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Effects of mTOR-STAT3 on the migra=tion and invasion abilities of hepatoma cell and mTOR-STAT3 expression in liver cancer

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ABSTRACT

Objective: To investigate the effects of mTOR-STAT3 pathway on the invasion and migration of hepatoma cell. **Methods:** mTOR and STAT3 expresssion in the hepatocellular carcinoma cell line HepG2 and normal liver cell line L02 were detected by reverse transcription PCR (RT-PCR) and western blotting. The migration and invasion abilities of cells and expression of STAT3 were detected by scratch adhesion test and transwell migration assays, after siRNA transfection blocking mTOR expression of HepG2 cells. **Results:** The HepG2 cells expression is higher compared with normal cells L02 expression. Western blotting assay showed the mTOR expression was blocked, while STAT3 expression was also decreased, after the siRNA transfection of HepG2 cells. The migration (scratch adhesion test) and invasion (transwell assays) abilities of HepG2 cells which the mTOR expression was blocked by siRNA interference were significantly decreased ($P<0.05$). **Conclusion:** mTORSTAT3 expression in hepatoma cells HepG2 was significantly higher than that in normal liver cells. mTOR blocking can reduce the expression of STAT3, which is also closely related to the invasion and metastasis of liver cancer cells.

1. Introduction

Liver cancer is the most common malignant tumors, which including hepatocellular carcinoma and metastatic liver cancer. China is a high prevalence area of liver cancer with the high incidence rate and mortality. Primary hepatocellular carcinoma (HCC) is one of the most common clinical malignant tumor, it is the second largest cause of cancer related death. The number of new cases in china every year is occupy 55% of the world, which has greatly threatened human health[1-3]. Conventional treatment in clinical are surgery, chemotherapy and radiotherapy. Surgery is the preferred treatment for liver cancer, which with low resection rate and poor prognosis, only small portion of patients with satisfactory results. There is cirrhosis with most of the liver cancer patients, and also associated with hypersplenism and peripheral hypocytosis. Liver cancer patients′ also unbearable to systemic chemotherapy; Radiation therapy is usually only for liver function is still good and without complication (such as cirrhosis, ascites, jaundices) and without distant metastasis, so the range for radiation therapy is relatively narrow. Other optional treatments include immunotherapy and traditional Chinese medicine, but these treatments have poor effect, so the clinical application is relatively limited[4-6].

The earliest occurrence of liver cancer metastasis is in the liver and easily invasive the hepatic portal vein and its branches then form a tumor thrombu. Tumor thrombus exfoliated and caused multiple metastases in the liver. The extrahepatic metastasis include blood metastasis (lung metastasis has the highest metastaic rate), lymph node metastasis and implantation metastasis[7,8]. There

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are two main forms of mammalian target of rapamycin (mTOR), which can participate in a series of intracellular physiological and pathological processes: mTOR-Raptor complex and the mTOR-Rictor complex. mToR-Raptor complex was found earlier than the mTOR-Rietor complex, which can regulate protein synthesis in cells and affect the growth and proliferation of cells[9,10]. Signal transduction and activator of transcription 3 (STAT3) is a bifunctional protein that mainly exists in cytoplasm. It can couple to tyrosine phosphorylation signal channel and play an important role, such as regulate the expression of a variety of proteins related to the cell proliferation and apoptosis. Excessive activation of STAT3 in cells can promote cell proliferation and inhibit apoptosis, it can also promote angiogenesis and involved in the regulation of tumor cells immune escape. Excessive activation of STAT3 is considered to be one of the important mechanisms of tumorigenesis, the abnormal activation of STAT3 also showed in multiple hepatoma cell strains[11-14].

In this study, we blocked the expression of mTOR and observed its effect on regulating the STAT3, and further observed the effect on liver cancer cell invasion and metastasis, try to find new direction for the treatment of liver cancer and the inhibition of liver cancer metastasis.

