

## Original Article

# Biological effects of lentivirus-mediated silencing of minichromosome maintenance protein 7 with shRNA on the liver cancer MHCC-97H cells

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**Abstract:** Objective: This study was to specifically silence the minichromosome maintenance protein 7 (MCM7) expressions with lentivirus-mediated RNA interference technique in liver cancer MHCC-97H cells and its biological consequences were investigated. Methods: Human MCM7 sequence was used for the design of shRNA targeting MCM7 which was then introduced to lentivirus, followed by transfection into MHCC-97H cells. Real time quantitative PCR and Western blot assay were performed to detect the mRNA and protein expression of MCM7 in these cells. MTT assay was performed to detect cell proliferation, flow cytometry to detect cell cycle and apoptosis, scratch-wound assay to detect cell migration ability, and transwell invasion assay to evaluate the invasion of these cells. Results: We successfully constructed LV-mcm7-RNAi expressing MCM7 shRNA. PCR and Western blot assay showed the mRNA and protein expression of MCM7 reduced significantly when compared with negative control group (LV-NC-RNAi) and blank control group ( $P < 0.05$ ). As compared to blank control group and negative control group, the cell proliferation reduced dramatically ( $P < 0.01$ ), cells were mainly arrested in G0/G1 phase and apoptotic cells increased markedly in LV-mcm7-RNAi group. Moreover, cells transfected with LV-mcm7-RNAi showed significant reductions in the invasion and migration as compared to other groups ( $P < 0.05$ ). Conclusion: Lentivirus mediated silencing of MCM7 with shRNA in MHCC-97H cells may inhibit the malignant behaviors of MHCC-97H cells (suppressed proliferation and compromised invasiveness), which is related to the cell cycle arrest and increase in apoptosis.

**Keywords:** Liver cancer MHCC-97H cells, RNA interference, minichromosome maintenance protein 7

## Introduction

Minichromosome maintenance protein 7 (MCM7) is a key component of the pre-replication complex involved in the initiation of eukaryotic DNA replication and essential for the initiation of eukaryotic DNA replication [1, 2]. It ensures that DNA undergoes a single round of replication per cell cycle by a licensing mechanism [3]. In normal tissues, MCM7 is not expressed or has a low expression, but highly expressed in multiple malignancies. The up-regulated MCM7 expression has been found to be an important event in the occurrence of some malignancies (such as Endometrial cancer [4], melanoma [5], esophageal adenocarcinoma [6], colorectal adenocarcinoma [7], oral squamous cell carcinoma [8], glioblastoma [9] and prostate cancer [10]. In this study, RNA interference (RNAi) tech-

nique was employed to silence MCM7 expression in liver cancer cells and its effects on the proliferation, invasion and metastasis of liver cancer cells were investigated. Our findings may provide evidence for the pathogenesis of liver cancer and the therapy of liver cancer targeting MCM7.

