

The effect and mechanism of metallothionein MT1M on hepatocellular carcinoma cell

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Abstract. – OBJECTIVE: Liver cancer is one of the most common digestive system malignant solid tumors. Its incidence and mortality rates keep high, causing serious mental and economic burden. So far, the exact mechanism of liver cancer onset has not been fully elucidated. Metallothionein (MT) widely exists in various types of organisms with highly conserved structure. It contains the short peptide of cysteine and sulfur protein with high affinity to heavy metals, including cadmium, zinc, and copper. MT1M is an important member of MT family that has been verified to participate in regulating hepatocellular carcinoma, thyroid cancer, cervical cancer, and other tumors. However, MT1M expression and mechanism in hepatoma cells have not been fully elucidated.

MATERIALS AND METHODS: Hepatoma cell line HepG2 was divided into control and MT1M group. MT1M plasmid was constructed and transfected to MT1M group. Real-time PCR was used to test MT1M expression. MTT assay was applied to detect HepG2 proliferation. Flow cytometry was performed to determine HepG2 apoptosis. Caspase-3 activity was measured. Western blot was used to detect Bcl-2 and Bax protein levels.

RESULTS: MT1M expression significantly increased after MT1M plasmid transfection compared with control ($p < 0.05$). MT1M group showed inhibited HepG2 proliferation, declined HepG2 apoptosis, enhanced Caspase-3 activity, reduced Bcl-2 protein level, and upregulated Bax protein compared with control ($p < 0.05$).

CONCLUSIONS: MT1M can suppress HepG2 proliferation and induce HepG2 apoptosis through downregulating Bcl-2, upregulating Bax, and enhancing Caspase-3 activity.

Key Words:

Liver cancer, MT1M, Apoptosis, Proliferation, Bcl-2, Bax.

Introduction

Liver cancer can be divided into two types as primary and secondary. Primary liver cancer is one of the most common digestive system malignant solid tumors that originate from liver epithelial or mesenchymal tissues. It accounts for the fifth incidence and second mortality rate worldwide^{1,2}. There are numerous risk factors for primary liver cancer, including genetic, physical, chemical, environmental, and lifestyle factors. Primary liver cancer development is a complex and multi-step process^{3,4}. Hepatitis B virus (HBV), alcohol, aflatoxin, sex hormone, hepatitis C virus (HCV), liver cirrhosis, water pollution, nitrosamines substances, and trace elements are all related to liver cancer pathogenesis, of which HBV infection is closely associated with its oncogenesis^{5,6}. As our country has high hepatitis B incidence, liver cancer incidence is high in our country⁷. Since the pathogenesis and specific molecular mechanism of primary liver cancer has not been fully clarified, it is a hot spot of research on liver cancer occurrence, development, diagnosis, and treatment^{8,9}. Clarifying the molecular pathogenesis of primary liver cancer is in favor of its prevention and treatment.

Metallothionein (MT) is a type of low molecular weight metal-binding protein widely exists in organisms. Its chemical structure is rich in cysteine, but not histidine and aromatic amino acids^{10,11}. Multiple stress factors, cytotoxic drugs, cytokines, metal, and drugs, can induce MT generation¹². Recent research showed that the biological function of MT was not only confined to heavy metal detoxification, but also included microelement storage, transport, and metabolism. It can

regulate microelement concentration in the body, and participate in hormone and cell metabolism regulation, ionizing radiation antagonism, γ -aminobutyric acid neuron activity regulation, free radicals scavenging, UV induced reaction regulation, strengthen stress ability, and cell growth, differentiation, and proliferation regulation^{13,14}. Studies have shown that MT was also involved in tumor occurrence and development process. MT1M is an important member of MT family that has been confirmed to be involved in regulating liver cancer¹⁵. However, MT1M expression and mechanism in liver cancer cell are still unclear.

