Overexpression of Mortalin in hepatocellular carcinoma and its relationship with angiogenesis and epithelial to mesenchymal transition

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Abstract. Mortalin is highly expressed in a variety of human tumors and associated with tumor metastasis. However, the relationship among the overexpression of Mortalin, epithelial to mesenchymal transition (EMT) and neovascularization is largely unknown. The aim of the present study was to investigate the expression of Mortalin in human HCC cell lines, clinical HCC specimens and its association with angiogenesis and EMT. The results of our study showed that the expression levels of Mortalin in cell lines with higher metastatic potential were significantly higher compared to those with lower metastatic potential. Compared with paracarcinomatous tissues and normal liver tissues, the expression of Mortalin was significantly increased in HCC tumor tissues. The expression of Mortalin was correlated with invasion and metastasis, Edmondson grade and TNM stage. A significant positive correlation was found between the expression of Mortalin and Vimentin, and tumors with high expression of Mortalin had a tendency to higher MVD compared to those with low expression of Mortalin. Using shRNA-mediated Mortalin knockdown, we found that decreased expression of Mortalin was accompanied by a reduction of Vimentin expression. Our findings demonstrated that the overexpression of Mortalin is correlated with the metastatic phenotype of HCC cells and can promote EMT, but cannot induce angiogenesis in HCC. The decreased expression of Mortalin is accompanied by an inhibition of EMT in the HCC cell lines.

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Introduction

Hepatocellular carcinoma (HCC) is currently the sixth most common malignancy and the third leading cause of cancer-related death worldwide, with approximately 695,900 deaths in 2008 (1,2). Although hepatectomy, liver transplantation, and ablation are the available treatment options for patients with HCC, high postoperative metastasis or recurrence rate remain the major obstacles that influence long-term survival, while most recurrences are due to invasion-related spreading. Therefore, there is an urgent need for a better understanding of the molecular mechanisms that contribute to HCC metastasis and for new potential therapeutic strategy.

Different molecular events and phenotypic changes are involved in cancer cell disintegration and migration into distant organs or tissues. The epithelial-to-mesenchymal transition (EMT), which is characterized by the loss or downregulation of epithelial markers (E-cadherin) and upregulation of mesenchymal markers (Vimentin), is a crucial step in tumor invasion and metastasis (3,4). Furthermore, HCC is a hypervascular tumor and neovascularization is a common phenomenon. The development of neovasculature plays an important role in the growth and metastasis of HCC (5).

Mortalin, which is also known as heat shock protein 75 (HSP75), is low or undetectable in the normal tissues, while highly expressed in several epithelial carcinomas (6,7). Overexpression of Mortalin interacts with the wild-type tumor suppressor protein p53 and modulates the Ras-Raf-MAPK pathway and then increase the malignancy of tumor cells (8-10). Therefore Mortalin appears to play a crucial role in tumorigenesis and metastasis. Recently, it was reported that Mortalin overexpression in HCC was associated with venous infiltration and advanced tumor stages and thus Mortalin was implicated as a tumor marker for predicting early recurrence (11). Nevertheless, there are no previous studies on the role of Mortalin in HCC, EMT and angiogenesis.

In the present study, we observed that the high expression of Mortalin is an important regulator inducing EMT, but it does not promote angiogenesis in HCC. Furthermore, we demonstrated that the decreased expression of Mortalin is

accompanied by an inhibition of EMT in the HCC cell line MHCC97H.

Materials and methods

Cell culture. Five human hepatoma-derived cell lines (Hep3B, MHCC97H, HepG2, MHCC97L and HCCLM3) with various metastatic potentials and a normal liver cell line L02 were purchased from Liver Cancer Institute of Zhongshan Hospital, Shanghai, China. MHCC97-L, MHCC97-H and HCCLM3 belonged to the same genetic background and had a stepwise increasing metastatic potential (12,13). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA) with 10% (vol/vol) fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 U/mll streptomycin at 37˚C in a humidified incubator supplemented with 5% CO_2.

Patients and tissue samples. Tumor and paracarcinomatous tissues for immunohistochemistry were obtained from 96 patients undergoing curative hepatectomy for HCC at Department of Hepatic Surgery, Anhui Provincial Hospital Affiliated to Anhui Medical University from June, 2007 to June, 2010. Frozen HCC tumor and corresponding paracarcinomatous tissues were obtained from 13 patients for qPCR and western blotting. Ten normal liver samples were acquired from the patients who had received an operation due to hepatic trauma as the controls. No patient had received adjuvant therapies before surgery. These patients included 76 males and 24 females with a mean age of 56.4 years (range, 21-80 years). Tumor size was measured as the largest dimension of the tumor by gross examination; the pathological tumor stage was defined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer; tumor differentiation was defined according to the Edmondson grading system (14). All tissue diagnoses were confirmed by three experienced pathologist who were unaware of patients' clinical and laboratory data. Clinicopathological parameters of the HCC patients are described in Table I. The study protocol conformed to the ethical guidelines of the declaration of Helsinki. Ethics approval for the study of human subjects was obtained from the research ethics committee of Anhui Provincial Hospital Affiliated to Anhui Medical University and written informed consent was obtained from all the patients.