## Materials and methods

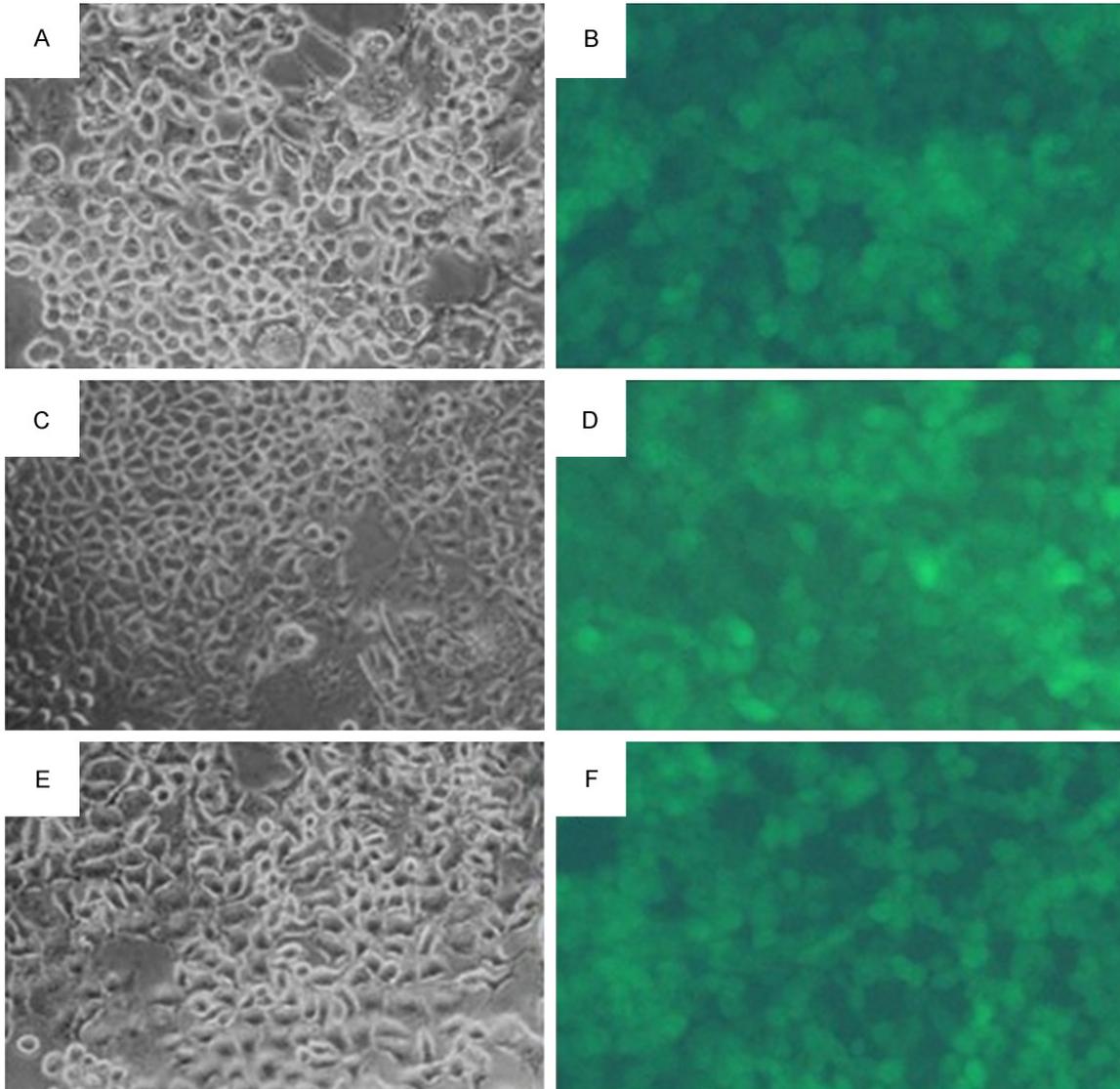
### Materials

Human liver cancer MHCC-97H cells were purchased from the Shanghai Aiyuan Biotech Co., Ltd. Lentivirus expressing MCM7 shRNA (LV-mcm7-RNAi) and negative control lentivirus (LV-NC-RNAi) were prepared in the Genechem Co., Ltd. DMEM, fetal bovine serum (FBS; Gibco, USA), Trizol (TAKARA), Taq DNA Polymerase

## Lentivirus-mediated silencing of MCM7 with shRNA on MHCC-97H cells

**Table 1.** Three shRNAs targeting MCM7 and 1 negative control shRNA were designed

NO.	Accession	Target Seq	CDS	GC%
MCM7-RNAi (23977-1)	NM_005916	GGACTCAATTTGTGAGAAT	511..2670	36.84%
MCM7-RNAi (23978-1)	NM_005916	GAGTTGGTGACTCAATTT	511..2670	42.11%
MCM7-RNAi (23979-1)	NM_005916	TGAACAAGAGTGAGGATGA	511..2670	42.11%
Negative Control, NC		TTCTCCGAACGTGTCACGT		



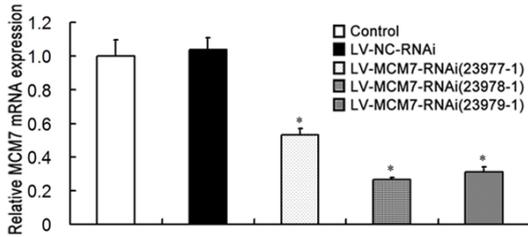
**Figure 1.** Detection of transfection efficiency by fluorescence microscope. A, B: MHCC-97H cells after transfection with LV-MCM7-RNAi (23977-1) for 72 h; C, D: MHCC-97H cells after transfection with LV-MCM7-RNAi (23978-1) for 72 h; E, F: MHCC-97H cells after transfection with LV-MCM7-RNAi (23979-1) for 72 h.

