ORIGINAL RESEARCH

Research on Mfn2 Gene Expression in Hepatocellular Carcinoma and its Antitumor Mechanism

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ABSTRACT

Objective • To detect the expression level of the Mfn2 gene in hepatocellular carcinoma (HCC) and adjacent normal liver tissues and further analyze its anticancer effects.

Methods • The expression levels of Mfn2, GLS1 and the autophagy-related proteins lc3b and Beclin1 in liver cancer and adjacent tissues in patients with liver cancer were detected by real-time–quantitative polymerase chain reaction (RT–qPCR). The HepG2 human HCC cell line was cultured *in vitro*, and the Mfn2 protein was stably expressed through transfection of a high Mfn2 expression plasmid. The Cell-Counting Kit-8 (CCK-8) method was used to observe the effect of Mfn2 overexpression on the activity of HepG2 cells. Furthermore, RT–qPCR and Western blotting were performed to detect the effects of Mfn2 overexpression on the protein expression of GLS1, Beclin1 and lc3b.

Results • Compared with tissues adjacent to cancer tissues, the mRNA levels of Mfn2, GLS1, Beclin1 and lc3b in liver cancer tissues were lower. Compared with normal hepatocytes, the expression of Mfn2, Beclin1 and lc3b in HCC cells was decreased, but the expression of GLS1 was increased. Compared with the control group (NC) transfected with empty plasmid, Mfn2 overexpression led to significant time-dependent inhibition of HepG2 cell activity and GLS1 protein expression ($P < .05$). In addition, Mfn2 overexpression induced autophagy by triggering the expression of autophagy-related proteins Beclin-1 and lc3b in HCC cells (all $P < .05$). The effect of transfection with a high-dose Mfn2 plasmid was more obvious than that of transfection with a low-dose Mfn2 plasmid (all $P < .05$).

Conclusions • The expression of Mfn2, GLS1, Beclin1 and lc3b in HCC was lower than in normal liver tissue. The expression of Mfn2, Beclin1 and lc3b in HCC cells was decreased, but the expression of GLS1 was increased. Overexpression of Mfn2 inhibited GLS1 gene expression by inhibiting the activity of HCC cells and promoted the expression of Beclin1 and lc3b to induce autophagy, thereby exerting an anticancer effect. Further research is needed to clarify the mechanism of Mfn2 activity. (*Altern Ther Health Med.* 2022;28(6):132-137)

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INTRODUCTION

As a global health problem, liver cancer is the fourth leading cause of cancer-related death.[1] Hepatocellular carcinoma (HCC) is the most common primary liver cancer (accounting for approximately 75% of liver cancers) and the most common cause of death in patients with chronic liver disease. It was estimated that approximately 841 080 new cases and 781 631 cancer-related deaths occur every year worldwide. In the majority of patients, HCC is secondary to liver cirrhosis. Moreover, any cause of liver disease, including alcohol consumption, chronic viral hepatitis and nonalcoholic steatohepatitis, might increase the risk for HCC.[3] Because liver cancer is typically diagnosed in the later stages, the effect of clinical treatment is limited.[4] Therefore, it is necessary to better understand the mechanism of the development and progression of liver

cancer to find targets for the diagnosis and treatment of HCC and to improve the prognosis.

Autophagy is a catabolic response of cells to external pressure that helps maintain cell homeostasis.[5] Clinically, the occurrence and development of many kinds of malignant tumors, such as gastric cancer and lung cancer, are related to the level of autophagy, but the specific mechanism of liver cancer is not clear.[6] Mfn2 belongs to the mitochondrial fusion protein (Mfn) family and is an indispensable protein in the process of mitochondrial outer membrane fusion.[7] Mfn2 plays an important role in many cellular processes, such as cell proliferation, apoptosis, autophagy and endoplasmic reticulum (ER) stress.[8] In HCC, Wang, et al found that Mfn-2 triggered ER and mitochondrial Ca2+ influx and induced hepatoma cell apoptosis.[9] De Sousa, et al found that Mfn2 was related to the activity of HepG2 cells.[10] Specifically, cellular activity decreased as the content of Mfn2 was decreased. Glutaminase (GLS) in mitochondria plays an important role as a key enzyme in glutamine metabolism.[11] The percentage of liver cancer tissues with renal-type GLS1 expression was as high as 90%,[12] but the specific role of GLS1 in the occurrence and development of liver cancer has not been clarified, and related research on GLS1 expression in all stages of liver cancer has not been reported.