2. Materials and methods

2.1. Main materials

The HepG2 and L02 cells were purchased from Shanghai Cell Bank. mTOR antibody, STAT3 antibody, β-actin antibody were purchased from ABcam Company, correpondding anti antibody were purchased from Beijing Zhongshan Jinqiao Company. RNA extraction Trizol, transfection reagent Lipo-fectamine 2000 were purchased from Invitrogen Corporation. RT-PCR kit was purchased from Fermentas company.

2.2. Experimental methods

2.2.1. Cell transfection and grouping

Human hepatoma cell line HepG2 was cultured in RPMI1640 medium containing 10% fetal bovine serum, then was placed in 5% CO_2 37 ℃ constant temperature incubator. According to 434-452 sites of mTOR gene, the oligonucleotide template and the nonsense templates was designed and synthesized (negative control nonsense templates had no homology with any coding sequence) (Table 1), oligo-1 and oligo-2, oligo-3 and oligo-4 of each template were complement to DNA duplexes. Two double-stranded DNA were transcribed to produce the siRNA sense strand and antisense strand respectively, after double-stranded siRNA was obtained, 20 bp DNA Ladder Marker was used as a reference and was identified by agarose gel electrophoresis. During transfection, the siRNA/liposome complex was placed at room temperature for 20 min, then the cells were added to be transfected, then they were mixed and observed after 24 h culture. Groups were as follows (n=3): HepG2 cells without any treatment as control group, transfected negative control sequence HepG2 as negative control group, transfected mTOR-siRNA HepG2 as experimental group. After 48 h of transfection, cells were collected by trypsin digestion.

Cells in each group were collected after 48 h transfection. Conventional method Trizol reagent was used for total RNA extraction and reverse transcription was performed. mTOR gene primer sequence upstream: 5′CGCTGTCATCCCTTTATCG3′; primer downstream sequence: 5′ATGCTCAAACACCTCCACC3′. Internal reference GAPDH primer upstream sequences: 5′GCACCGTCAAGGCTGAGAA3′; primer downstream sequence: 5′AGGTCCACCACTGACACGTTF3′. PCR conditions were set as follows: 95 ℃ 10 min, 95 ℃ 15 s, 53 ℃ 30 s, 72 ℃ 30 s, 40 cycles. Parallel experiments were repeated three times.

2.2.2. Western blotting

Cells were collected and protein was extrected

Table 1
Targeting mTOR siRNA transcriptional template sequences.

Sequence name	Sequence
siRNA	oligo-1 5′-AAGATAGTTGGCAAATCTGCCTATAGTGAGTCGTATTAGGATCC-3′
	oligo-2 5′-GGATCCTAATACGACTCACTATAGGCAGATTTGCCAACTATC-3′
	oligo-3 5′-AAGGCAGATTTGCCAACTATCTATAGTGAGTCGTATTAGGATCC-3′
	oligo-4 5′-GGATCCTAATACGACTCACTATAGATAGTTGGCAAATCTGCC-3′
siRNA-control	oligo-1 5′-AAGGCCATAATCAAGAAATACTATAGTGAGTCGTATTAGGATCC-3′
	oligo-2 5′-GGATCCTAATACGACTCACTATAGTATTTCTTGATTATGGCCTT-3′
	oligo-3 5′-AAGTATTTCTTGATTATGGCCTATAGTGAGTCGTATTAGGATCC-3′
	oligo-4 5′-GGATCCTAATACGACTCACTATAGGCCATAATCAAGAAATACTT-3′

byconventional method. After protein quantification, SDS-PAGE separation and PVDF transfer, Western blot method was used to detect mTOR and STAT3 expressions.

