehyde and embedded in paraffin for pathological studies.

Hematoxylin-eosin staining and immunohistochemistry (IHC)

Sections were deparaffinized three washes in xylene followed by rehydration with gradient concentrations of ethanol. For conventional pathological examination, sections were stained with hematoxylin and eosin (H&E). IHC for MIF was performed following a standard method. Endogenous peroxidase activity was

Table 1. Highly expressed proteins in HCCLM9 compared with MHCC97L by LC-MS/MS identification

No.	Accession	Protein name	M. W.	Molecular function	Biological progress
1	P14174	Macrophage migration inhibitory factor	12,476	Chemoattractant activity	Inflammatory response
2	O60361	Putative nucleoside diphosphate kinase	15,529	ATP binding	Nucleotide metabolism
3	O15511	Actin-related protein 2/3 complex subunit 5	16,320	Actin binding	Cytoskeleton organization
4	Q9GZV4	Eukaryotic translation initiation factor 5A-2	16,793	Translation elongation factor	Protein biosynthesis
5	P63241	Eukaryotic translation initiation factor 5A-1	16,832	Translation elongation factor	Protein biosynthesis
6	P15531	Nucleoside diphosphate kinase A	17,149	Kinase	Differentiation
7	P22392	Nucleoside diphosphate kinase B	17,298	Transcriptional activator	Transcription regulation
8	P10620	Microsomal glutathione S-transferase 1	17,599	Glutathione transferase	Glutathione metabolic
9	P23528	Cofilin-1	18,502	Actin binding	Anti-apoptosis
10	P61204	ADP-ribosylation factor 3	20,601	GTP binding	Protein transport
11	P84077	ADP-ribosylation factor 1	20,697	GTP binding	Protein transport
12	P62424	60S ribosomal protein L7a	29,996	Ribonucleoprotein	Ribosome biogenesis
13	Q9BTT0	Acidic leucine-rich nuclear phosphoprotein 32	30,692	Phosphatase inhibitor	
14	P40926	Malate dehydrogenase	35,503	Oxidoreductase	Tricarboxylic acid cycle
15	Q13011	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase	35,816	Isomerase activity	Lipid metabolism
16	P09525	Annexin A4	35,883	Phospholipase inhibitor	Anti-apoptosis
17	P07355	Annexin A2	38,604	Phospholipid binding	Vesicle fusion regulation
18	P14618	Pyruvate kinase isozymes M1/M2	57,937	Kinase	Glycolysis
19	P10809	Heat shock protein 60	61,055	Cell surface binding	Host-virus interaction
20	Q12931	Heat shock protein 75	80,110	Molecular chaperone	Stress response
21	P08238	Heat shock protein HSP 90-beta	83,264	Molecular chaperone	Stress response
22	P07900	Heat shock protein HSP 90-alpha	84,660	Molecular chaperone	Stress response
23	P55072	Transitional endoplasmic reticulum ATPase	89,322	Hydrolase	Transport
24	Q9UBF2	Coatomer subunit gamma-2	97,622	Protein binding	Protein transport
25	Q9Y678	Coatomer subunit gamma	97,718	Protein binding	Protein transport
26	P19367	Hexokinase-1	102,486	Hexokinase activity	Glycolysis
27	P12814	Alpha-actinin-1	103,058	Bundling protein	Focal adhesion
28	P53675	Clathrin heavy chain 2	187,030	Structural molecule activity	Mitosis
29	Q00610	Clathrin heavy chain 1	191,615	Structural molecule activity	Mitosis
30	P35579	Myosin-9	226,532	Protein anchor	Cell shape
31	Q14204	Dynein heavy chain	532,408	Motor protein	Transport

blocked with 3% hydrogen peroxide for 20 min. Antigen retrieval was performed by heating the sections in 0.01 M citrate buffer in a microwave oven. Nonspecific binding was blocked by incubating the tissue sections with 2% bovine serum albumin (BSA, sigma, USA) for 30 min. Slides were then incubated for 1 h at 37°C with MIF antibody mentioned above at a dilution of 1:200. After 3 washes with TBS, bound antibody was detected with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:500) for 1 h at 37°C. Immunocomplexes were detected with diaminobenzidine (DAKO, Denmark, UK) as a chromogen resulting in deposition of a brown reaction product. After counterstaining with Gill hemotoxylin, sections were dehydrated and mounted for microscopic viewing. A negative control section was performed with the same procedure, except that antibody dilution buffer was used to substitute for the primary anti-MIF antibody.