Therefore, we speculate that Mfn2 may regulate autophagy by affecting cell energy metabolism and thus plays a role in the occurrence and development of liver cancer. Because GLS1 is a key enzyme in tumor energy metabolism and is highly expressed in tumor tissues, in this study, GLS1 levels were measured to determine the level of tumor energy metabolism. Therefore, this study aimed to detect the expression levels of Mfn2, GLS1 and autophagy-related factors in HCC and adjacent tissues and analyze the mechanisms of their anticancer effects.

MATERIALS AND METHODS

Patients

Clinical data and liver pathological tissues of patients with HCC who underwent surgical resection and pathological diagnosis in Dalian Central Hospital in China between October 2019 and May 2021 were collected. Patients receiving other treatments before surgical resection, including radiotherapy, chemotherapy or transcatheter arterial chemoembolization, were excluded from the study. Ultimately, 20 patients were included in the study; 11 men (mean age 60 years) and 9 women (mean age 57 years).

This study was approved by the ethics committee of Dalian Central Hospital, and all patients signed an informed consent form.

Cell Culture

The normal human hepatocyte line 7702 (HL-7702) was obtained from the laboratory of Dalian Central Hospital in China. The HepG2 HCC cell line was purchased from ATCC (Manassas, Virginia, USA).

RPMI-1640 (GIBCO, Grand Island, New York, USA) and DMEM (GIBCO, USA) containing 10% fetal bovine serum (GIBCO, USA) and 1% penicillin–streptomycin (a dual antibiotic; GIBCO, USA), were used for cell culture. Cells were placed in an incubator at a constant temperature with 5% CO_2 and relative humidity. The cells were subcultured for subsequent experiments when they reached 80% to 90% confluency.

Plasmid Transfection

An Mfn2 overexpression plasmid was constructed by Shanghai Jima Pharmaceutical Technology Co., Ltd. Lipo6000™ transfection reagent (Biyuntian Beyotime) was used to transfect an empty plasmid vector (negative control [NC]), a plasmid with Mfn2 (mfn2-l) at a low concentration (0.5 μg/ml) and a plasmid with Mfn2 (mfn2-l) at a high concentration (1 μg/ml) into HepG2 cells.

After 6, 24 and 48 hours of transfection, the cells were placed under an inverted fluorescence microscope to screen the clones positive for green fluorescent protein (GFP) to obtain a stable transfection strain with high Mfn2 expression. In addition, the mRNA level of Mfn2 was detected to verify the effectiveness of Mfn2 plasmid transfection. The cells were used in experiments 48 hours after transfection.

Cell Activity was Determined by the Cell-Counting Kit-8 Method

HepG2 cells stably transfected with NC, mfn2-l and mfn2-h were inoculated into 96-well plates at a density of 10^4 cells/well, and 10 μL of Cell Counting Kit-8 (CCK-8) solution (Dojindo, Japan) was added after 24, 48 and 96 hours. After being placed in the incubator for 2 hours, the absorbance (OD) was measured at 450 nm with a spectrophotometer to calculate the relative activity of the cells.

Gene Expression in Tissue Was Measured by Real-Time Fluorescence Quantitative Polymerase Chain Reaction

The total messenger RNA (mRNA) of tissues and cells was extracted with TRIzol solution, and the content was determined with a spectrophotometer. After the quantified mRNA was reverse transcribed into complementary DNA (cDNA) with a Reverse Transcription Kit (Takara, Japan), SYBR Green (Takara, Japan) fluorescence staining was used, and the samples were placed in a Roche LightCycler 480 for real-time fluorescence quantitative polymerase chain reaction (RT–qPCR) to test the relative expression level of the genes. The following primer sequences were used:

- Mfn2 preprimer (5'- 3'): CCTGTGTGGCACAAGTTGGA, postprimer: GCACAGAGGCTACCAACTCATTTC;
- GLS1 preprimer: GAAGGCACAGACATGGTTGGTA, postprimer: ATTGGGCAGAAACCACCATTAG;
- Beclin1 preprimer: CCAGATGCGTTATGCCCAGAC, postprimer: CATTCCATTCCACGGGAACAC;
- LC3B preprimer: GATGTCCGACTTATTCGAGAGC, postprimer: TTGAGCTGTAAGCGCCTTCTA; and
- β-actin preprimer: TGGCACCCAGCACAATGAA, postprimer: CTAAGTCATAGTCCGCCTAGAAGCA.