2.2.3. HepG2 cells basement membrane invasive ability detection

Transwell upper chamber surface was coated by Matrigel 5 μg, and was air dried at 4 ℃, basement membrane was hydrated, and 500 μL culture medium (containing FBS or chemokines) was added in Transwell lower chamber. Transfected cells and non-transfected cells were collected after 48 h, cell density was adjusted to 1×10^6/mL. 200 μL was then added to each Transwell chamber. The Transwell plate was cultured in 5% CO_2 37 ℃, after 24 h the chamber was removed and the cells were washed with PBS. Noninvasive cells were wiped off with a cotton on matrix gelatin and the surface of upper chamber, fixed with methanol, stained with crystal violet and washed with PBS. Five fields of vision were selected randomly from each slice for 200 times microscopic observation and the number of invasion cells which through basement membrane were counted. Mean value was regarded as the invasion and metastasis number. This test was repeated for three times.

2.2.4. Scratch adhesion test

After grouping, cells in 24-well plate were seeded. When the cells grew to 90% confluence, three straight lines were drawn for the cells in each well with 10 μL pipette tip. They were washed with PBS for three times, then cultured in the medium for 24 h. Five fields of vision were selected randomly from cells in each well for microscopic observation. There were 3 parallel holes at every group and each well was repeated twice.

2.3. Statistical analysis

The measurement data were expressed as mean±SD values and the difference between each group were analyzed by SPSS/Win13.0 software (SPSS, Chicago, IL). One-Way ANOVA was applied in the comparison between groups, P<0.05 has statistical significance.

3. Results

3.1. Hepatoma cells and normal liver cells mTOR and STAT3 expression

The results showed that the expression of HepG2 cells was higher than L02 cells (P<0.05) (Figure 1).

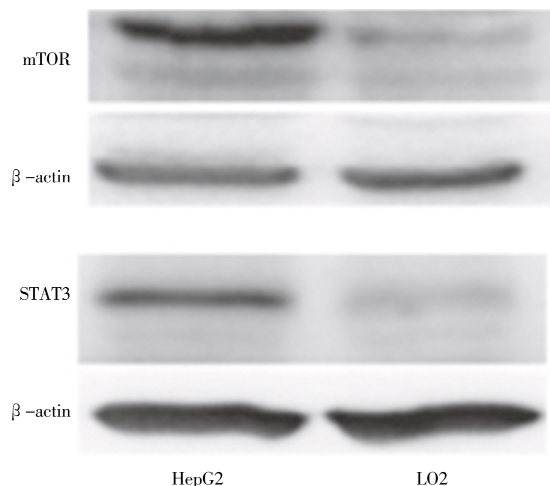

Figure 1. Hepatoma cells and normal liver cells mTOR and STAT3 expression.

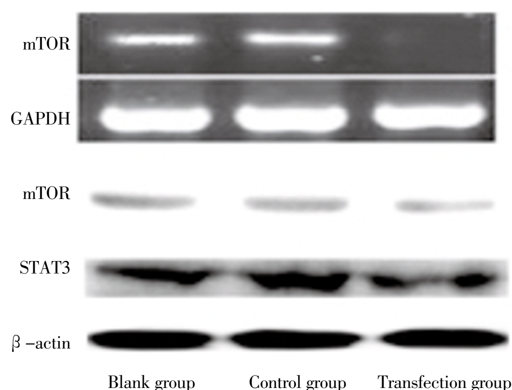

Figure 2. The mTOR and STAT3 expression of hepatoma cells and normal liver cells.

3.2. Transfection cells mTOR expression

This showed successful transfection and siRNA interference blocked the expression of mTOR. Compared with the control group, the Western blot detection showed that the STAT3 protein expression was also significantly decreased (P<0.05) after blocking the expression of mTOR.

3.3. mTOR blocking on HepG2 invasion and metastatic ability

After transfection blocking of the mTOR expression, use Transwell assay to detect the change of HepG 2 cell basement membrane invasion ability. As shown in Figure 3, the number of passed membrane cells in the blank group, the control group and the transfection group were 289±17, 291±16, 67±12. Compared with the blank group and the control

group, the number of passed membrane cells decreased significantly ($P<0.05$).Compared the blank group with the control group, there is no statistically significant ($P>0.05$).