Statistics analysis

Statistical analyses were performed with SPSS software version 17.0 (SPSS Inc. Chicago, IL) and two-tailed $P<0.05$ was considered as statistically significant.

Results

Differential protein expression profile between MHCC97L and HCCLM9 cell lines

Subcellular proteins from MHCC97L and HCCLM9 cells were separated by SDS-PAGE and analyzed by LC-MS/MS. Subsequently, the mass spectrum acquisition and database searching were performed. A total of 31 proteins had significantly higher expression in HCCLM9 cell line than MHCC97L cell line (**Table 1**). These up-regulated proteins were associated with stress response, transcription and translation, cytoskeleton, metabolism and pro-

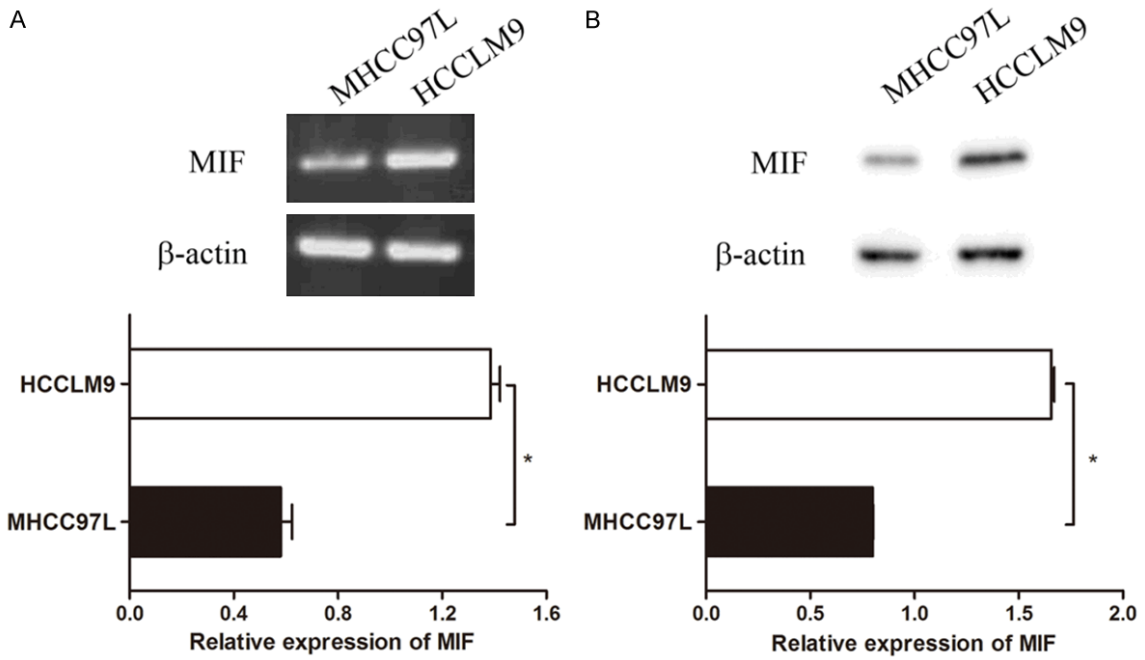

Figure 2. Expression of MIF mRNA and protein in non-metastatic and metastatic HCC cell lines. A. MIF mRNA expression was stronger in metastatic cell line HCCLM9 compared with non-metastatic cell line MHCC97L. Gene expression level was shown relative to the expression level of β-actin. B. Comparison of MIF protein levels by western blot (top panel) and quantification using Image Lab software (Bio-Rad, USA) (bottom panel). β-actin was used as a loading control. The quantification data were means ± SD of 3 independent experiments. **P*<0.05 indicated that statistically significant differences between MIF expression in HCCLM9 and MHCC97L.

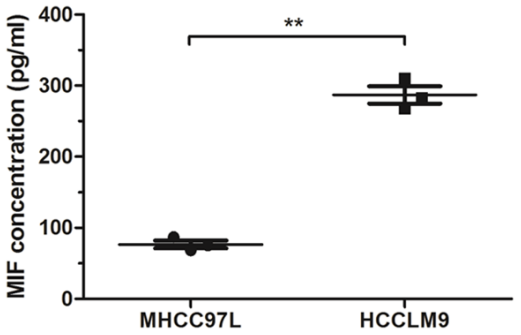

Figure 3. Secreted MIF in the supernatant from MHCC97L and HCCLM9 cells. Data from three independent experiments are pooled together. Results are means (SEM). ***P*<0.01.

tein transport. Macrophage migration inhibitory factor (MIF) was chosen as a candidate biomarker for subsequent validation.