RNA extraction, reverse transcription and real-time quantitative PCR (qPCR). Total RNA was extracted from six cell lines, normal liver tissues, snap-frozen HCC tumor and corresponding paracarcinomatous tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quality of mRNA was evaluated by the OD260/OD280 ratio and samples were used only when the ratio was between 1.8 and 2.0. cDNA was synthesized according to the instructions of the Revert Aid First Strand cDNA synthesis kit (Invitrogen). The sequences of Mortalin-specific primers were designed as follows: forward, 5'-GAGAGACAGGGGTTGATTTGAC-3'; reverse, 5'-GCA CAGATGAGGAGAGTTCACA-3'. In addition, the sequences of Vimentin-specific primers were designed as follows: forward, 5'-CCGACACTCCTACAAGATTTAGA-3'; reverse, 5'-CAAAGATTTATTGAAGCAGAACC-3'. For standardization of RNA quality control, the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was quantified by using the primer set 5'-AAGGTCATCCCTG AGCTGAAC-3' (forward) and 5'-ACGCCTGCTTCACCACC TTCT-3' (reverse). Real-time quantitative PCR was performed at 95˚C for 5 sec and 60˚C for 30 sec for 40 cycles using SYBR Green PCR Master Mix (Applied Biosystems) and Applied Biosystems 7500 Real-Time PCR System. Relative gene expression level was calculated by using the $2^{-\Delta\Delta Ct}$ method. After 40 cycles of PCR, a melting curve analysis was performed to monitor PCR product purity and then the amplicons were analyzed on a 1.0% agarose gel containing ethidium bromide.

Western blot analysis. Six cultured cell lines in an exponentially growing phase were washed thoroughly twice with PBS and then proteins of cell lines, liver tissues, snap-frozen HCC and corresponding paracarcinomatous tissues were extracted using Total Protein Extraction kit (KeyGen, China). After centrifugation, protein concentrations of lysate were quantified using BCA method. Then, equal amounts of protein samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (0.45 μm, Millipore, MA, USA). Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h and then incubated with primary antibody of rabbit anti-Mortalin antibody (ab53098) (Abcam, Cambridge, MA, USA), rabbit anti-Vimentin antibody (ab92547) (Abcam) and rabbit anti-β-actin antibody (ab133626) (Abcam) at 4˚C overnight. After 1.5 h of incubation in horseradish peroxidase-labelled secondary antibodies (ZB-2308, ZSGB-BIO), the blots were detected using ECL western blot kits (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The density of the bands was quantified with an ImageJ Software (National Institute of Health, USA). The expression of β-actin was used as an internal control.

Histopathological and immunohistochemical analyses. Immunohistochemisty was used to detect the expression of Mortalin in 10 normal liver samples and 100 HCC tumor and corresponding paracarcinomatous samples; Vimentin and CD34 were only examined in HCC tumor tissues. Briefly, formalin-fixed and paraffin-embedded tissues were cut into sections with a thickness of 2 μm. Sections were stained with hematoxylin and eosin (HE) for histological examination. Sections were dewaxed in xylene and rehydrated in a graded series of alcohols. Thereafter, antigen retrieval was performed by microwaving in sodium citrate buffer (10 mM, pH 6.0) for 15 min and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Subsequently, rabbit anti-Mortalin antibody (ab53098) (Abcam), mouse anti-Vimentin antibody (ZM-0260, ZSGB-BIO), mouse anti-CD34 (ZM-0046, ZSGB-BIO) antibody were, respectively, incubated at 4˚C overnight. Following 30 min of incubation in horseradish peroxidase-conjugated secondary antibody (PV-6000, ZSGB-BIO), sections were stained in 3, 3-diaminobenzidine tetrahydrochloride (DAB) (ZLI-9017, ZSGB-BIO) solution under microscopic observation and then counterstained

Table I. Clinicopathological factors and the expression of Mortalin in 100 patients with HCC.

Characteristics	Case	Mortalin expression		χ^2	P-value
		Low	High		
Age (years)					
≤55	28	3	25	2.421	0.210
>55	72	20	52		
Gender					
Male	76	19	57	0.322	0.570
Female	24	4	20		
Tumor size (cm)					
≤5	38	5	33	3.352	0.067
>5	62	18	44		
Tumor nodules					
Single	68	18	50	0.898	0.343
Multiple	32	5	27		
Tumor capsula					
Complete	70	13	57	2.584	0.108
None	30	10	20		
AFP (ng/ml)					
≤400	31	7	24	0.004	0.947
>400	69	16	53		
$ICGR_{15}$ (%)					
≤10	79	17	62	1.697	0.193
>10	21	6	15		
HBsAg					
Positive	84	20	64	0.014	0.907
Negative	16	3	13		
Liver cirrhosis					
Present	87	21	66	0.004	0.951
Absent	11	2	9		
Child-Pugh grade					
A	79	15	64	3.420	0.064
B	21	8	13		
Edmondson grade					
I-II	67	20	47	4.272	0.039
III-IV	33	3	30		
TNM stage					
I-II	64	21	43	8.188	0.004
III-IV	36	2	34		
Invasion and metastasis					
Absent	61	19	42	4.742	0.029
Present	39	4	35		

$ICGR_{15}$, indocyanine green retention rate at 15 min.

lightly with hematoxylin. The positive controls were used with sections of breast cancer and the negative controls were processed with PBS instead of primary antibody.

