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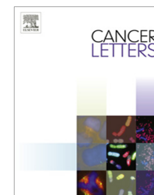

Membrane palmitoylated protein 3 promotes hepatocellular carcinoma cell migration and invasion via up-regulating matrix metalloproteinase 1

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ABSTRACT

Membrane associated guanylate kinase (MAGUK) family, has been extensively studied in cellular adhesion and signal transduction at sites of cell–cell contact. Recently, growing attention has been paid to its role in the initiation and progression of various cancers. However, its role in hepatocellular carcinoma (HCC) has been rarely investigated. In this study, we found that membrane palmitoylated protein 3 (MPP3), a member of MAGUK family, was significantly up-regulated in both high metastatic potential cell lines and clinical tissue samples of HCC, and the most significant increase was observed in the tumors invading the portal veins. Higher level of MPP3 correlated with poorer survival of patients with HCC. Forced expression of MPP3 significantly enhanced HCC cell migration and invasion, whereas knockdown of this gene inhibited this oncogenic effect. Mechanismly, we found that MPP3 promoted HCC cell migration and invasion via up-regulating matrix metalloproteinase 1 (MMP1). These findings indicate that MPP3 play an important role in HCC metastasis by promoting cell migration and invasion, suggesting that it may serve as a novel prognostic marker and molecular target for therapy of HCC.

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1. Introduction

Hepatocellular carcinoma (HCC) is a major health problem worldwide, ranking as the fifth most common cancer in the world and the third most common cause of cancer-related death [1]. Cancer metastases, both intrahepatic and extrahepatic, are major factors in the mortality of HCC patients. However, the molecular mechanisms underlying HCC metastasis remain largely unclear.

Membrane palmitoylated protein 3 (MPP3) is a member of membrane associated guanylate kinase (MAGUK) family, located on chromosome 17q21 [2]. Members of this family are defined by a basic core of three different protein interaction modules, a PSD95/DLG/ZO-1(PDZ)domain, an Src homology3 (SH3) interaction module, and an enzymatically inert guanylate kinase (GK) domain [3]. The modular organization of MAGUKs enables them to function as scaffolds in different cell junction structures, where they have a general role in creating and maintaining specialized membrane domains and act as molecular scaffolds for the signaling pathway components [4,5].

Although MAGUKs were found as the human homologues of a Drosophila tumor suppressor gene, *Disc large* [6], a series of previous studies reported that MAGUKs were involved in tumor progression as oncogenes. For examples, DLG1 interacts with human virus oncoprotein E4-ORF1 and functions as a oncoprotein to promote Ras-mediated PI3K activation [7]. The overexpression of CARMA1 presumably is associated with the development of gastric B-cell lymphoma [8]. CARMA3 is crucial for EGFR-induced activation of NF-κB and tumor progression [9] and overexpression of CARMA3 in non-small-cell lung cancer is linked for tumor progression [10]. ZO-1 is overexpressed in pancreatic ductal adenocarcinoma (PDAC) and raises the possibility that this overexpression may confer a metastatic advantage to pancreatic cancer cells [11]. Also, studies implied that MPP3 might participated in the process of human tumorigenesis. MPP3 associates with nectin-1 mediated by its PDZ domain and this association increases cell surface expression and ectodomain shedding of nectin-1 [12]. In addition, the expression of nectin-1on cell surface is up-regulated in highly migratory and invasive carcinoma [13], suggesting there might be

Abbreviations: MPP3, membrane palmitoylated protein 3; MAGUK, membrane associated guanylate kinase; MMP1, matrix metalloproteinase 1; HCC, hepatocellular carcinoma; PVTT, portal vein tumor thromb; mRNA, messenger RNA; PCR, polymerase chain reaction; SD, standard deviation; RNAi, RNA interference; siRNA, small interfering RNA; β2-MG, β2-microglobulin.

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a relationship between MPP3 and tumor metastasis. However, the function of MPP3 in cancer is still unclear.

In this study, we found that MPP3 was frequently overexpressed in HCC, and promoted HCC cells migration and invasion via up-regulating MMP1. Our study strongly highlights the significance of MPP3 in HCC metastasis and therefore provides a potential drug target in liver cancer therapy.