MIF over-expressed in high metastatic potential cell line HCCLM9

RT-PCR was conducted to evaluate the mRNA level of MIF in MHCC97L and HCCLM9 cells. The results showed that MIF mRNA was 2.4 fold higher in HCCLM9 cells than that in MHCC97L cells (**Figure 2A**). MIF expression at the protein level was also determined with western blot. It was observed that MIF protein level in HCCLM9 cells was 2 fold up-regulated compared with MHCC97L cells (**Figure 2B**).

HCCLM9 secreted high levels of MIF

The expression level of MIF in the supernatant was measured in MHCC97L and HCCLM9 cells after 24 hour incubation with serum-free culture medium. MIF expression was significantly higher in HCCLM9 cell line than in MHCC97L cell line (302.2 ± 14.5 *vs* 86.2 ± 6.6, *P*<0.01; **Figure 3**), which indicated that MIF was a secreted protein and might be a candidate biomarker for HCC metastasis.

Orthotopic xenografts in mice to evaluate the level of MIF in vivo

H&E staining was performed on paraffin sections to evaluate the establishment of animal model (**Figure 4**). Besides liver tumors in all ani-

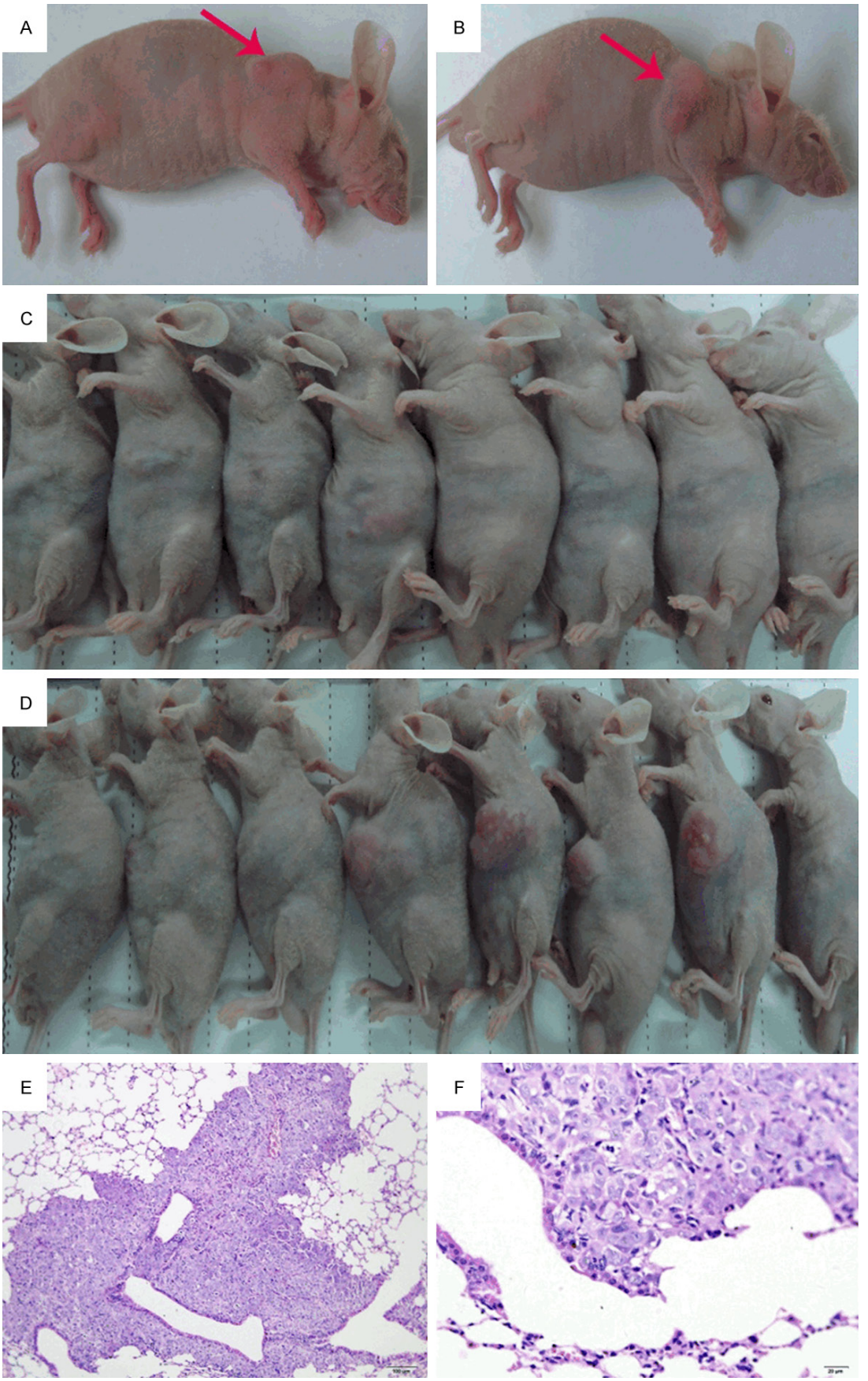
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Figure 4. Establishment of animal model with HCC cell lines MHCC97L and HCCLM9. (A) Subcutaneous tumor in MHCC97L group; (B) Subcutaneous tumor in HCCLM9 group; (C) Orthotropic implantation in MHCC97L group; (D) Orthotropic implantation in HCCLM9 group; (E and F) Lung metastasis in HCCLM9 group; Magnification, ×100, scale bar=100 μm for (E); Magnification, ×400, scale bar=20 μm for (F).