Mortalin and Vimentin staining were assessed by a semi-quantitative scoring system including the intensity of staining and the proportion of stained cells (15). At low-power (x40) microscope, staining intensity of tissue sections were scored (0, no staining; 1, weak staining appearing as light yellow; 2, moderate staining appearing as yellowish-brown; 3, strong staining appearing as brown); at high-power (x400) microscope, >5 fields in one section were scrutinized and then the percentage of positive stained cells was calculated (0, none; 1, <10%; 2, 10%-50%; 3, >50%). The final score of each section was obtained: [(score for staining intensity) x (score for percentage of positive cells)]. For category analysis, immunoreactivity of tumor cells was distinguished between high (total score ≥4) and low (total score <4). CD34 is an antigen present in the vascular endothelial cells. We used antibodies against CD34 to stain vascular endothelial cells and then calculated the microvessel density (MVD) (16). The field of maximal CD34 expression was found in tumor tissues. Within the field, the area of maximal angiogenesis was selected and microvessels were counted on a x200 magnification field. The immunohistochemical results were scored by three pathologists who were blinded to clinical data.

Plasmid extraction and RNA interference. The small hairpin RNAs (shRNA) (Genechem, China) against Mortalin were designed by inserting oligos with a hairpin loop into the GV115 vector. The target sequences of Mortalin shRNA (NM_004134, CTGGAATGGCCTTAGTCAT) were synthesized by Shanghai GeneChem Co. Bacteria containing the plasmid were inoculated on solid culture medium with Amp resistance in the incubator. A bacterial colony was picked and then inoculated in liquid culture medium on a vibration shaker at 37˚C (shaker speed 150 rpm) overnight. Harvest overnight bacterial culture by centrifuging 12,000 g for 2 min. Plasmid was extracted using the Qiagen Plasmid Mini kit (Qiagen, China) according to the manufacturer's instructions, then dissolved in a suitable volume of TE buffer. MHCC97H cells at ~$3x10^5$/ml were seeded in a 6-well plate and placed in antibiotic-free DMEM containing 10% FBS. When the cells confluence reached 90%, transient transfection of MHCC97H cells was performed. A mixture containing 10 μl/well Lipofectamine 2000 (Invitrogen), 10 μl/well plasmid and 480 μl/well OPTI-MEM were added to plates. After 6 h, OPTI-MEM was replaced by DMEM containing 10% FBS.

Three groups were established in this experiment: blank group (no interference), NC (negative control) group (transfected with NC shRNA) and shRNA group (transfected with Mortalin shRNA). Cells were harvested 24, 48, 72 and 96 h after transfection and subjected to MTT assay, flow cytometry, qPCR and western blot analyses. Simultaneously, a GFP (green fluorescent protein) plasmid was used to determine transfection efficiency and cell viability was determined by the MTT assay (MTT Cell Proliferation and Cytotoxicity Assay kit) and flow cytometry (Annexin V/PI apoptosis kit). All experiments were performed in triplicate to confirm the reproducibility.

Table II. Relative quantity of Mortalin mRNA and protein in six cell lines.

	Hep3B	MHCC97H	HepG2	L02	MHCC97L	HCCLM3
mRNA	0.45±0.08	1	0.14±0.06	0.04±0.01	0.05±0.02	2.38±0.27
Protein	0.72±0.11	1.21±0.13	0.35±0.07	0	0	2.14±0.29

MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Twenty-four hours post-transfection cells ($2.0x10^4$/ml, 200 μl/well) were cultured in 96-well plates with DMEM among three groups. MTT (0.5%) (Beyotime, China) with a volume of 20 μl was added to each well for 4 h and then DMEM was aspirated from wells as far as possible without disturbing the cells and crystals on the plastic surface. Subsequently, each well was added with a 200-μl dimethyl sulfoxide (DMSO) to dissolve crystals and the plate agitated on a plate shaker for 10 min. The optical density (OD) measurements were carried out in an enzyme-linked immunosorbent assay (ELISA) reader at the wavelength of 490 nm. The reader was calibrated to 0 absorbance using DMEM without cells.

Flow cytometry. Twenty-four hours post-transfection, cells ($2.0x10^5$) were collected by centrifugation among three groups and then suspended in 500 μl 1X binding buffer. Subsequently, 5 μl Annexin V-FITC and 10 μl propidium iodide (PI) (Annexin V/PI apoptosis kit, Multi Sciences) were added to each sample. Samples were gently mixed and incubated at room temperature for 5 min in the dark. The cells were analyzed on a Canto-II™ reader (BD Biosciences) and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis. Statistical analyses were performed using the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The difference in Mortalin mRNA and protein expression level among six cell lines and tissue samples were examined using Student's t-test. Fisher's exact or χ^2 tests were performed to analyze correlations among different protein expressions or between protein expressions and various clinicopathological parameters. P<0.05 was considered statistically significant.