2. Materials and methods

2.1. Cell lines and cell culture

Four human HCC cell lines were used in this study: SMMC-7721 (from the author's institution) and MHCC-97L, MHCC-97H, and HCC-LM3 [14–16], human HCC cell lines with low or high lung metastatic potential, which were obtained from Liver Cancer Institute, ZhongShan Hospital, Fudan University. All cell lines were routinely maintained in high glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a humidified incubator under 5% CO_2.

2.2. Human tissue samples and patients

From June 2006 to May 2009, clinicopathologic data and surgical resection specimens were obtained from Qidong Liver Cancer Institute (Jiangsu province, China), Zhongshan Hospital (Shanghai, China) and Eastern Hepatobiliary Surgery Hospital (Shanghai, China). All patients with HCC underwent surgical resection of tumors. One grouped HCC consist of a normal liver tissue, a primary HCC tissue and a portal vein tumor thrombus tissue from the same individual and 30 grouped HCCs were collected from 30 patients. Recurrence or intrahepatic metastasis or extrahepatic metastasis after surgery were their causes of death. Mean-time of follow-up or median time to death was 687 or 817 days, respectively. The tumor specimens were immediately snap-frozen in liquid nitrogen and stored at −80 °C for further analysis. Informed consent was obtained from each subject or subject's guardian after approval by the appropriate hospital Ethics Committee.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues or cultured cells using Trizol reagent and was reverse transcribed by using SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative real-time PCR was carried out by using SYBR Green Supermix kit and Premix Ex Taq™ kit (Takara, Dalian, China) with the iCycler detection system (Bio-Rad, Hercules, CA, USA). The primers were listed in Table 3. Blank controls with no cDNA templates were done to rule out contamination. The specificity of PCR product was confirmed by melting curve analysis and gel electrophoresis. All the gene expression levels were normalized to that of the housekeeping gene β2-microglobulin (β2-MG). Relative expression levels of objective gene normalized by β2-MG were calculated by the $2^{\triangle Ct}$, where △Ct (critical threshold) = Ct of objective gene − Ct of β2-MG, and then transformed to log2. Each reaction was repeated independently at least three times.

2.4. Wound-healing assay

Cell migration was evaluated using the scratch wound assay. Briefly, Cells were cultured for 2 days to form a tight cell monolayer and then serum starved for 16 h. Following the serum starvation, the cell monolayer was wounded with a 200 μL plastic pipette tip. The remaining cells were washed twice with culture medium to remove cell debris and incubated at 37 °C with normal serum – containing culture medium. At the indicated times, six randomly selected fields of migrating cells at the wound front were photographed and the migrated area at each time point compared with time 0 h was measured using ImageJ 1.42 software. Experiments were repeated independently three times.

2.5. Cell migration and invasion assay

The migration assay was performed with transwell inserts that had 6.5 mm polycarbonate membranes and pores 8.0 μm in size (Corning Inc., NY, USA). Briefly, 1×10^5 cells were resuspended in serum-free medium and added to the upper chamber. Culture medium containing 10% FBS was used as a chemoattractant in the lower chamber. The cells were incubated for 18 h in a humidified incubator at 37 °C. The cells that migrated through the membrane pores to the lower surface of the membrane were fixed and stained with crystal violet. Stained cells in each field were photographed, and the number of stained cells per chamber was counted in six randomly selected fields. Each experiment was performed in triplicate.

The invasive potential of the cells was examined with a BioCoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA, USA) that contained 8.0 μm membrane pores coated with matrigel matrix. In brief, 3×10^5 cells were seeded in the upper chamber and allowed to migrate through the extracellular matrix to the lower chamber for 36 h. The invaded cells were fixed, stained, and counted as the same as cell migration assay.

2.6. Establishment of MPP3 stably expressing cell and MPP3 stable knockdown cell

MPP3 cDNA was subcloned into mammalian expression vector pcDNA3.1 Myc-His/B(−)(Invitrogen) containing Myc and His tags and neomycin resistance gene for establishment of stable cell lines. For stable transfection, at 24 h after transfection with pcDNA3.1Myc-His/B(−)-MPP3 or pcDNA3.1Myc-His/B(−), SMCC-7721 cells were passaged at 1:6 into new dishes and were selected with 600 μg/mL G418 (Invitrogen) for 14 days. Individual colonies expressing MPP3 were isolated and confirmed by Western blotting and maintained on the same selection medium. Control colonies stably transfected with pcDNA3.1Myc-His/B(−) were also generated in parallel.