Materials and Methods

Main Instruments and Reagents

HepG2 cell line was purchased from ATCC Cell Bank (Manassas, VA, USA). High glucose Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained by HyClone (South Logan, UT, USA). Eukaryotic expression plasmid pCMV was bought from Clontech (Mountain View, CA, USA). Dimethyl sulfoxide and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) powder were purchased from Gibco (Rockville, MD, USA). Enzyme-ethylene diamine tetraacetic acid (EDTA) was from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was from Pall Life Sciences (Cortland, NY, USA). EDTA was purchased from HyClone (South Logan, UT, USA). Western blot related chemical reagents were from Beyotime (Suzhou, China). ECL reagent was from Amersham Biosciences (Little Chalfont, UK). Rabbit anti-human Bcl-2, Bax monoclonal antibodies, and mouse anti-rabbit horseradish peroxidase (HRP) tagged IgG secondary antibody were from Cell Signaling Technology (Danvers, MA, USA). Polymerase chain reaction (PCR) amplification kit and PCR product purification kit were from Promega (Madison, WI, USA). Plasmid extraction kit and restriction enzymes were from Roche (Basel, Switzerland). RNA extraction kit and reverse transcription kit were from Axygen (Tewksbury, MA, USA). Annexin V-FITC apoptosis detection kit was got from BD (San Jose, CA, USA). Other common reagents were purchased from Sangon (Shanghai, China). FACSCalibur™ flow cytometry instrument was from BD (San Jose, CA). ABI 7700 Fast fluorescence quantitative PCR reaction apparatus was from ABI (Thermo Fisher Scien-

tific, Waltham, MA, USA). LabSystem Version 1.3.1 microplate reader was from Bio-Rad (Hercules, CA, USA). Bechtol was got from Suzhou Suta purification equipment engineering Co., Ltd., (Jiangsu Sheng, China).

Methods

HepG2 Cell Cultivation and Grouping

HepG2 cell line stored in liquid nitrogen was resuscitated in 37°C water bath and 1000 rpm centrifugation for 3 min. Next, the cells were resuspended in 2 ml fresh medium containing 10% fetal bovine serum (FBS), 90% high glucose DMEM, 100 U/ml penicillin, and 100 μ g/ml streptomycin and cultured in 37°C and 5% CO₂ for 24-48 h. The cells in 3-8th generation and logarithmic phase were randomly divided into two groups, including control and MT1M group that were transfected by MT1M plasmid.

MT1M Plasmid Construction and Transfection

MT1M sequence was designed by Primer 6.0 and synthesized by Invitrogen (Carlsbad, CA, USA). pCMV carrier was inserted. The primers sequences were as follows, forward, 5'-ATTGAATTCGGATGGACCCCAACTGCTC-3', reverse, 5'-ATTCTCGAGTCAGGCACAGCA-GCTG-3'. Human liver cDNA library was selected as template, together with pCMV-MT1M containing site to construct cDNA. PCR product was further purified and digested to connect to pCMV plasmid. After double digestion, reconstructed pCMV-MT1M plasmid was confirmed by sequencing.

Real-time PCR

Total RNA was extracted by TRIzol and reverse transcribed to cDNA. The primers used were designed by Primer 6.0 and synthesized by Invitrogen (Table I). Real-time PCR was applied to test target gene expression. Reaction condition: 55°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was used as internal reference. 2^{- Δ Ct} was applied to calculate relative expression level.

MTT Assay

HepG2 cells in logarithmic phase were digested and seeded into 96-well plate at 3000/well. The cells were divided into different groups and cultured for 72 h with five replicates. Next, the

Table 1. Primer sequence.

Gene	5'-3'	5'-3'
GADPH	ACCAGGTATCTGCTGGTTG	TAACCATGATGTCAGCGTGGT
MT1M	ATTGAATTCGGATGGACCCCACTGCTC	ATTCTCGAGTCAGGCACAGCAGCTG

plate was treated by 20 μ L 5 g/L MTT solution and incubated for 4 h. After removing the supernatant, the plate was added with 150 μ L dimethyl sulfoxide (DMSO) for 10 min and read at 570 nm to calculate proliferation rate.

Flow Cytometry

HepG2 cells were digested and seeded in 50 mL culture flask at 5×10^5 /mL. After cultured for 48 h, the cells were collected and washed by phosphate-buffered saline (PBS) at 1000 rpm for 5 min. Next, the cells were fixed in pre-cooling 75% ethanol at 4°C overnight and washed by PBS again. Next, cells were added with 800 μ L phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 100 μ g/mL propidium iodide (PI) (3.8% sodium citrate, pH 7.0), and 100 RnaseA (10 mg/mL) at 37°C for 30 min. At last, the sample was tested by flow cytometry and analyzed by FCS Express 3.0 software (Glendale, CA, USA).

Caspase 3 Activity Detection

Caspase 3 activity was detected by kit according to the manual. The cells were digested by enzyme and centrifuged at 600 g and 4°C for 5 min. Then, the cells were cracked on ice for 15 min and centrifuged at 20000 g and 4°C for 5 min. After added with 2 mM Ac-DEVD-pNA, the sample was read at 405 nm.