(Promega), rabbit anti-human MCM polyclonal antibody (Abcam), primers for MCM (Shanghai Sangong Co., Ltd) AnnexinV/PI apoptosis assay kit, cell cycle detection kit (Nanjing Keygentech Co., Ltd) and Transwell chamber (Chemicon) were used in the present study.

### Cell culture and preparation

MHCC-97H cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in a humidified environment of 5% CO<sub>2</sub>. When the cell conflu-

## Lentivirus-mediated silencing of MCM7 with shRNA on MHCC-97H cells



**Figure 2.** MCM7 mRNA expression detected by real time fluorescence quantitative PCR. \* $P < 0.05$ , compared with NC group or blank control group.

ence reached  $>80\%$ , cells were digested with 0.25% trypsin and passaged.

### Construction of MCM7 shRNA expressing vector and lentivirus packaging

MCM7 gene was used as a template and its siRNAs were designed. A total of three shRNAs targeting MCM7 and 1 negative control shRNA were designed (**Table 1**). The target sequence was connected to GV118 (U6-MCS-Ubi-EGFP), followed by identification by sequencing and subsequent lentivirus packaging.

### Grouping

Cells were divided into 3 groups: control group, negative control group (LV-NC-RNAi) and interference group (LV-mcm7-RNAi).

### Infection of cells with lentivirus

The titer of lentivirus was determined with serial dilution method. Then, MHCC-97H cells were seeded into 96-well plates, followed by addition of  $1 \times 10^8$  TU/ml lentivirus (10  $\mu$ l), 5  $\mu$ g/ml polybrene and complete medium. Cells were incubated in an environment of 5%  $\text{CO}_2$  at 37°C for 24 h. The medium was refreshed, followed by culture for additional 48 h. Cells were observed under a fluorescence microscope to evaluate the transfection efficiency.

### Detection of MCM7 mRNA expression by real-time PCR

Total RNA was extracted from cells in each group with Trizol and the RNA concentration was determined by UV spectrophotometry. Then, RNA was reverse-transcribed into cDNA. Primers used in PCR were designed with Primer 5. Primers for MCM7 were as follows: 5'TCAGCGTCACTGGTATTTCTTG'3 (forward)

and 5'TCATCCTCACTCTTGTTTCATCTTCA'3 (reverse). Primers for GAPDH were as follows: 5'CGCTGAGTACGTCGTGGAGTC'3 (forward) and 5' GCTGATGATCTTGAGGCTGTTGTC'3 (reverse). Real time-PCR was performed under following conditions: pre-denaturation at 95°C for 10 min, and then a total of 40 cycles at 95°C for 15 s and at 60°C for 60 s. Both MCM7 and GAPDH were expanded by PCR, there were 3 wells in each group and averages were obtained. The threshold cycle number (Ct value) was recorded, and  $2^{-\Delta\Delta\text{Ct}}$  method was used to determine the mRNA expression of MCM7 which was normalized to that of GAPDH. Ct refers to the number of cycles after the fluorescence signals reach the threshold and  $\Delta\text{Ct}$  is calculated as follow:  $\Delta\text{Ct} = \text{mean Ct (MCM7)} - \text{mean Ct (GAPDH)}$ .  $\Delta\text{Ct}$  of negative control group was used as a reference and  $\Delta\Delta\text{Ct}$  was calculated as follow:  $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{experiment}} - \Delta\text{Ct}_{\text{negative control}}$ . Then,  $2^{-\Delta\Delta\text{Ct}}$  was calculated as the relative mRNA expression of MCM7.