ShRNA constructs were provided by W. Hahn (RNAi consortium) in lentiviral cassettes. A shRNA with high MPP3 knockdown efficiency was used (5′-CCGGAACCCTATATTATATTTGTAACTCGAGTTACAAATATAATATAGGGTTTTTTTG-3′), an shRNA with no effect on MPP3 levels was used as a control (5′-CCGGAATTCTCC-GAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATTTTTTTG-3′). As described previously [17], lentivirus was made using a three-plasmid packaging system. Briefly, shRNAs in the pLKO.1-puro vector were co-transfected into 293T cells along with expression vectors containing the *gag*/*pol*, *rev* and *vsvg* genes. Lentivirus was harvested 48 h after transfection, and 5 mg/mL polybrene was added. Subconfluent MHCC-97H cells were infected with harvested lentivirus, and were selected in 2 mg/mL puromycin. Individual colonies of stable knockdown of MPP3 and control colonies were isolated and confirmed by Western blotting and maintained on the same selection medium.

2.7. RNA interference (RNAi)

The cells were transfected with annealed double-stranded MMP1 small interfering RNA (MMP1 siRNA) (5′-GGAGAAAUAGUGGCCCAGUGGUUGAtt-3′) or a non-specific scrambled control siRNA (5′-ACAGACUUCGGAGUACCUGtt-3′) (synthesized from Shanghai GeneChem Co., Shanghai, China) at a final concentration of 100 nM using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions.

2.8. Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS), and protein extracts from cells was prepared in RIPA buffer (10 mM Tris, pH 7.5, 0.1 M NaCl, 1 mM EDTA, and 0.1% Triton X-100). The protein was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and electrotransferred to an nitrocellulose membranes (Millipore, MA). The membranes were blocked with TBST buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween-20, and 0.2% Triton X-100) containing 5% skimmed milk, then incubated with the primary antibodies against different proteins at 4 °C overnight, followed by incubation with peroxidase – conjugated secondary antibody. Bands were visualized by an enhanced chemiluminescence (GE Healthcare). The antibodies in Western blotting analysis included anti-MPP3 (Novus Biologicals, Littleton, CO, USA), anti-MMP1 (Calbiochem, San Diego, CA, USA), anti-β-actin (Sigma–Aldrich, St. Lous, MO, USA).

2.9. Immunohistochemistry (IHC)

Paraffin blocks were sectioned at the thickness of 4 mm. The wax was melted at 65 °C overnight. The sections were deparaffinized in xylene, rehydrated in graded alcohol series and boiled in 0.01 M citrate buffer (pH 6.0) for 2 min in an autoclave. Endogenous peroxidase activity was blocked using hydrogen peroxide (0.3%), which was followed by incubation with normal goat serum to reduce non-specific binding. Tissue sections were incubated with MPP3 rabbit polyclonal antibody (1:30 dilution) (Novus Biologicals) and MMP1 rabbit monoclonal antibody (1:50 dilution) (Epitomics, Burlingame, CA, USA) followed by biotin-conjugated goat antimouse immunoglobulin and horseradish peroxidase (HRP)-conjugated streptavidin (DAKO, Glostrup, Denmark). Aminoethylcarbazole was used as chromogenic substrate and red precipitate was identified as positive staining. The specimens were counterstained with haematoxylin and mounted with glycerol gelatin. Each batch of IHC contained the slides of a positive and a negative control to ensure the staining quality.

Under the low-power field, each slide was evaluated randomly at 10 different areas containing tumor cells by two independent investigators blinded to the clinicopathologic data. At least 100 tumor cells were examined per field. Two scoring systems, staining intensity and percentage of stained cells, were included in our study. The staining intensity was scored on a semi-quantitative four-point scale as follows: 0, equivalent to the negative control; 1, weak cytoplasmic stain

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