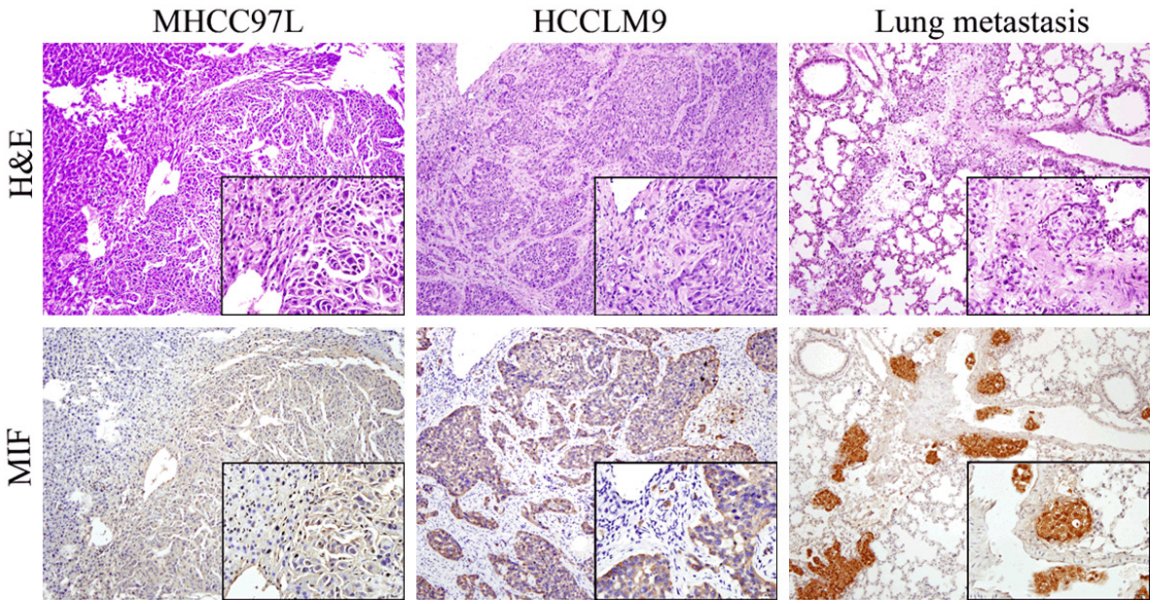

Figure 5. Histological analysis of MIF in liver tumors and lung metastasis of orthotopic graft mouse model. In MHCC97L group, there was weak immunoreactivity in cancer cells. In HCCLM9 group, there was marked brown staining in cancer cells. Note that the H&E and immunohistochemistry staining were from the same region. Magnification, ×100; scale bar=100 μm; inserts, magnification, ×400; scale bar=20 μm.

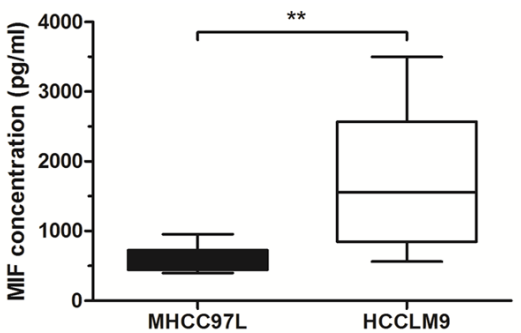

Figure 6. Serum MIF concentration in mice with orthotopic xenografts MHCC97L and HCCLM9. Data from three independent experiments are pooled together. Results are means (SEM). $^{**}P<0.01$.