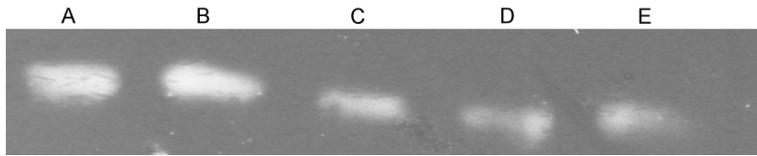
### Detection of MCM7 protein expression by western blot assay

Total protein was extracted from cells in each group and protein concentration was determined with BCA method. Protein solution was mixed with 2 $\times$  loading buffer at a ratio of 1:1 and boiled for 10 min. This mixture was subjected to 10% SDS-PAGE, and proteins were transferred onto PVDF at 100 mA for 90 min. The membrane was blocked in non-fat milk for 4 h and then treated with rabbit anti-human MCM7 antibody at 4°C over night. This membrane was washed in PBST thrice and then treated with goat anti-rabbit secondary antibody for 2 h at room temperature. After washing, visualization was done with ECL, and bands were observed and analyzed with a gel analysis system. The optical density (OD) of bands was determined. The relative protein expression of target protein was calculated as follow: relative expression of target protein =  $\text{OD}_{\text{target protein}} / \text{OD}_{\beta\text{-actin}}$ , and  $\beta$ -actin served as an internal reference.

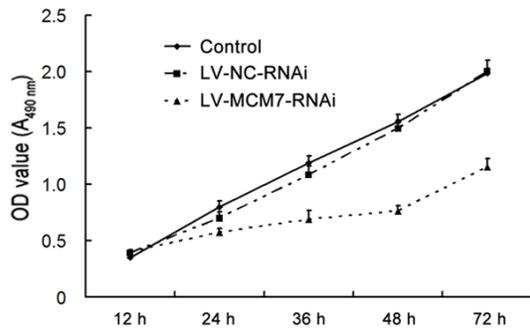
### Detection of cell proliferation of MTT assay

MHCC-97H cells in logarithmic growth phase were harvested, and cell density was adjusted to  $1 \times 10^5$ /mL. Then, these cells were seeded into 96-well plates (100  $\mu$ L/well) and there were 4 wells in each group. Cells were incubat-

## Lentivirus-mediated silencing of MCM7 with shRNA on MHCC-97H cells



**Figure 3.** MCM7 protein expression determined by Western blot assay after specific silencing of MCM7. A, B: Negative control group; D-F: Blank control group.



**Figure 4.** Cell growth curve of MHCC-97H cells after MTT assay.

ed at 37°C in an environment of 5% CO<sub>2</sub> for 24 h, 48 h, 72 h and 96 h. After addition of 5 mg/mL MTT (20 µL/well), cells were incubated for 4 h and the supernatant was removed. DMSO (100 µL/well) was added to each well and cells were further incubated for 10 min with constant shaking to resolve purple crystals. The absorbance (A) was measured at 540 nm and cell growth curve was delineated.

### Detection of cell cycle

MHCC-97H cells in logarithmic growth phase were harvested, seeded into 6-well plates (1×10<sup>5</sup>/well) and incubated in an environment of 5% CO<sub>2</sub> for 48 h. After digestion with trypsin, 1×10<sup>6</sup> cells were collected from each group and washed with pre-cold PBS thrice, followed by centrifugation at 1000 r/min for 5 min. The supernatant was removed, and 100 µL of RNase was added, followed by incubation at 37°C for 30 min. Following addition of 500 µL of PI, cells were incubated in dark for 30 min at room temperature and subjected to flow cytometry. Experiment was done three times.

### Detection of cell apoptosis

MHCC-97H cells in logarithmic growth phase were harvested, seeded into 6-well plates (1×10<sup>5</sup>/well) and incubated in an environment

of 5% CO<sub>2</sub> for 48 h. After digestion with trypsin, 1×10<sup>6</sup> cells were collected from each group and washed with pre-cold PBS twice. Cells were re-suspended in 500 µL of binding buffer, followed by addition of 5 µL of AnnexinV-FITC and 5 µL of PI. Cells were incubated on ice for 15 min in

dark and then subjected to flow cytometry for the detection of apoptotic cells.

### Detection of cell migration in vitro

After transfection for 72 h, MHCC-97H cells were digested and seeded into 6-well plates followed by incubation in a humidified environment of 5% CO<sub>2</sub> at 37°C. When the cell confluence reached 90%, the medium was removed, and a wound was made with a 100 µL pipette tip, followed by washing with PBS thrice. Cells were incubated with serum-free DMEM for 24 h, and representative photographs were captured.