Results

The expression level of Mortalin mRNA and protein in HCC and normal liver cell lines. The expression of nodal mRNA was detected in all cell lines. The expression of Mortalin protein was detected in Hep3B, MHCC97H, HepG2 and HCCLM3, but not in MHCC97L and L02 (Fig. 1). The mean expression level of Mortalin mRNA and protein are shown in Table II. It was found that among the three human HCC cell lines with the same genetic background, the expression level of Mortalin was the highest in HCCLM3, while it was the lowest in MHCC97L (P<0.05).

The expression level of Mortalin mRNA and protein in HCC tumor, paracarcinomatous and normal liver tissues. Mortalin was detected in 10 normal liver samples and all 13 HCC

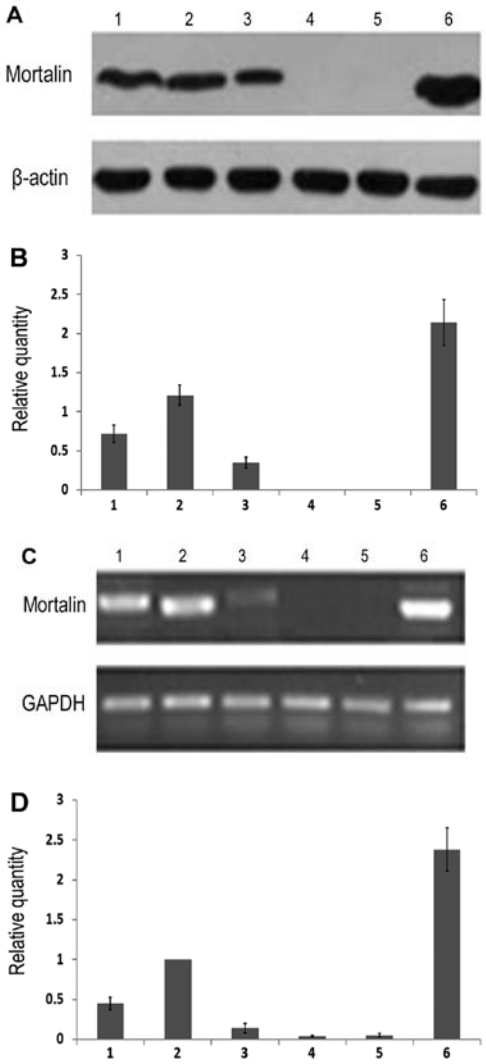

Figure 1. Expression of Mortalin protein and mRNA in liver cell lines. 1, Hep3B; 2, MHCC97H; 3, HepG2; 4, L02; 5, MHCC97L; 6, HCCLM3. (A) The expression of Mortalin protein in six cell lines using western blotting. (B) Relative quantity of Mortalin protein expressed in six cell lines. (C) The expression of Mortalin mRNA in six cell lines using qPCR. (D) Relative quantity of Mortalin mRNA expressed in six cell lines.

tumors and corresponding paracarcinomatous samples. The mean mRNA and protein level of Mortalin in HCC tumor, paracarcinomatous and normal liver tissues were 1, 0.36±0.14, 0.18±0.07 and 2.58±0.21, 0.22±0.09, 0.05±0.02, respectively (Fig. 2). The results showed that the expression level of Mortalin in HCC tumor tissues was significantly higher than that in paracarcinomatous and normal tissues (P<0.05).

Immunohistochemical expression of Mortalin in HCC tumor, paracarcinomatous and normal liver tissues. We found that