Protein Expression Detected by Western Blot

RIPA lysis buffer (Biyuntian, China) was used to extract cell protein. After 30 minutes of lysis on ice, the supernatant was extracted by centrifugation at 12000 g for 20 minutes, and then the protein concentration was measured with a BCA Protein Concentration Determination Kit (Biyuntian, China). An SDS–PAGE gel was poured, and an equivalent amount of 20 μg protein was added to the gel for electrophoresis. An equivalent amount (20 μg) of G protein was added to the gel for electrophoresis and then transferred to a PVDF membrane (Millipore-Sigma, St Louis, Missouri, USA). Blocking solution (Biyuntian, China) was used to treat the membrane at room temperature for 1 hour. After washing the membrane with TBST-Tween 20 3 times, the corresponding primary antibodies against Mfn2 (1:1000, a12771, ABclonal, China), GLS1 (1:1000, a11043, ABclonal, China), Beclin1 (1:1000, a10101, ABclonal, China), lc3b (1:1000, a19665, ABclonal, China) and β-actin (1:10000, AC004, ABclonal, China) were added, and the membranes were incubated at 4°C in a refrigerator shaker overnight. The next day, the membrane was incubated at room temperature with the corresponding HRP-conjugated goat anti-rabbit secondary antibody (1:5000, As014, ABclonal, China) for 1 hour, and then the protein bands were developed with a fluorescence imaging system containing an enhanced chemiluminescence (ECL) solution (Biyuntian, China).

Statistical Analysis

All the measurement data in this study are expressed as mean ± standard deviation. All cell experiments were independently repeated 3 times. IBM® SPSS 20.0 software was used for statistical analysis. Paired t test was performed for clinical sample comparisons, and Student's t test was performed to compare different groups in the cell experiments. Under a single treatment, one-way ANOVA was used for intragroup comparisons. Differences were regarded as statistically significant when $P < .05$.

RESULTS

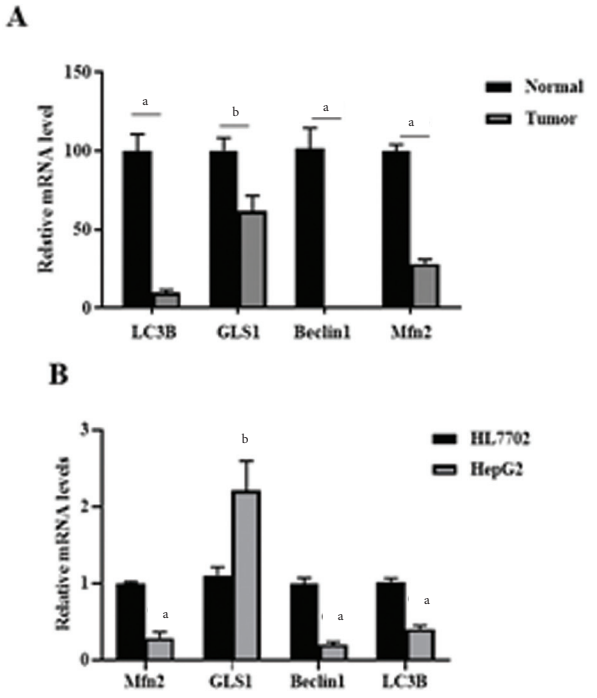
Expression of Mfn2, GLS1, Beclin1 and lc3b in HCC Tissues and Cells

Gene expression in the HepG2 HCC and HL-7702 normal liver cell lines, liver cancer tissue and adjacent normal liver tissue was detected. As shown in Figure 1A, the mRNA levels of the Mfn2, GLS1, Beclin1 and lc3b genes in the liver cancer tissues were significantly lower than in the adjacent normal liver tissues ($P < .05$). In addition, as shown in Figure 1B, the changes in Mfn2, Beclin1 and lc3b mRNA in HCC cells were consistent with those in liver cancer tissues compared with normal hepatocytes ($P < .05$), but the expression of GLS1 was increased in the HCC cells ($P < .05$).