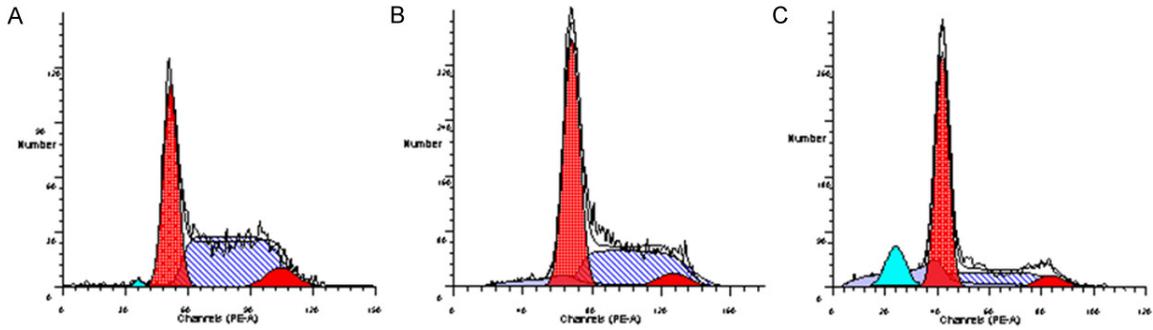
### Detection of cell invasiveness in vitro

The Transwell chamber was placed in plates, and 300 µL of serum-free medium was added into upper chambers, followed by incubation for 30 min at room temperature for the re-hydration of matrigel. The residual medium was removed. After transfection for 72 h, cells were digested with trypsin and re-suspended in serum free medium at a density of 5×10<sup>5</sup>/ml. The lower chambers were placed into 24-well plates and cell suspension was added to upper chambers (200 µL/chamber), and medium containing 10% FBS was added to lower chambers (500 µL/chamber), followed by incubation at 37°C in an environment of 5% CO<sub>2</sub> for 24 h. The upper chambers were taken out of plates and placed upside down. After washing in PBS, these chambers were fixed in methanol for 10 min and stained with hematoxylin for 10 min. A cotton swab was used to remove the cells without migration. Cells migrating across the membrane were observed under a microscope and counted in 10 randomly selected fields.

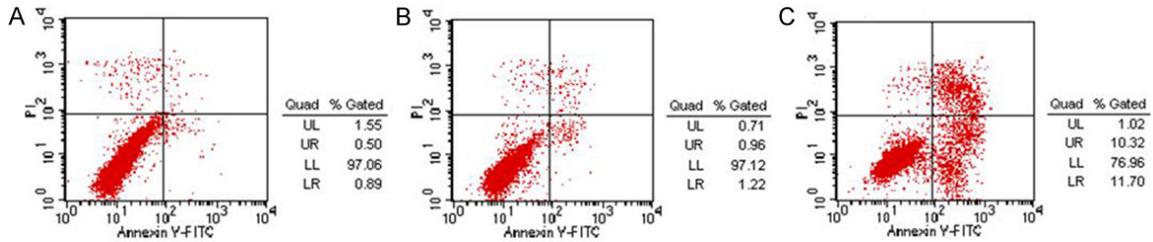
### Statistical analysis

Statistical analysis was performed with SPSS version 16.0. Qualitative data are expressed as

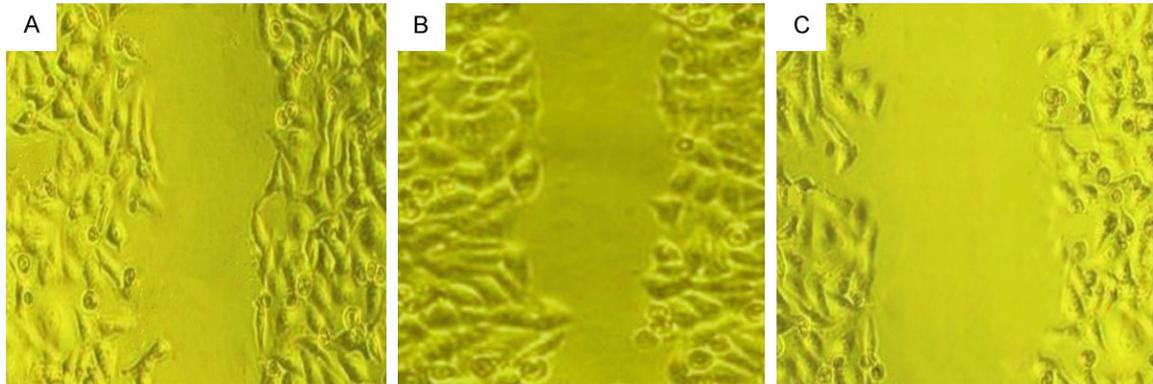
## Lentivirus-mediated silencing of MCM7 with shRNA on MHCC-97H cells