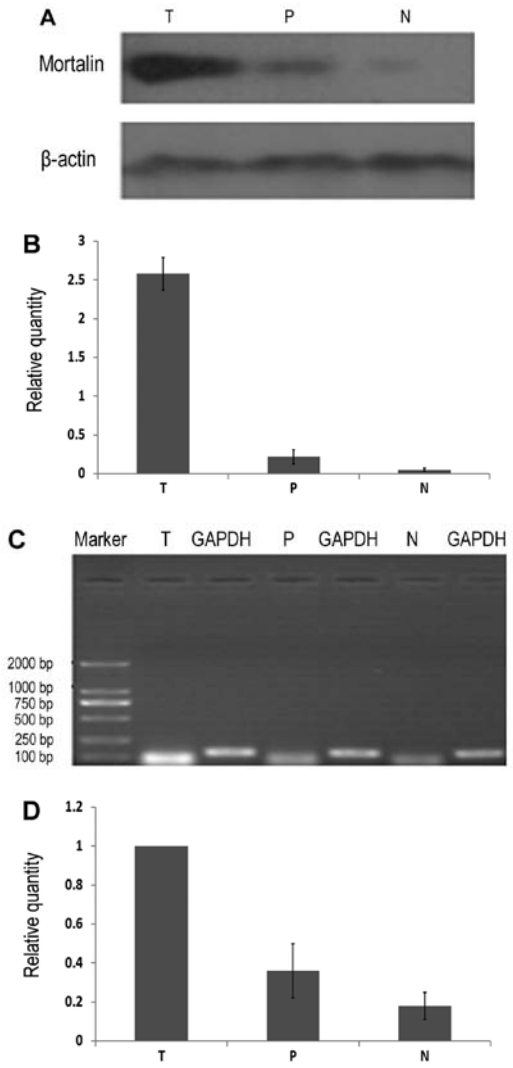

Figure 2. Expression of Mortalin protein and mRNA in liver tissues. T, HCC tumor tissue; P, paracarcinomatous tissue; N, normal tissue. (A) Expression level of Mortalin protein in liver tissues using western blotting. (B) Relative quantity of Mortalin protein expressed in liver tissues. (C) Expression level of Mortalin mRNA in liver tissues using qPCR. (D) Relative quantity of Mortalin mRNA expressed in liver tissues.

positive expression of Mortalin was mainly cytoplasmic, some tumor cells stained strongly, while others exhibited slight or no staining at all (Fig. 3B-D). Mortalin expression was detected in 77% (77/100) of HCC tumor tissues, which was significantly higher than 19% (19/100) in the paracarcinomatous tissues and 1% (1/10) in the normal liver tissues (χ^2=67.388, P<0.001; χ^2=16.669, P<0.001, respectively) (Table III). Association between the expression of Mortalin and clinicopathological characteristics was evaluated and was found that the high expression of Mortalin was positively correlated with poor Edmondson grade (χ^2=4.272, P=0.039), advanced TNM stages (χ^2=8.188, P=0.004) and status of invasion and metastasis (χ^2=4.742, P=0.029), but there was no significant correlation with other variables (Table I).

Immunohistochemical expression of Mortalin, Vimentin and MVD in HCC tumor tissues. Vimentin is an important hallmark of EMT; high expression of Vimentin was observed

Table III. Immunohistochemical expression of Mortalin in HCC, paracarcinomatous tissues and normal liver tissues.

	Mortalin expression			
Liver tissue	Low	High	χ^2	P-value
HCC tumor tissue	23	77		
Paracarcinomatous tissue	81	19	67.388	<0.001
Normal tissue	9	1	16.669	<0.001

Table IV. Correlation between Mortalin and Vimentin expression in 100 HCC patients with HCC.

	Vimentin expression			
Mortalin expression	Low	High	r	P-value
Low	16	7	0.236	0.018
High	32	45		

mainly in the cytoplasmic staining (Fig. 3G and H). Among all patients with HCC, 52% (52/100) patients had high expression of Vimentin. Spearman's rank correlation test was used to analyze the relation between Mortalin and Vimentin; a significant positive correlation was found between the expression of Mortalin and Vimentin (r=0.236, P=0.018) (Table IV). Angiogenesis is an important process of growth and metastasis in HCC; MVD was detected by using an antibody against CD34 (Fig. 3E and F). MVD in HCC tumor tissues ranged from 0 to 139/200 x field (median, 32/200 x field). We found that tumors with high expression of Mortalin had a tendency to higher MVD than those with the low expression of Mortalin (39.4±42.5 vs. 29.7±16.9, t=1.630, P=0.106).

shRNA transfection in MHCC97H cells. GFP fluorescence showed that plasmid Mortalin shRNA was successfully transfected into MHCC97H cells (Fig. 4). MTT and flow cytometry assays were applied to determine cell viability 24 h after transfection among the three groups. OD values were not significantly different among the three groups (P>0.05) (Fig. 5). The result showed that only 2.5 and 3.5% cytotoxicity was present compared to the blank group. Similarly, flow cytometry analysis showed that early apoptosis (Annexin V^+/PI^-) rates among the three groups were 0.8, 4.5 and 9.2% (Fig. 6), indicating that transfection did not cause severe cell damage. To demonstrate the level of inhibition after transfection, qPCR assay was performed. As shown in Fig. 7A, Mortalin mRNA level of cells were 2.52±0.37, 1.97±0.28, 1.12±0.25 and 0.55±0.13 in shRNA group after 24, 48, 72 and 96 h, respectively. Simultaneously, the expression level of Mortalin mRNA in blank group and NC group were 2.90±0.31, 2.83±0.22, 2.95±0.27, 2.90±0.23 and 2.88±0.41, 2.80±0.15, 2.93±0.30, 2.91±0.09, respectively. The expression level of Mortalin mRNA in shRNA group was significantly lower than that in blank group and NC group (P<0.05, respectively). These results showed that Mortalin