mals, pulmonary metastases occurred in 8 of 8 mice (100%) of HCCLM9 group in comparison to 0 of 8 (0%) of MHCC97L group. As expectedly, enhanced MIF expression was observed in liver tumors of HCCLM9-nude mice compared with MHCC97L-nude mice (**Figure 5**). Moreover, abundant MIF expression was predominantly found in lung metastases of HCCLM9 group (**Figure 5**). The level of MIF in sera was also higher in HCCML9 group than that in MHCC97L group (1708.0 ± 358.0 *vs* 586.8 ± 68.2, $P<0.01$; **Figure 6**), which indicated that MIF might be an ideal biomarker for HCC metastasis prediction. Overall, we found that MIF levels were upregulated in highly metastatic HCCLM9 cells compared to lowly metastatic MHCC97L cells according the *in vivo* study.

Discussion

Although the diagnostic and treatment improved over the past years, metastasis and recurrence remain major challenges and root cause for poor outcome in HCC. Therefore, it is important to develop new strategies such as biomarkers to predict metastasis or recurrence in an early-stage. We have established an HCC cell model system, MHCC97L and HCCLM9, with the same genetic background but remarkably different metastatic potential. Based on this cell model system, comparative proteomics method was used to obtain the differential proteome profile in the current study. We identified

31 proteins over-expressed in high metastatic potential HCC cell line HCCLM9 comparing with low metastatic potential HCC cell line MHCC97L, and the molecular function and biological process were also listed in **Table 1**.

Furthermore, some of the proteins have been indentified and validated to be correlated with tumor metastasis. For instance, annexin A4 was over-expressed in renal clear cell carcinoma and it seemed to be related to the metastatic potential of this type of tumor [19]. Emoto et al [20] found that Annexin A2 was over-expressed in advanced gastric carcinomas and it was significantly correlated with lymph node metastasis and venous invasion. Over-expression of heat shock protein 60 (Hsp60) could promote metastasis in pancreatic carcinoma, large bowel carcinoma and prostate carcinoma [21]. The level of cell surface heat shock protein 90 (Hsp90) was shown to increase in cancer cells and correlated with metastatic activity, and a neutralizing antibody against extracellular was shown to inhibit melanoma metastasis *in vivo* and to result in prolonged survival in murine xenograft model systems [22, 23]. Moreover, pyruvate kinase isozymes M1/M2 was also identified in agreement with our previous work [12].

Among these proteins, macrophage migration inhibitory factor (MIF) attracted our attentions as it was reported to be a probable link between inflammation and cancer [14, 24]. MIF was initially defined as an inflammatory cytokine derived from activated T-lymphocytes and inhibited the migration of macrophages. Subsequent studies demonstrated that MIF not only played essential role in innate immunity, but also involved in adaptive immune response [25, 26]. In recent years, MIF has been reported to be over-expressed in various cancers and involved in processes fundamental to tumorigenesis such as proliferation and evasion of apoptosis [14, 27].

We further validated the MS data using RT-PCR, western blot and ELISA *in vitro*, and the results showed that the expression level of MIF was significantly higher in HCCLM9 with high metastasis property (**Figures 2** and **3**). We also performed verification in a spontaneous lung metastasis mouse model *in vivo,* and the similar phenomenon was observed as *in vitro* (**Figures 4**-**6**). These results indicated that MIF might play a role in the process of metastasis which was coincident with previous reports. Meyer-Siegler et al found that androgen independent prostate cancer cells required MIF activated signal transduction pathways for both proliferation and invasion, which could be abolish by MIF suppression [28]. In human lung adenocarcinoma cells, both the migratory and invasive potential were decreased by siRNA mediated knockdown of MIF or MIF small molecule antagonist, and in the contrast, over-expression of MIF induced a dramatic enhancement of cell migration [29]. Sun et al analyzed the cellular effects of MIF siRNA on tumor invasion and metastasis and revealed that MIF promoted tumor invasion and metastasis via the Rho-dependent pathway [30]. According to these data, MIF might not only be a biomarker for predicting metastasis, but also be a therapeutic target although which needed to be further investigated.

In conclusion, our current study has identified a list of proteins might be associated with HCC metastasis using comparative proteomics method. We focused on MIF and observed its correlation with HCC metastasis *in vitro* and *in vivo*. However, we did not elucidate the precise role of MIF played in the process of tumor metastasis within the present study. Further investigations are in process to illustrate the mechanism by which MIF could promote HCC cell migration and invasion, and evaluate if MIF could be the potential therapeutic target for metastasis of HCC.

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Disclosure of conflict of interest

None.

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