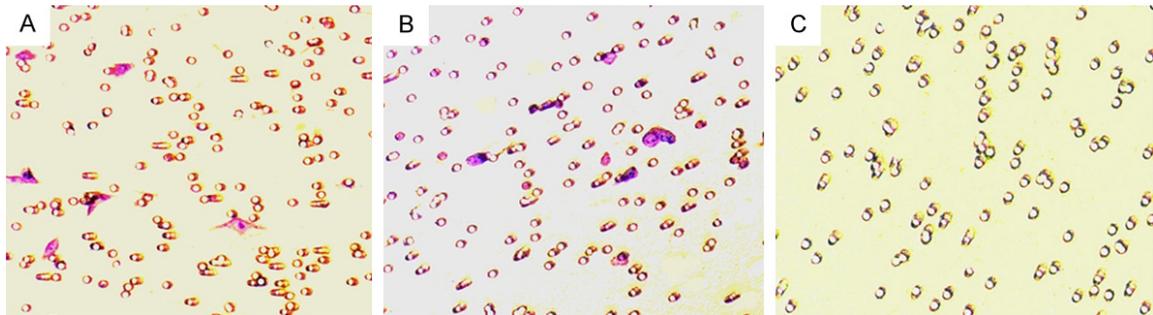
**Figure 5.** Detection of cell cycle by flow cytometry. A: Control group; B: LV-NC-RNAi group; C: LV-MCM7-RNAi group.



**Figure 6.** Detection of apoptotic cells by flow cytometry. A: Control group; B: LV-NC-RNAi group; C: LV-MCM7-RNAi group.



**Figure 7.** Detection of cell migration by wound scratch assay. A: Control group; B: LV-NC-RNAi group; C: LV-MCM7-RNAi group.



**Figure 8.** Detection of cell invasion in vitro by Tanswell invasion assay. A: Control group; B: LV-NC-RNAi group; C: LV-MCM7-RNAi group.

## Lentivirus-mediated silencing of MCM7 with shRNA on MHCC-97H cells

mean  $\pm$  standard deviation (SD) and compared with one-way analysis of variance (ANOVA) among groups. A value of  $P < 0.05$  was considered statistically significant.

### Results

#### *Transfection efficiency of MHCC-97H cells by lentivirus*

After transfection with different LV-mcm7-RNAi lentiviruses for 24 h, medium was refreshed with complete medium, followed by further incubation for 48 h. The GFP expression became stable and showed green fluorescence under a fluorescence microscope. Results showed the transfection efficiency was  $>95\%$ , the reporter gene was confirmed, and it was feasible to perform PCR. As shown in **Figure 1**, after transfection with different LV-mcm7-RNAi lentiviruses, the GFP was even in different groups and there was no marked difference in the fluorescence density among groups, suggesting that the transfection efficiency was comparable among these groups.

#### *mRNA expression of MCM7 after specific silencing of MCM7*

Real time fluorescence quantitative PCR was employed to detect the mRNA expression of MCM in MHCC-97H cells after silencing of MCM7 with different lentiviruses. When compared with NC group and blank control group, the mRNA expression of MCM7 reduced significantly by more than 50% (74.47% and 73.50% in LV-MCM7-RNAi (23978-1) group;  $P < 0.05$ ). Thus, LV-MCM7-RNAi (23978-1) was selected as an optimal lentivirus. In addition, the mRNA expression of MCM7 was comparable between negative control group and blank control group ( $P > 0.05$ ) (**Figure 2**).

