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ORIGINAL CONTRIBUTION

The relationship between Metallothionein-1F (MT1F) Gene and Hepatocellular carcinoma

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To investigate the expression of MT1F gene in hepatocellular carcinoma tissue and the growth suppression effect of exogenous introduction of MT1F gene on liver cell line HepG2 and to explore the potential application of MT1F gene in gene therapy of tumor. Eukaryotic expression vector of pCMV-MT1F plasmid was introduced into HepG2 line which expressed no MT1F protein originally with lipofectamine transfection method. The cell growth curve, soft agar colony formation rate and tumorigenicity in SCID mice were examined to demonstrate the growth suppression effect of exogenous MT1F gene on HepG2 cell line .The MT1F mRNA and MT1F protein were also detected in 60 pairs of surgical specimens of hepatocellular carcinoma by in situ *hybridization and immunohistochemistry. The transfected HepG2 cell line grew more slowly than control HepG2 as shown by cell growth curves, the soft agar colony formation rate (3.8 percent vs. 7.4 percent, $p < .01$) and the average growth rate of tumor in SCID mice (30.9 ± 6.9 vs. 70.3 ± 5.6, $p < .01$) .The expression level of MT1F mRNA and protein significantly increased in paracancerous tissue, normal tissue than in cancer tissues (75 percent, 70 percent vs. 16.7 percent by ISH and 66.7 percent, 60 percent vs. 10 percent by IHC, $p < .01$). Exogenous MT1F gene shows the strong effect of growth inhibition on HepG2 cell line. In the liver cancer tissue, MT1F shows down-regulated expression that supports the inhibited function of MT1F in cancer growth and suggests MT1F may have an important role in gene therapy of hepatocellular carcinoma.*

INTRODUCTION

Hepatocellular carcinoma (HCC)[d] is one of the most common malignant tumors in China, especially Qidong, a HCC high-risk area. Its pathogenesis and development are closely related with some of proto-oncogenes and their products [1]. MT1F (BC027262) is known as a member of metallothionein family characterized by rapid and transient expression in response to stimulation, modulating gene transcription positively or negatively depending on the cell types and playing an important role in the cell growth [1, 2]. But its relationship with HCC is not well understood so far. In order to evaluate the growth inhibition of MT1F on HepG2 and analyze the expression of MT1F in HCC tissue, the relationship between MT1F and hepatocellular carcinoma is explored by the methods of gene transfection, tumorigenicity in

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[d] *Abbreviations:* CMV, cytomegalovirus; DAB, diaminobenzidine; HCC, Hepatocellular carcinoma; MT1F, Metallothionein-1F; PBS, phosphate-buffered saline; RSV, respiratory syncytial virus.

severe combined immunodeficient (SCID) mice and *in situ* hybridization, immunohistochemistry, etc.

MATERIALS AND METHODS

Cell lines and tissues

Human hepatoma cell lines HepG2 was obtained from Shanghai Cell Research Institute, These cells were cultured with Dulbecco's Modified Eagle medium (DMEM) supplemented with 10 percent heat-inactivated fetal bovine serum (Sigma, United States) at 37°C 5 percent CO_2 incubator unless otherwise noted.

Sixty pairs of HCC and adjacent non-tumor counterparts were obtained at time of surgical resection from Qidong Liver Cancer Institute, twenty normal human liver tissues adjacent to hepatic metastatic tumors of colon cancer were obtained at the time of surgery and served as controls. There were fixed in 40 ml/l neutral formalin with 1/1000 diethyl pyrocarbonate (DEPC, Sigma Chemical Company, United States) embedded in paraffin, and the sections were cut in 5 μm.

Eukaryotic expression vector of pCMV-MT1F plasmid

The plasmid was constructed by us. The final construct contains the neogene (5.5 kb fragment) driven by the respiratory syncytial virus (RSV) promoter and the MT1F gene driven by the human cytomegalovirus (CMV) promoter. The plasmid was confirmed by amplification, purification and tested by endonuclease cutting.

Gene transfection

The eukaryotic expression vector of pCMV-MT1F plasmid was transfected to human HepG2 cell line by the lipofectamine (Gibco Company, United States) according to the manufacturer's instructions. After transfection, the cells were trypsinized and reseeded at 1:2 ratio for selection culture with G418 at 600 mg/l. Four weeks later, the resistant colonies were formed.

