Original Article

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

Yu Xu1,2*, Xiaohua Leng1*, Long Wu4, Jinxuan Hou1,3, Yan Li5

1Hubei Key Laboratory of Tumor Biological Behaviors & Hubei Cancer Clinical Study Center, Zhongnan Hospital of Wuhan University, Wuhan, China; Departments of 2Radiation and Medical Oncology, 3Oncology, Zhongnan Hospital of Wuhan University, Wuhan, China; 4Department of Radiochemotherapy, General Hospital of Wuhan University, Wuhan, China; 5Department of Surgical Oncology, Beijing Shijitan Hospital of the Capital Medical University, Beijing, China. *Equal contributors.

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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
MIF in HCC metastasis

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% CO₂ 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. Louis, 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% bromophenol 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 (5×10⁶ 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 mm³) 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% phenetobarbital 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

<table>
<thead>
<tr>
<th>No.</th>
<th>Accession</th>
<th>Protein name</th>
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<th>Molecular function</th>
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<td>Translation elongation factor</td>
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</table>

MIF in HCC metastasis

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-
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-
MIF in HCC metastasis

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.

Maligns, 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.

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.

Address correspondence to: Dr. Jinxuan Hou, Department of Oncology, Zhongnan Hospital of Wuhan University, Hubei Key Laboratory of Tumor Biological Behaviors & Hubei Cancer Clinical Study Center, 169 Donghu Road, Wuchang District, Wuhan 430071, China. Tel: +86-27-67812860; Fax: +86-27-67812892; E-mail: jhou@whu.edu.cn; Dr.
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