#### *Detection of MCM7 protein expression after specific silencing of MCM7*

After transfection with different lentiviruses, the MCM7 protein expression reduced significantly when compared with blank control group ( $P < 0.05$ ). LV-MCM7-RNAi (23978-1) was the most effective lentivirus in the silencing of MCM7, and the MCM7 protein expression was reduced by 73.50%. However, there was no significant difference in the MCM7 protein expression between negative control group and blank

control group ( $P > 0.05$ ) (**Figure 3**). On the basis of results from real time PCR and Western blot assay, LV-MCM7-RNAi (23978-1) was the most effective lentivirus in the silencing of MCM7 expression and could inhibit the mRNA expression and protein expression in MHCC-97H cells.

#### *Detection of cell proliferation*

When compared with blank control group and LV-NC-RNAi group, the cell growth was significantly inhibited in LV-MCM7-RNAi group. A significant reduction in A was observed in LV-MCM7-RNAi group at 24 h after transfection ( $P < 0.05$ ), and the inhibition of cell growth became more evident over time (**Figure 4**).

#### *Detection of cell cycle by flow cytometry*

When compared with LV-NC-RNAi group ( $50.61 \pm 9.22$ ) and control group ( $48.92 \pm 3.40\%$ ), the proportion of cells in G0/G1 phase increased markedly, but that in S phase reduced dramatically in LV-MCM7-RNAi group ( $P < 0.05$ ). This suggests that cells in LV-MCM7-RNAi group were arrested in G0/G1 phase and failed to enter the S phase (**Figure 5**).

#### *Detection of apoptotic cells by flow cytometry*

The apoptosis index of MHCC-97H cells in LV-MCM7-RNAi group was  $12.08 \pm 1.37\%$ , which was significantly higher than that in negative control group ( $0.89 \pm 0.08\%$ ) and blank control group ( $1.22 \pm 0.05\%$ ) ( $P < 0.05$ ). However, there was no marked difference between negative control group and blank control group ( $P > 0.05$ ) (**Figure 6**).

#### *Detection of cell migration in vitro*

Wound scratch assay showed MHCC-97H cells migrated into the wound under a microscope. After culture for 24 h, the migration distance in LV-MCM7-RNAi group was significantly shorter than that in control group and LV-NC-RNAi group. This suggests that specific inhibition of MCM7 is able to suppress the migration ability of MHCC-97H cells (**Figure 7**).

#### *Detection of cell invasion in vitro*

Transwell invasion assay showed the number of migrated cells in control group, LV-NC-RNAi group and LV-MCM7-RNAi group was  $58 \pm 7.33$ ,  $49.8 \pm 6.38$  and  $0.3 \pm 0.03$ , respectively.

## Lentivirus-mediated silencing of MCM7 with shRNA on MHCC-97H cells

Statistical analysis showed the number of cells migrating across the membrane in LV-MCM7-RNAi group reduced markedly as compared to LV-NC-RNAi group and control group ( $P < 0.01$ ). However, the number of migrated cells was comparable between LV-NC-RNAi group and control group ( $P > 0.05$ ) (Figure 8).

### Discussion

Malignant tumor is characterized by the uncontrollable autonomous growth resulting in sustained division and proliferation. In recent years, studies on the regulation of cell growth have proposed that the initiation of DNA replication plays a central role in the regulation of cell growth. MCM is a key component of the pre-replication complex involved in the initiation of eukaryotic DNA replication and plays important roles in the initiation of eukaryotic DNA replication and extension. MCM2-7 binds to the origin of DNA replication and then forms a pre-replication complex together with other recruited initiation factors, which allows the replication of chromatin. In addition, the MCM4/6/7 dimeric complexes have the helicase activity and ATP hydrolytic activity and are able to denaturalize the double-stranded DNA which allows the extension after replication [2]. After replication initiation, MCM2-7 departs from chromatin and will not assemble the pre-replication complex during the replication, which ensures that DNA undergoes a single round of replication per cell cycle [3, 11]. There is evidence showing that the ATPase of MCM7 is a key component for the DNA helicase activity and ATP hydrolytic activity. Some factors regulating the cell cycle (such as Rb, Cdc6, P27<sup>kip1</sup> and others) may interact with MCM7 to inhibit DNA replication and block the progression of cell cycle. In normal cells, the protein and mRNA expression of MCM changes with the progression of cell cycle. MCM expression reaches a peak in G1/S phase and reduces significantly or even becomes undetectable in G0 phase or when cells become differentiated or senescent [2].