Western Blot

The cells were cracked on ice for 15-30 min and ultrasonicated for 4×5 s to extract protein. After centrifuged at 10,000 g and 4°C for 15 min, the protein was moved to a new Eppendorf (EP) tube and stored at -20°C. The protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After blocked by 5% skim milk for 2 h, the membrane was incubated in Bcl-2 primary antibody at 1:1000 and Bax primary antibody at 1:500 overnight. The membrane was incubated with secondary antibody at 1:2000 for 30 min and washed by phosphate-buffered saline and

tween (PBST). At last, the membrane was treated with chemiluminescent agent for 1 min and imaged on X-ray. Protein image processing system and Quantity one software were used for data analysis. All experiments were repeated for four times.

Statistical Analysis

All the statistical analyses were performed on SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were presented as $\bar{x} \pm s$. One-way ANOVA was used for mean comparison. LSD was performed for post-hoc test. $p < 0.05$ was considered as statistically significance.

Results

MT1M Expression in Hepatocellular Carcinoma Cells

Real-time PCR was applied to test MT1M mRNA expression in hepatocellular carcinoma cells. The results showed that MT1M mRNA expressed low in control group. In contrast, MT1M plasmid transfection significantly elevated MT1M mRNA expression ($p < 0.05$) (Figure 1).

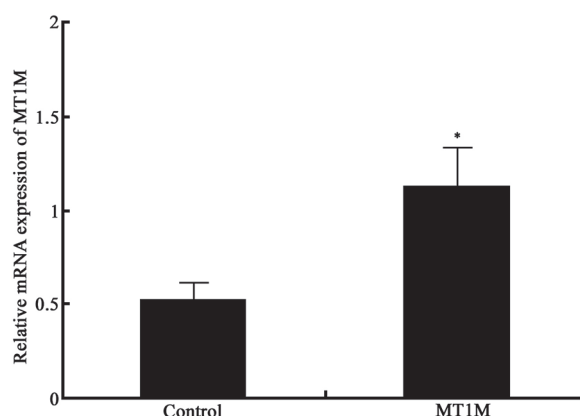


Figure 1. MT1M expression in HepG2. The expression of MT1M mRNA was significantly increased after MT1M plasmids was transfected. * $p < 0.05$, compared with control.

MT1M Impact on HepG1 Proliferation

MTT assay was used to detect HepG2 proliferation after MT1M transfection for 48 h. The result showed that MT1M significantly inhibited HepG2 proliferation compared with control ($p < 0.05$) (Figure 2), suggesting that MT1M had inhibitory effect on HepG2 proliferation.

MT1M Effect on HepG2 Apoptosis

To evaluate the apoptosis of HepG2, flow cytometry was performed. We found that MT1M markedly induce HepG2 apoptosis compared with control ($p < 0.05$) (Figure 3 and 4).

MT1M Impact on Caspase 3 Activity in HepG2

Caspase 3 detection kit was also used to validate the apoptosis of HepG2. The result demonstrated that the growing expression of MT1M can elevate Caspase 3 activity in HepG2 cells ($p < 0.05$) (Figure 5), revealing that MT1M can promote HepG2 apoptosis through enhancing Caspase 3 activity.

MT1M impact on Bcl-2 and Bax Protein Levels in HepG2

We also detected Bcl-2 and Bax protein levels in HepG2 after MT1M transfection by using Western blot. The results exhibited that in MT1M transfection group, the level of Bcl-2 protein declined, but level of Bax protein was up regulated, leading to the decreasing ratio of Bcl-2 and Bax ($p < 0.05$) (Figure 6 and 7).

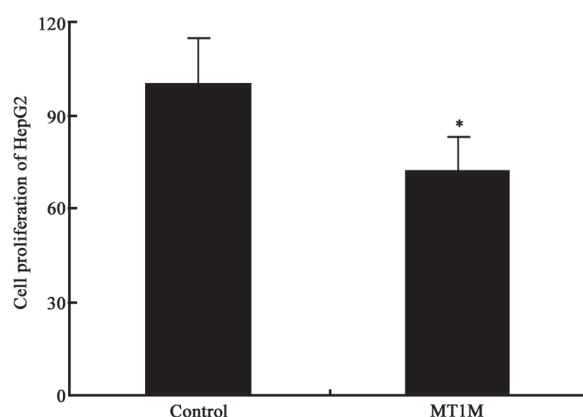


Figure 2. The effect of MT1M on HepG1 proliferation. The proliferation of HepG1 was inhibited as expression of MT1M was up regulated. * $p < 0.05$, compared with control.