Effectiveness of Plasmid Transfection in HepG2 Cells

GFP-positive cells stably expressing the plasmid vector were observed by fluorescence microscopy 6, 24 and 48 hours after plasmid transfection. As shown in Figure 2, with the

Figure 1. Expression of Mfn2, GLS1, Beclin1 and lc3b in HL-7702 and HepG2 hepatocellular carcinoma (HCC) cells. (A) The expression of Mfn2, GLS1, Beclin1 and lc3b in HCC and adjacent normal liver tissues. (B) Expression of Mfn2, GLS1, Beclin1 and lc3b in the HL-7702 normal human hepatocyte cell line and the HepG HCC cell line.



NOTE: Normal, normal liver tissue adjacent to cancer; Tumor, liver cancer tissue.

[a]$P < .001$, comparison with the normal tissue group or HL-7702 cell group

[b]$P < .01$, comparison with the normal tissue group or HL-7702 cell group

Figure 2. Determination of plasmid expression in HepG2 cells. The expression of GFP was observed by fluorescence microscopy 6, 24 and 48 hours after transfection.

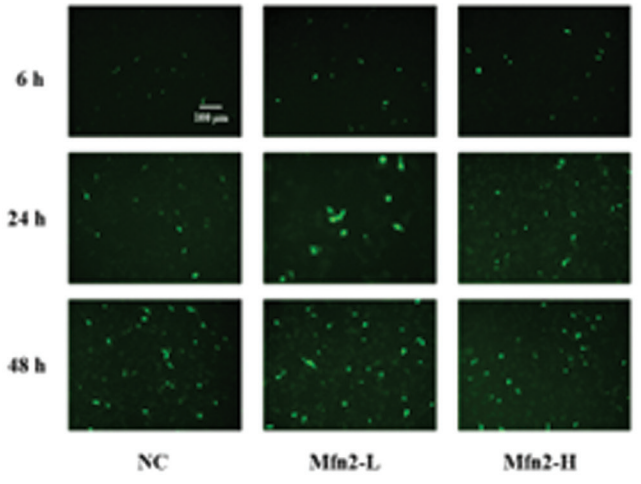
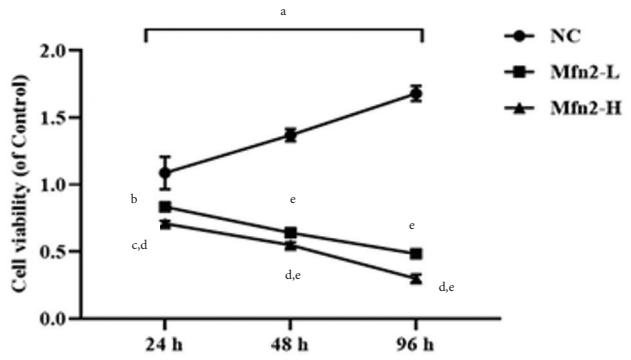
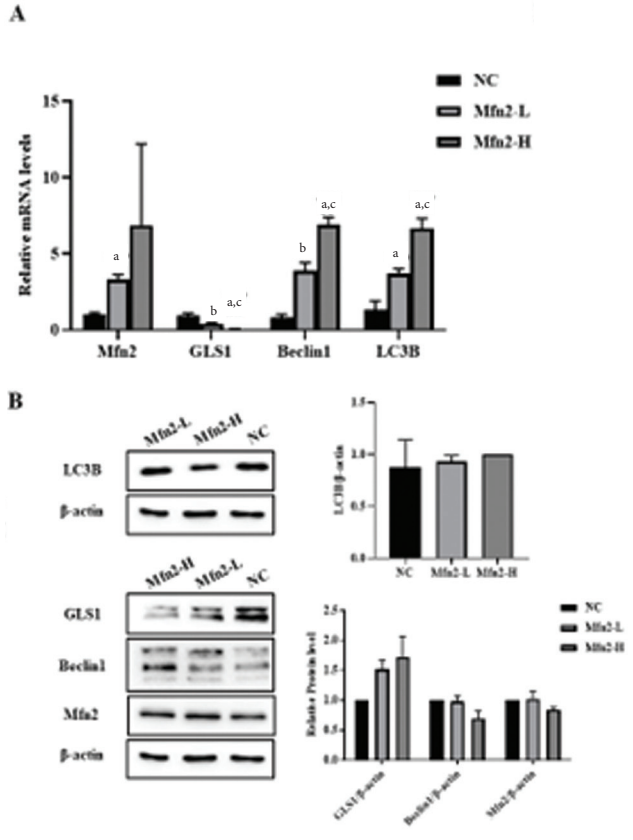