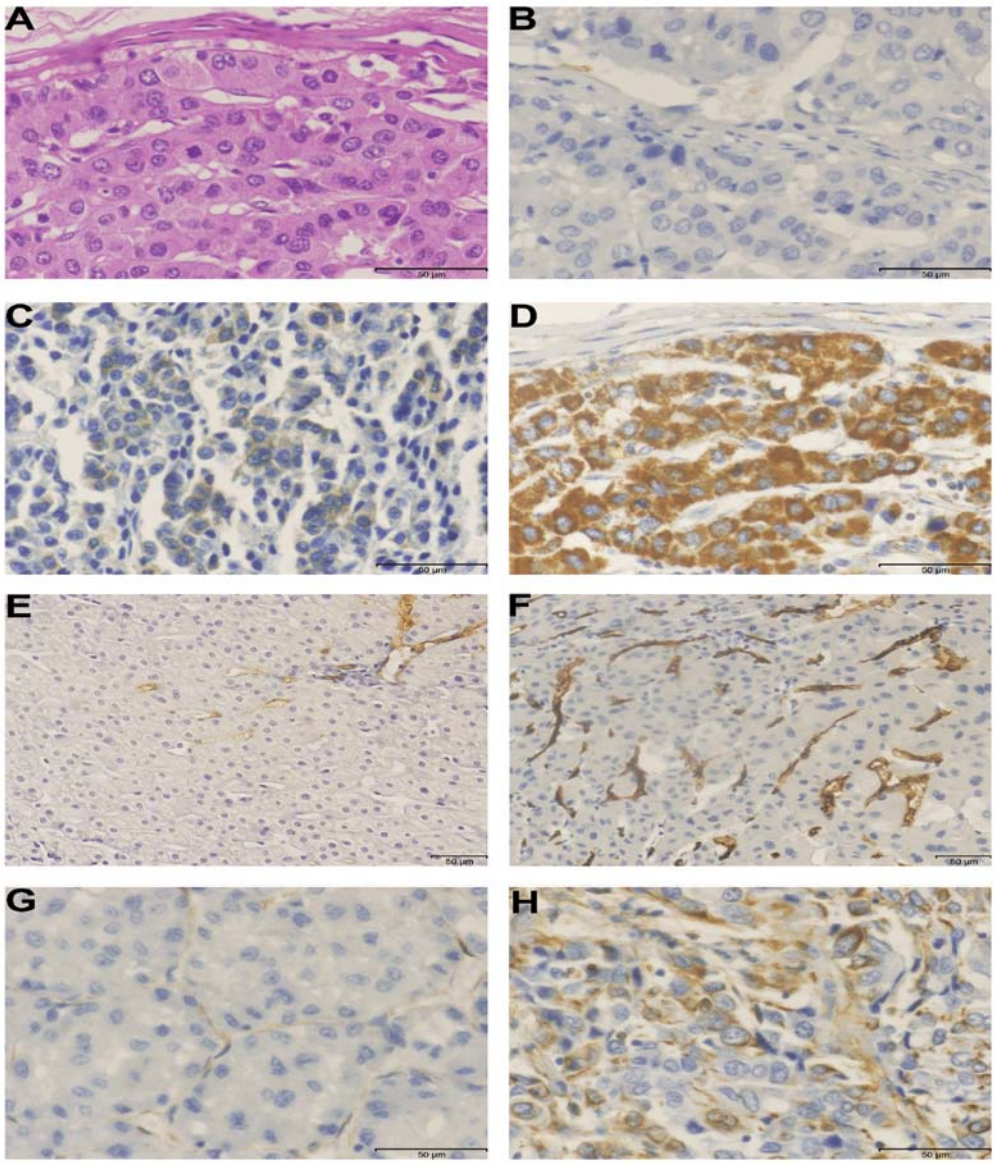

Figure 3. Immunohistochemical staining of Mortalin, CD34 and Vimentin in HCC tumors. (A) HCC tumor, hematoxylin-eosin staining (x400). (B) Negative expression of Mortalin (x400). (C) Low expression of Mortalin (x400). (D) High expression of Mortalin (x400). (E) Low expression of MVD (x200). (F) High expression of MVD (x200). (G) Low expression of Vimentin (x400). (H) High expression of Vimentin (x400).

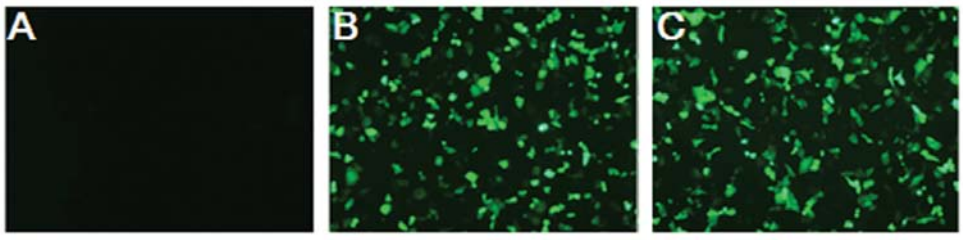

Figure 4. Green fluorescence in MHCC97H cells 24 h after transfection (x200) in the blank group (A), NC group (B) and siRNA group (C).

shRNA was an effective sequence to suppress the target gene and the capability of interference increased gradually upto 96 h after transfection.