Discussion

At present, though there are numerous treatment methods for liver cancer, including surgery, radiotherapy, chemotherapy, immunotherapy, and interventional therapy, the actual clinical effect is poor. Liver cancer is easy to appear metastasis, resulting in high-recurrence rate, poor prognosis, low survival, worse quality of life, and heavy burden. Searching for an effective molecular target for liver cancer treatment is of great significance for liver cancer prevention and treatment⁸.

MT, existing in almost all organisms, is highly conservative in evolution process, mainly including MT-1, MT-2, MT-3, and MT-4. MT-1

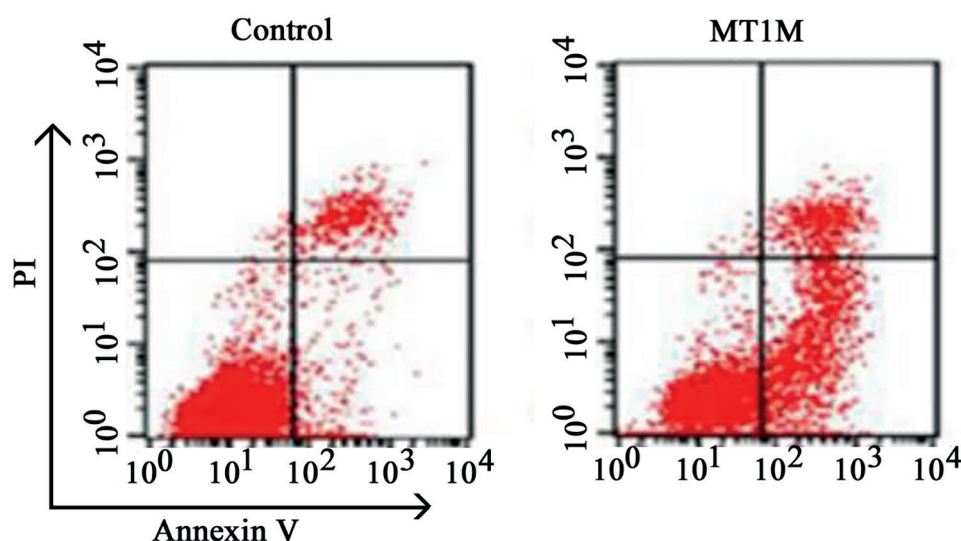


Figure 3. The effect of MT1M on HepG2 apoptosis. MT1M induced the apoptosis of HepG2.

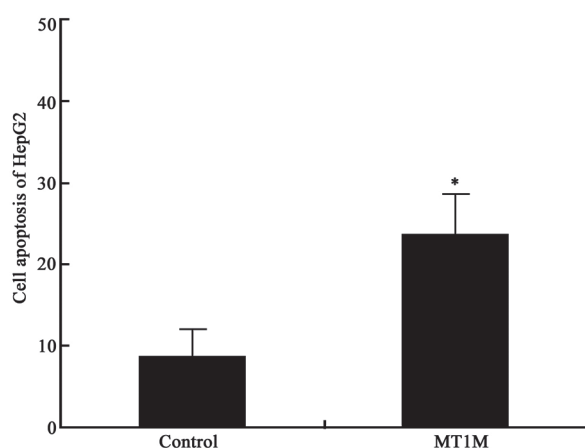


Figure 4. The effect of MT1M on HepG2 apoptosis. MT1M promoted the apoptosis of HepG2. * $p < 0.05$, compared with control.

and MT-2 mainly distribute in liver and kidney, MT-3 exists in central nervous system containing astrocytes and neurons, and MT-4 expresses in the stratified squamous epithelium of skin, tongue, and digestive tract^{16,17}. Clinical studies confirmed that in addition to heavy metal detoxification, superoxide anion free radical, hydroxyl radical, and DPPH free radicals clearance, MT also plays an important regulating role in tumor cells. It may strengthen the body resistance ability through regulating heavy metal or adapting to poor environment. MT was found downregulated in thyroid tumor cells, and breast cancer cells, thus promoting tumor cells growth and proliferation¹⁸. Therefore, MT is expected to become a new target for tumor treatment, and its

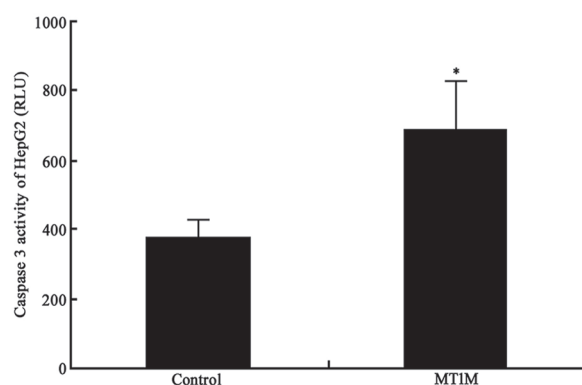


Figure 5. The impact of MT1M on Caspase 3 activity of HepG2. The Caspase 3 activity of HepG2 was induced as the expression of MT1M increased. * $p < 0.05$, compared with control.