Detection of transfected cell neogene with polymerase chain reaction

Extracting the cell DNA followed by performing polymerase chain reaction (PCR) to amplify 327 bp of neogene with primer 1 (5′-ACAAGATGGATTGCACGCAGG-3′) and primer 2 (5-TTCTCGGCAGGAG-CAAGGTGAG-3′). The cycling procedure was denatured at 95°C for 1 min, annealed at 55°C for 1.5 min, extended at 72°C for 1 min, and after 30 cycles, lengthened at 72°C for 5 min, The untransfected HepG2 cell line was used as a negative control.

Western blot and immunocytochemistry

The MT1F polyclonal antibody prepared by us: The cDNA of MT1F was amplified into prokaryatic expression vector and transformed into BL21 for expression. By Niaffinity Chromatography, the product was identified to be a single component by SDS-PAGE. The rabbits were immunized with purified product. Western blot analyzing showed high titer rabbit anti-human MT1F polyclonal antibody [3].

Cells (3×10^5) were harvested and dissolved by the addition of sodium dodecyl sulfate (SDS)-containing lysis buffer. The lysate was used for SDS-polyacrylamide gel electophoresis on a 7.5 percent gel. The proteins were electrophoretically transferred from the gel to NC membrane. The transferred membrane was treated with polycolonal antibody against MT1F protein (1:200). Followed by detection with peroxidase-labeled goat anti-rabbit (1:1000, Dako, United States). The resulting complexes were detected with the ECL reagent (Amersham Company, United Kingdom) according to the manufacturer's instructions. The MT1F protein was detected

by immunocytochemistry. The untransfected HepG2 cell line was used as a negative control.

Detection of biological features in transfected cells

Growth curve assays Cells (1×10^5) were seeded in each well of a 24-well plate and were allowed to grow for varying periods, The trypsinized cells were counted by cell counter plate, The growth assays were made by counting in triplicate on every other day of culture for up to 7 days followed by constructing the cell growth curves.

Soft agar culture: The anchorage independent growth was examined by seeding 1000 cells in 0.3 percent agar medium into 60 mm plates previously lined with 0.5 percent agar medium, The plates (in triplicate and repeated twice) were cultured at 37°C, 5 percent CO_2 incubator for 14 days. The average number of colony formation (any colony containing more than 50 cells was counted as a colony) and the colony formation rate (the number of colonies/the number of seeded cells) were calculated.

Tumorigenicity in SCID mice Two groups were divided randomly. The experimental group that consisted of six mice was used for hepG2 cells with transfected MT1F injection. The control group of five mice was used for hepG2 cells without transfected MT1F injection. Trypsinized cells (5×10^6) were injected into the subcutaneous sites on the shoulders of SCID mice. Animals were inspected at regular intervals for the appearance of visible tumors to measure the time of first appearance. Thirty days later, the mice were sacrificed and the tumors were carefully removed by blunt dissection. The tumors were weighed and their average growth rates were measures as mg/day.

MT1F in situ *hybridization*

The expression of MT1F was detected by digoxigenin-labeled gene probe. The human breast tissue and the mouse brain tissue were used as the positive control. Either the sections detected with incubation solution instead of the probe or the sections detected with ribonucleases (Rnase) (10 mg/ml) before MT1F detection was designed for the negative control. The positive expression showed the brown staining signal in the cytoplasm.

Immunohistochemistry: MT1F was analyzed by using MT1F rabbit polyclonal antiserum with the SABC method following treatment with 3,3'-diaminobenzidine (DAB) staining. The human breast tissue and the mouse brain tissue were used as the positive control. Negative control was designed using phosphate-buffered saline (PBS) instead of MT1F rabbit polyclonal antiserum in detection. The positive expression showed the brown staining signal in nuclei.

STATISTICAL ANALYSIS

The data were statistically analyzed using Student's *t* test and the difference of results was analyzed by U test and T test.