To find the relationship between Mortalin and Vimentin, qPCR and western blot assays were applied. Vimentin mRNA level of cells in shRNA group after 24, 48, 72 and 96 h were 1.56±0.21, 1.02±0.27, 0.58±0.16 and 0.31±0.07, respectively (Fig. 7A). 24, 48, 72 and 96 h after transfection, the expression level of Mortalin protein and Vimentin protein in shRNA group were 2.09±0.37, 1.48±0.23, 0.73±0.11, 0.25±0.06 and 1.03±0.21, 0.54±0.14, 0.21±0.07, 0.11±0.02, respectively (Fig. 7B). We found that the decreased expression of Mortalin

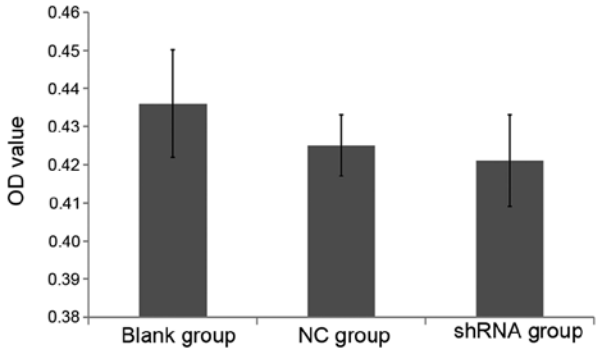

Figure 5. Cell viability (OD value) was measured by MTT assays 24 h after transfection in the blank group, NC group and siRNA group, respectively.

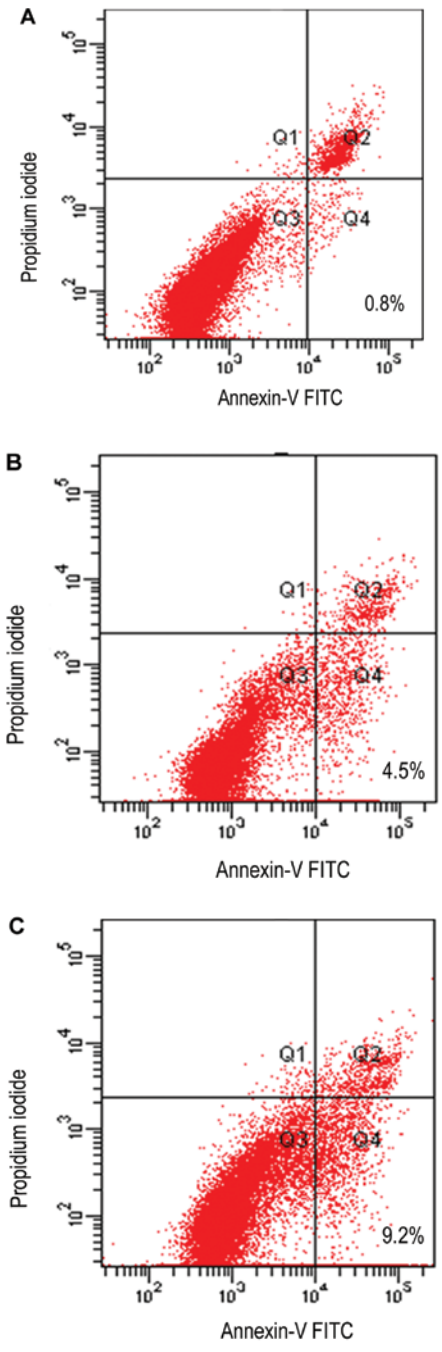

Figure 6. Cells in the blank group (A), NC group (B) and siRNA group (C) were analyzed by flow cytometry 24 h after transfection.

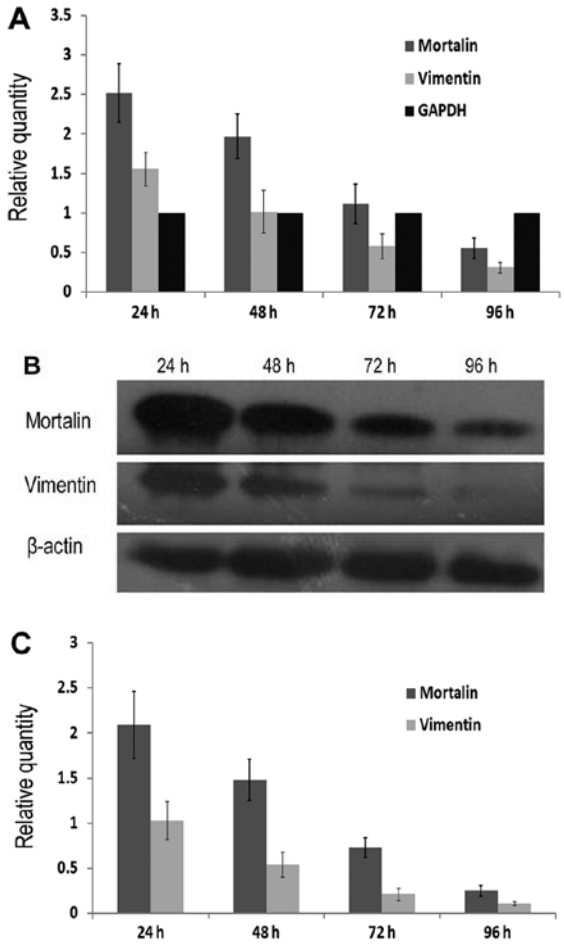

Figure 7. The expression level of Mortalin and Vimentin in MHCC97H cells. 1, shRNA group for 24 h; 2, shRNA group for 48 h; 3, shRNA group for 72 h; 4, shRNA group for 96 h. (A) Relative quantity of Mortalin mRNA and Vimentin mRNA expressed in MHCC97H cells. (B) The expression of Mortalin protein and Vimentin protein were measured by western blotting. (C) Relative quantity of Mortalin protein and Vimentin protein expressed in MHCC97H cells.

was accompanied by a reduction of Vimentin expression (Fig. 7C). This observation supported our conclusion that inhibition of Mortalin expression could decrease the expression of Vimentin and had a suppressive function in epithelial-to-mesenchymal transition.