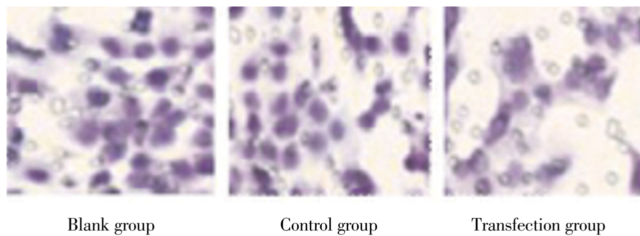

Figure 3. Transwell cell invasion ability detection (200×).

After transfection of siRNA blocked the expression of mTOR, scratch adhesion test showed the scratch width of the blank group, control group and transfection group were 1.35 ±0.29, 1.26±0.38, 0.63±0.42 mm respectively, Compared with the blank group and the control group, the migration of the cells in MTOR siRNA transfection group was significantly decreased, scratch healing speed was significantly decreased ($P<0.05$), while compare the scratch width of the blank group with the control group, the differences between two groups have no statistically significant ($P>0.05$).

4. Discussion

With the gradual deepening study of the mTOR signals pathway recently, MTOR signaling pathway conduction was found closely related with a variety of tumor occurrence and development, especially in the process of cell growth and proliferation of mTOR signaling pathway. However, the mechanism of mTOR gene on liver cancer cells invasion and metastasis is still not clear. In this study, we design and synthesize siRNA sequences which aiming at mTOR gene, and use it to transfected human hepatoma cell line HepG2, observed its effect on liver cancer cell invasion and metastasis. After 48 h of siRNA transfection, detected the expression of mTOR at the gene level and protein level respectively. The results showed that: mTOR with high expression in the non-transfected mTOR-siRNA blank control group, while after the mTOR siRNA transfection, successfully blocked its expression at the mRNA level and protein level. This indicates that the design of siRNA sequences successfully blocked the expression of mTOR. The mTOR siRNAture we designed can be used as tools for the further study of liver cancer occurrence and development, invasion and metastasis[15-17].

In the basic characteristics of malignant tumors, invasion and metastasis are the important aspect, which are also the main factors lead to cancer recurrence and mortality. During the treatment of liver cancer, the key factors include the control of tumor invasion and metastasis[18-21]. Recent studies showed that STAT3 signaling pathway plays an important role in the incidence and the development of cancer, especially in the promotion of tumor cell proliferation, inhibition of tumor cell apoptosis, promote tumor cell invasion and metastasis and immune escape, STAT3 signaling pathway is particularly important[17,22-25]. In this study, we further demonstrated the role of mTOR in the regulation of STAT3. After blocking mTOR, STAT3 expression was significantly reduced, which provides more powerful evidence that mTOR as therapeutic target of liver cancer metastasis.

In this study, we used Transwell invasion assay in vitro to detect cell invasion. The numbers of passed membrane cells were used as characterization to evaluate the changes of HepG2 cells invasive ability before and after siRNA transfection. The results showed that the mTOR siRNA transfection cells can successfully block the expression of mTOR. Compared cells invasive ability of transfected cells with non-transfected cells, the cell invasion was significantly decreased after siRNA blocked mTOR expression. This indicates mTOR is an important regulatory factor in the process of invasion and metastasis of HCC, which plays an important regulatory role. This effect may because it can regulate STAT3.

In conclusion, this study further confirmed mTOR and STAT3 with high expression in liver cancer cells. After blocking mTOR expression, the expression of STAT3 also reduced, further validates mTOR can regulate STAT3. And the experimental results showed that mTOR-STAT signaling down-regulation significantly inhibited the invasion and metastasis of liver cancer cells, which provide potential new ideas for the liver cancer anti-metastatic treatment. Follow-up studies also need to study the STAT3 further downstream signal transduction after blocking mTOR, so that can have deeper researches in the role of mTOR/STAT3 signaling pathways in liver metastasis.

Conflict of interest statement

We declare that we have no conflict of interest.

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