RESULTS

Identification of plasmid

The eukaryotic expression vector of PCMV-MT1F plasmid was tested by PCR amplification. purification and restriction endonuclease and confirmed to be consistent with the plasmid map. With single digestion of EcoRI, a 6.1 kb band was obtained which represents the whole length of the plasmid DNA. With double digestion of EcoRI combining with Xho I, both 5.5 kb and 0.673 kb bands were obtained which represented the fragment of the vector and MT1F gene fragment respectively.

Gene transfection

HepG2 was transfected with pCMV-MT1F plasmid by lipofectamine transfection method. Four weeks after G418 selec-

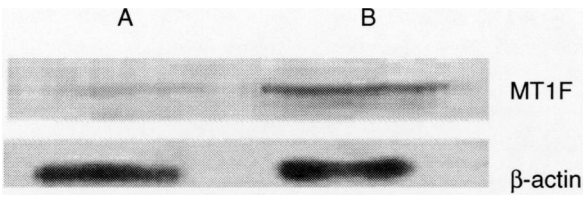

Figure 1. Western blot analysis of MT1F, (A) HepG2 was transfected with pCMV plasmid by lipofectamine transfection; (B) HepG2 was transfected with pCMV-MT1F plasmid by lipofectamine transfection

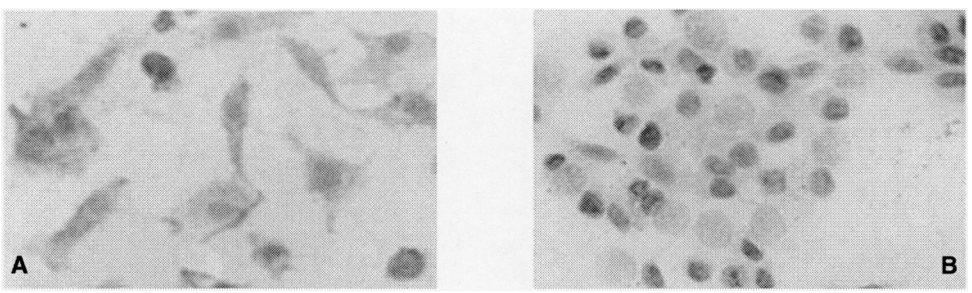

Figure 2. The transfected HepG2 was positively brown stained in the cytoplasmic or nucleus (A) compared to the negative staining for control (B).

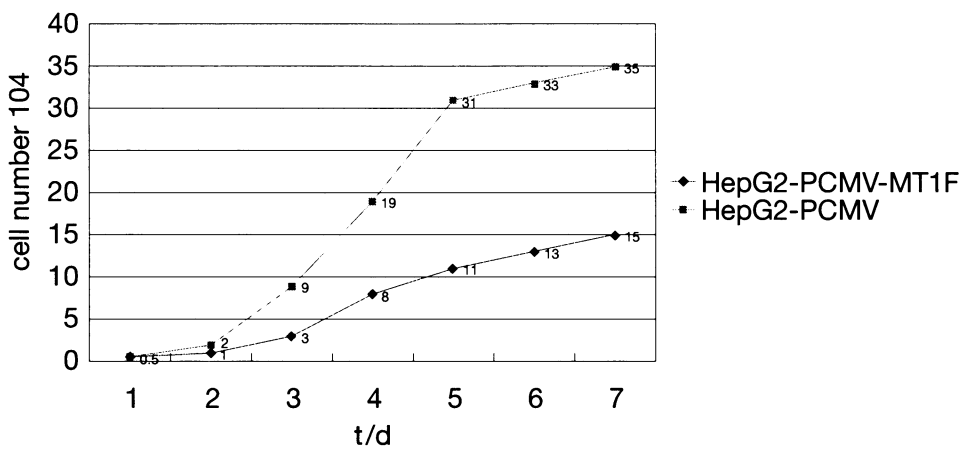

Figure 3. Hep G2 cell growth curves.