In recent years, the role of MCM7 in the occurrence, progression, invasion, prognosis and other malignant behaviors of tumors has been a focus in most studies. In studies on melanoma [5, 12], esophageal cancer [13], and oral squamous cell carcinoma [8], results have

revealed that MCM7 expression increases from normal proliferation, dysplasia and malignant proliferation, suggesting that high MCM7 expression is related to the occurrence and development of different malignancies. High MCM7 expression meets the requirements for the division and proliferation of malignant cells. Thus, MCM7 may become a specific marker of malignancies. High MCM7 expression usually predicts a poor prognosis. In oral squamous cell carcinoma patients, MCM7 was highly expressed, which was negatively related to the survival time, and patients with poorly differentiated malignant cells had a higher expression of MCM7 [8]. In patients with prostate cancer [13] and medulloblastoma [14, 15], high MCM7 expression is involved in the invasion and metastasis of malignancies, and thus it may serve as a prognostic factor in these patients. In lung cancer patients [16], MCM7 was found to be an independent prognostic factor and more reliable in the evaluation of prognosis of lung cancer as compared to MCM2 and Ki-67. In our previous study [17], DNA microarray was employed to screen the genes related to hepatocellular carcinoma (HCC), and results showed MCM7 as a member of MCM family was highly expressed in HCC tissues and the poorer the differentiation of HCC cells, the higher the clinical stage, the more the number of malignant lesions and the more incomplete the HCC capsule, the higher the MCM7 expression was. In addition, the MCM-7 expression was consistent with the serum AFP level. These findings imply that MCM7 expression play important roles in the occurrence and development of HCC. Immunohistochemistry [18] showed MCM7 protein expression was only found in liver cancer, but absent in normal liver and adjacent normal tissues. Moreover, the MCM7 expression was positively related to the tumor size, the integrity of tumor capsule, AFP level, degree of tumor differentiation and clinical stage of liver cancer, and MCM7 positive patients had a lower survival rate as compared to those negative for MCM7. The positive rate of MCM7 was 72.7% in liver cancer and still as high as 56.5% in AFP negative liver cancer. These findings indicate that MCM7 is crucial for the occurrence and development of liver cancer, but the specific mechanism is still unclear. Thus, it is necessary to investigate the role and exact mechanism of MCM7 in the pathogenesis of liver cancer.

## Lentivirus-mediated silencing of MCM7 with shRNA on MHCC-97H cells

In the present study, we constructed shRNA targeting MCM7 and introduced into recombinant lentivirus which was then transfected into human liver cancer MHCC97-H cells. Then, the proliferation, apoptosis, invasion and metastasis of these cells investigated after specific silencing of MCM7. Fluorescence microscopy was performed to evaluate the transfection efficiency. Results showed the transfection efficiency was as high as 90% after transfection with different lentiviruses. Western blot assay and RT-PCR showed the mRNA and protein expression of MCM7 was reduced by more than 50% after transfection of lentivirus expressing MCM7-shRNA as compared to negative control group and blank control group, and the reduction was the most evident in LV-MCM7-RNAi (23978-1) group (73.50% and 74.65%, respectively). Thus, LV-MCM7-RNAi (23978-1) was the most effective to silence MCM7 expression in MHCC97-H cells. MTT assay showed the cell proliferation in LV-MCM7-RNAi group was slower than that in control group and LV-NC-RNAi group. Flow cytometry revealed that, as compared to LV-NC-RNAi group and control group, cells were arrested in G0/G1 phase, and cells in S phase also reduced markedly in LV-MCM7-RNAi group. Moreover, the apoptosis index in LV-MCM7-RNAi group increased markedly as compared to other groups. In wound scratch assay and Transwell migration assay, results showed the migration and invasion of MHCC97-H cells were significantly compromised after specific silencing of MCM7. Taken together, these findings indicate that silencing of MCM7 with specific shRNA is able to block cell cycle, significantly increase apoptotic cells and markedly inhibit cell growth in liver cancer cells. Thus, MCM7 may become an effective target in the gene therapy of liver cancer.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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