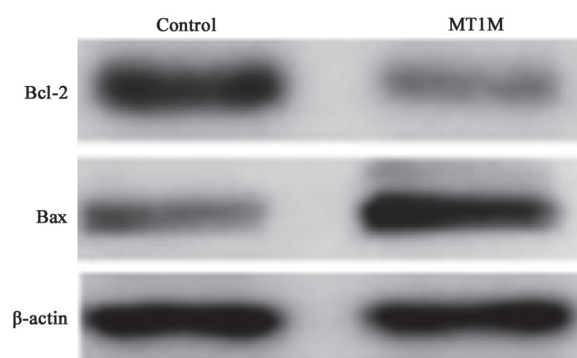


Figure 6. MT1M impact on Bcl-2 and Bax protein levels in HepG2. The level of Bcl-2 protein decreased while the level of Bax protein increased in MT1M group, compared that in control.

related mechanism is also paid attention. Recent study showed that MT1M gene in MT-1 was correlated with liver cancer tumorigenesis¹⁵, though the specific mechanism had not been clarified. Therefore, this study analyzed MT1M gene impact on hepatocellular carcinoma cell proliferation and apoptosis, and related mechanism through constructing MT1M gene eukaryotic expression plasmid and HepG2 transfection. The results confirmed that MT1M transfection can inhibit HepG2 proliferation and promote apoptosis, revealing that MT1M can regulate liver cancer occurrence and development. Apoptosis plays a key role in liver cancer occurrence and development regulation¹⁹. Cell apoptosis is the regulation to homeostasis that can repress onco-

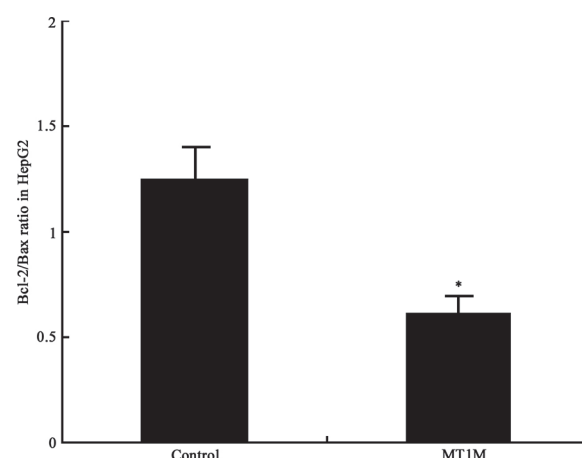


Figure 7. The impact of MT1M on the ratio of Bcl-2 and Bax in HepG2. The ratio of Bcl-2 and Bax in HepG2 transfected with MT1M declined compared to that in control. * $p < 0.05$, compared with control.

genesis and tumor excessive growth. Bcl-2 overexpression and Bax downregulation are closely associated with hepatocellular carcinoma cells anti-apoptosis and apoptosis imbalance²⁰. Bcl-2 overexpression causes damaged cells resistant to apoptosis, induces proliferation, and promotes tumor progression²¹. Bax overexpression can antagonize or inhibit Bcl-2 protein expression, activate apoptosis signal, and promote cell apoptosis. The balance of Bax and Bcl-2 determines apoptosis and anti-apoptosis competition. Its imbalance leads to abnormal cells behavior²². Caspase 3 mainly exists in the cytoplasm in the form of inactive precursor, while it can be activated apoptosis family as the strongest apoptosis regulation factor when the cells start apoptosis process²³. This study proved that MT1M can inhibit Bcl-2 expression, promote Bax expression, increase Caspase 3 activity, and regulate the balance of apoptosis and anti-apoptosis.

Conclusions

MT1M can suppress HepG2 proliferation and induce HepG2 apoptosis through downregulating Bcl-2, upregulating Bax, and enhancing Caspase-3 activity. This study provides new target and theoretical basis for clinical liver cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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