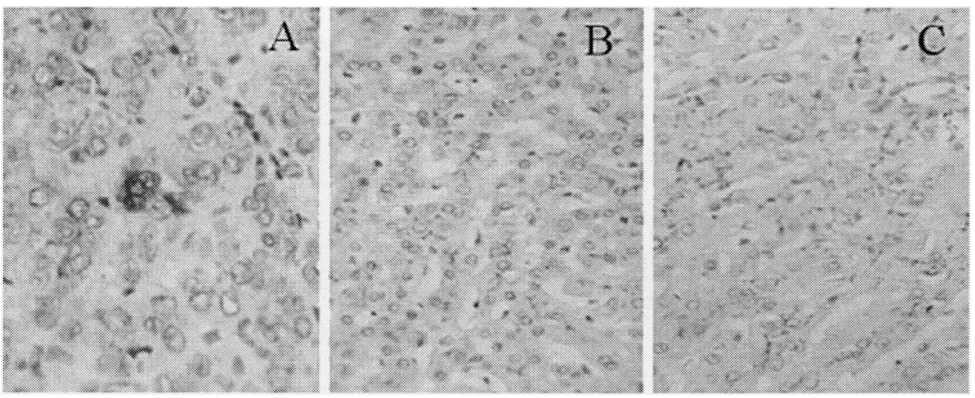

Figure 4. Immunohistochemical staining for MT1F; (A) For HCC, cytoplasmic and nuclear staining was dominant whereas membrane staining was rare, showing brown DAB × 200; (B) Para-cancerous liver tissue showed membrane or cytoplasmic staining like normal liver tissue, showing *brown* DAB × 200; (C) In normal liver tissue, the staining was mainly positive on the cellular membrane or cytoplasmic staining, showing brown DAB × 200.

tion culture, the resistant colonies were formed. PCR Indicated that 327 bp of neo-gene was shown in transfected HepG2 compared to the negative control, demonstrating that PCMV-MT1F plasmid had been introduced into HepG2 cell and integrated into the genomic DNA.

Detection of MT1F protein in transfected HepG2 cell

Western blot: MT1F protein was shown for transfected HepG2 compared to the negative band of the control HepG2 (Figure 1).

Immunocytochemistry: The transfected HepG2 was positively brown-stained in the cytoplasmic or nucleus compared to the negative staining for control. The results demonstrated that exogenous MT1F gene introduced into HepG2 expressed high level of MT1F protein (Figure 2).

Growth feature of transfected HepG2

Growth curves: The transfected HepG2 cell grew much more slowly in DMEM medium than the control HepG2 cell (Figure 3).

Soft agar growth assay: Small and few colonies were formed slowly in transfected HepG2 compared to large and numerous colonies formed quickly in control HepG2. The colony formation rates were 3.8 percent and 7.4 percent in transfected HepG2 and in control HepG2 respectively (Table 1) which demonstrated that the anchorage independent growth ability of HepG2 expressing MT1F was reduced.

Tumorigenicity in SCID mice: The tumorigenicity test showed that the tumors started to appear on the 25th day after injection in the six SCID mice injected with transfected HepG2 on the 13th day in four SCID mice injected with control HepG2. The tumors of HepG2 expressing MT1F grew slowly with an average growth rate of 30.9 mg/day, and the tumors of control HepG2 grew fast with an average growth rate of 70.3 mg/day (Table 2). The results demonstrated that the tumorigenicity in SCID mice of MT1F transfected HepG2 was inhibited.

Table 1. Soft agar assays in HepG2 cell line

Cell line	No.of Seeded cell	No. of colonies				CFR
		1	2	3	Mean	
HepG2	1000	70	74	78	74±2.4	74 percent
MT1F-HepG2	1000	36	40	38	38±1.2	38 percent[b]

[b]P < .01

Table 2. Tumorigenicity assays in hepG2 cell line.

Cell line	Tumorigenicity Rate	Growth rate of tumors (mg/day)						
		1	2	3	4	5	6	Mean
HepG2	4/4	80.3	75.0	56.3	60.7	—	—	70.3±5.6
MT1F-HepG2	6/6	21.0	10.3	25.4	56.7	40.0	31.8	30.9±6.9[a]

[a]P < .05

Table 3. Expression of MT1F mRNA and proteins in HCC and its para-cancerous tissue, normal tissue n (percent).

Group	n	ISH	IHC
Normal tissue	20	14 (70.0)	12 (60)
Para-cancerous tissue	60	45 (75.0)[a]	40 (66.7)[a]
Liver cancer	60	10 (16.7)[b]	6 (10.0)[b]

[a]p > .05 Normal tissue vs. para-cancerous tissue.
[b]p < .01HCC vs. para-cancerous tissue or normal tissue.