Discussion

HCC invasion and metastasis are closely related to adverse clinical outcome and shorter survival of cancer patients. Although great efforts have been made to clarify molecular mechanisms involved in HCC invasion and metastasis, the detailed mechanisms for HCC malignancy and metastasis are still unknown. It was reported that angiogenesis and EMT played important roles in the occurrence and progression of HCC (16,17). Mortalin, as a 'stress protein', was a highly conserved molecular chaperone and abundant in the same tumors and played a key role in cell cycle regulation, carcinogenesis and tumor progression (7,18,19). Simultaneously, Mortalin-p53 interaction resulted in inhibition of transcriptional activation and control of centrosome

duplication functions (9,20). In addition, Lu *et al* (21) found that inhibitor of Mortalin was able to induce tumor cell apoptosis. Therefore, Mortalin was suggested to serve as a therapeutic marker.

It was previously reported that Mortalin was overexpressed in leukemia, brain tumors, colon carcinoma and tumor cell lines (7,22). Yi *et al* (11) have demonstrated that overexpression of Mortalin was closely associated with venous infiltration and advanced tumor stages in HCC. These findings suggested that overexpressed Mortalin could increase malignancy and aggressive behavior and predicted early recurrence in HCC. Chen *et al* (23) found that expression of Mortalin was notably higher in the SMMC 7721 than in a normal liver cell line. Lu *et al* (21) showed that human HepG2 cells lacked mortalin-p53 interaction and were resistant to apoptosis, but cell apoptosis was significantly increased by Mortalin shRNA transfection. These findings showed the potential for clinical application of chemotherapeutic drugs in HCC treatment.

HCC is usually a hypervascular tumor and upregulation of Vimentin is considered as the essential step of EMT process. When a liver tumor was 1-2 mm in diameter, it could obtain nutrients through diffusion without an extensive vasculature. If tumor growth become much larger, angiogenesis must occur, also, metastasis is required for angiogenesis (24). CD34 is considered to be a marker for neovascularization, the expression of CD34 positive endothelial cells played an important role in the process of angiogenesis in HCC and metastasis. EMT was first discovered at key transition steps during embryogenesis and was the critical event that mediated tumor metastasis. Through activation of several specific transcription factors, tumor cells invariably adopt a mesenchymal phenotype to invade surrounding tissues and metastasize (4,25). Vimentin has been recognized as a marker for EMT and its overexpression has been strongly associated with metastatic phenotype and poor prognosis.

To our knowledge, this was the first study to investigate the relationship among Mortalin expression, angiogenesis and EMT by immunohistochemical staining. We discovered that Mortalin had a significant correlation with Vimentin, but the high expression of Mortalin group did not have significantly higher MVD than that in low expression of Mortalin group. This shows that Mortalin could not promote angiogenesis, but could contribute to the process of EMT. We also observed that the expression level of Mortalin in HCCLM3 with the highest metastatic potential was notably higher than in the other five cell lines with lower metastatic potential. This suggested that Mortalin expression correlated with the metastatic phenotype of HCC cells.

In three cell lines with the same genetic background and various metastatic potentials, HCCLM3, with the highest metastatic potential, exhibited the highest level of Mortalin and MHCC97L, with the lowest metastatic potential, having the lowest level of Mortalin. This showed that overexpression of Mortalin could possess metastasis-inducing capabilities. We analyzed expression of Mortalin and clinicopathological characteristics and found Mortalin was associated with Edmondson grade, TNM stage and tumor invasion and metastasis. By Mortalin shRNA transfection, we also found that decreased expression of Mortalin was accompanied by a reduction of Vimentin expression. Our conclusions are that low expression of Mortalin was able to inhibit EMT and decrease tumor progression and lose the metastasis-inducing capability.

Although the same studies have identified that HSP70 blocked TGF-β-induced EMT in HaCat cells (26) and HSP72 inhibited EMT by inhibiting Smad3 activation in renal epithelial cells (27), the mechanisms by which Mortalin (HSP75) promoted EMT and tumor metastasis are still unclear. To solve these problems, further experiments are required.

In conclusion, our study suggests that overexpression of Mortalin is correlated with metastatic phenotype of HCC cells and can promote EMT, but cannot induce angiogenesis in HCC. The decreased expression of Mortalin is accompanied by an inhibition of EMT in the HCC cell line. Using shRNA transfection, Mortalin knockdown may have potential for clinical application to decrease tumor metastasis and recurrence after curative surgery.

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