Figure 3. Effect of Mfn2 overexpression on the activity of HepG2 cells.



[a]$P < .001$, within-group comparison
[b]$P < .05$, compared with the NC group
[c]$P < .01$, compared with the NC group
[d]$P < .01$, compared with the Mfn2-l group
[e]$P < .001$ compared with the NC group

Figure 4. Effect of Mfn2 overexpression on the expression of Mfn2, GLS1, Beclin1 and lc3b in HCC cells. (A) The mRNA levels of Mfn2, GLS1, Beclin1 and lc3b genes were detected by RT–qPCR. (B) The expression of Mfn2, GLS1, Beclin1 and lc3b proteins was measured by Western blot.



[a]$P < .001$, compared with the NC group
[b]$P < .01$, compared with the NC group
[c]$P < .01$, compared with the Mfn2-l group

extension of transfection time, the expression of GFP in HepG2 cells gradually increased, and was highest at 48 hours, as indicated by the increase in fluorescence intensity.

Effect of Mfn2 Overexpression on the Activity of HCC Cells

The CCK-8 method was used to determine the effect of overexpressed Mfn2 on cell activity in this study. As shown in Figure 3, after the cells were inoculated on the cell plate, the activities of mfn2-l and mfn2-h in cells decreased significantly ($P < .05$) in a time-dependent manner ($P < .05$) compared with those in the NC group with the extension of culturing time. In addition, the inhibitory effect of transfection with a high concentration of Mfn2 on cell activity was more obvious with prolonged time ($P < .05$). The stably expressing transfected cells were incubated for 48 hours according to the experimental conditions in our subsequent experiments.

Effect of Mfn2 Overexpression on the Expression of Mfn2, GLS1, Beclin1 and lc3b in HCC Cells

As shown in Figure 4A, compared with those in the NC group, the mRNA levels of the Mfn2, Beclin1 and lc3b genes in the mfn2-l and mfn2-h groups increased significantly ($P < .05$), and the upregulated effect induced by mfn2-h was the most obvious ($P < .05$), while the expression changes in GLS1 showed the opposite trend (all $P < .05$). In addition, Figure 4B shows Mfn2, GLS1, Beclin1 and lc3b protein expression in cells. The protein expression changes were consistent with mRNA level changes (all $P < .05$).

DISCUSSION

HCC remains the leading cause of death worldwide. In recent years, determining the important mechanisms of HCC occurrence and development has shown great clinical therapeutic potential.[13,14] However, the basic pathogenesis of HCC has not been fully clarified. In this study, we explored the expression of Mfn2 and other genes in HCC tissues and HCC cells and specifically studied the effect of Mfn2 expression on HCC cell activity-, apoptosis- and autophagy-related genes. We found that the expression of Mfn2 was decreased in HCC tissues and cells, and the anticancer effect of Mfn2 might have been related to its inhibitory effect on cell activity and induction of apoptosis and autophagy.