MT1F expression among HCC tissue and its paracancerous tissue, normal tissue

The expression of MT1F mRNA and protein in HCC was lower than that of normal tissue or its paracancerous tissue ($p < .01$, HCC vs. normal or paracancerous tissue; $p > .05$, normal vs. paracancerous tissue) (Figure 4, Table 3).

DISCUSSION

As we know, the oncogenes and tumor suppressor genes are involved in the pathogenesis and development hepatocellular carcinoma [3-5]. Our studies indicate that MT1F gene as a tumor related-gene is correlated with the tumor development. It was reported that MT1F proteins were decreased and even disappeared in several

kinds of cancer tissues; furthermore, the MT1F mRNA expression was consistent with the expression level of MT1F protein that had been verified in the down-regulation of MT1F occurred at the transcription level in cancer cells. Southern blot analysis indicated no deletion, no rearrangement or mutation of MT1F on DNA level. The exogenous introduction of MT1F could inhibit the growth of tumor cells accompanied in a dose-dependent manner, for example, the ability of anchorage independent growth and tumorigenicity in SCID mice of human HT1080 fibrosarcoma without original MT1F was significantly inhibited after the exogenous introduction of MT1F [6-8]. If the antisense MT1F was introduced to the cells, it would inhibit the endogenous MT1F expression and promote the malignant transformation of the cells. In the present study, eukaryotic expression vector of PCMV-MT1F plasmid was introduced into HepG2 cell line that expressed no MT1F protein originally with lipofectamine transfection method. The introduction and expression of pCMV-MT1F plasmid into HepG2 cell line was confirmed by G418 selection culture by which colony formation persisted in next generations and by PCR amplification of neogene contained in the vector. Furthermore, the strong expression of MT1F protein in transfected HepG2 was detected by Western blot and immunocytochemistry, which verified the success of gene transfection. Growth inhibition of the transfected cells shown by the growth curves, the colony formation rates in the soft agar and tumorigenicity in SCID mice demonstrated that exogenous MT1F gene inhibited the growth of HepG2.

Recent studies suggest that the mechanism of suppression of tumor growth by MT1F is the MT1F protein has a zinc-finger domain which regulates the transcription of many downstream genes by binding to the GC rich element in the promoter region and modulates genes transcription and various biological effect [5]. MT1F can compete with transcription activator SP1 in binding to an overlapping consensus binding motif in the promoter region of GCE which commonly exists in oncogene and tumor suppressor gene and abolishes the function of SP1, leading to the transcription inhibition of downstream genes and growth inhibition of tumor cells [6-8]. In addition the exogenous MT1F may inhibit the growth by binding to the GEE of TGF-β1 and activating the transcription of TGF-β1and subsequently activating P21 gene or by down-regulating Bcl-2 gene to influence the tumor cells [7, 9, 10]. Some studies indicated that the activation of some oncogenes, e.g., wild type p53 gene [8], cell apoptosis [9], the TNF-α [11] and the concentration of calcium may be involved in the mechanism of growth inhibition property of MT1F. On the whole, various and complicated mechanisms may be involved in the suppressive property of MT1F to tumor growth [12-14].

MT1F is one of the metallothionein family which regulates the cellular growth and differentiation by activating Cyclin D1 to promote the cells from the G0/G1phase into the G2/M phase [12]. The mechanism of down-expression of MT1F in cancer tissues is not clear up to now. It was hypothesized that the high concentration of MT1F proteins produced by overstimulation of MT1F interacted with the promoter region of EBS and subsequently the gene transcription was inhibited which was concordant with the present results that showed strong expression of MT1F in normal tissue adjacent to hepatocellular carcinoma and weekly expression in cancer cells.

Since only down-regulation expression without gene mutation occurred in MT1F DNA level, it was different from some tumor suppressor genes such as p53 [13], p16 [14], and Rb [15] with mutation gene on DNA level. It is more convenient to introduce normal exogenous gene into the tumor cells than to repair mutation gene in gene therapy [11]. The present study substantiates that exogenous MT1F as a target gene has a potential application in gene therapy of hepatocellular carcinoma.

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