Autophagy is a self-degradation process that plays a crucial role in clearing misfolded or aggregated proteins, clearing damaged organelles (such as mitochondria, ER and peroxisomes) and eliminating intracellular pathogens.[15] As a multifunctional protein, Mfn2 participates in a variety of biological processes in the human body under normal physiological and pathological conditions, including mitochondrial fusion and transport, cell metabolism, apoptosis and autophagy.[16] It has been reported that knocking out Mfn2 expression in breast cancer and lung cancer enhanced cell migratory and invasive abilities. When melanoma cells with Mfn2 knocked out were injected into nude mice, significant lung metastasis was observed, indicating that Mfn2 inhibited tumor cell migration and

invasion *in vivo*.[17] Previous studies have shown that Mfn2 significantly inhibited the growth and proliferation of vascular smooth muscle cells by inhibiting Ras-Erk MAPK signaling pathway activation.[18] Other studies have investigated the functional roles of Mfn2 in various malignant tumors, such as HCC, bladder cancer and gastric cancer.[19-21] Clinical and epidemiological evidence has suggested that low expression of Mfn2 is associated with a poor prognosis in patients with one of many types of cancer.[22] However, the potential mechanism of Mfn2 antihepatoma effects is unclear. We detected the expression of Mfn2 in the early stage of tumor development in tissues and found that its level was lower than that in adjacent tissues. In addition, according to a 2013 study on the metabolic characteristics of liver cancer cells in untargeted tissue, the expression of GLS1 increased in liver cancer tissues, protecting the cancer cells to a certain extent from oxidative stress and apoptosis.

Considering these results, we measured the expression of Mfn2 in liver cancer tissues and HCC cells and found that it was decreased, similar to the results of previous studies.[19,22] In view of the low expression of Mfn2 in HCC, Mfn2 is expected to be used as a new biomarker for the clinical diagnosis of HCC in the future to identify HCC, which will help with early guided treatment and improve patient prognosis.

Inhibiting cell activity and inducing apoptosis and autophagy play are important in inhibiting the occurrence and development of cancer.[23,24] We found that overexpressed Mfn2 in HCC cells significantly inhibited cell activity and played an anticancer role. In addition, Beclin1- and LC3-related proteins participate in the initial steps of autophagy and are closely related to the occurrence and development of tumors.[25] Recent studies have shown that Beclin1 and LC3 can be used to predict the prognosis of malignant tumors. Beclin1 was related to the clinical prognosis in patients with cancer; specifically, the prognosis of patients with high Beclin1 expression was better than that of patients with low Beclin1 expression.[26] Our study also showed that the expression levels of the Beclin1 and lc3b genes in liver cancer tissues and HCC cells decreased, suggesting that the autophagy activation in the liver cancer tissues and HCC cells was decreased. When HCC cells overexpressed Mfn2, the expression of Beclin1 and LC3 increased significantly, suggesting that Mfn2 induces autophagy by upregulating the expression of these proteins, playing an anticancer role.

In addition, our study explored the role of GLS1 in liver cancer. It is known that glutamine metabolism is an important metabolic pathway for cancer cell survival, and both the glutamine uptake and decomposition rates are increased in tumors.[11] In cancer cells, GLS1 is a key enzyme that catalyzes glutamine to glutamate and ammonia, and GLS1 is highly expressed in colorectal cancer, prostate cancer and breast cancer.[27] Previous studies have shown that inhibiting GLS1 activity inhibited tumor growth.[28] Although low GLS1 expression was found in HCC, the expression level of GLS1 in HCC cells was significantly higher than in normal hepatocytes, suggesting that there might be differences in GLS1 expression within the human body. When cells overexpressed Mfn2, GLS1 expression was inhibited. In a previous study, Xi, et al found that GLS1 promoted the proliferation of hepatoma cells via the Akt/GSK3β/CyclinD1 pathway,[29] which supported our findings. Therefore, the results suggest that GLS1 might be involved in the occurrence and development of HCC and that the anticancer effect of Mfn2 might be achieved by mediating GLS1 expression. However, the potential mechanisms of these actions need to be further researched.

CONCLUSIONS

This study found that the expression of Mfn2, GLS1, Beclin1 and lc3b in liver cancer tissues was lower than in normal liver tissues. The expression of Mfn2, Beclin1 and lc3b in HCC cells decreased, while that of GLS1 increased. Overexpression of Mfn2 induced autophagy, playing an anticancer role by inhibiting the activity of HCC cells, and induced GLS1 gene expression, promoting Beclin1 and lc3b expression.

AUTHOR CONTRIBUTIONS

Yushuang Huang and Wei Gao contributed equally to this study.

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