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ORIGINAL ARTICLE

RACK1 Silencing Induces Cell Apoptosis and Inhibits Cell Proliferation in Hepatocellular Carcinoma MHCC97-H Cells

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Abstract This study aimed to explore the effects of *RACK1* gene silencing on the apoptosis and proliferation of hepatocellular carcinoma (HCC) MHCC97-H cells. After transfecting MHCC97-H cells with siRNA, *RACK1* gene silencing model was established. The cells were divided into blank group, siRNA group and empty plasmid group, respectively. The mRNA and protein expressions of *RACK1*, *cyclin D1* and *BAX* were determined by qRT-PCR and Western blotting. CCK-8 assay, flow cytometry and FITC-Annexin V/PI staining were used to determine cell viability, cell cycle and cell apoptosis, respectively. The results of qRT-PCR and Western blotting suggested that when compared with the blank group and the empty plasmid group, the mRNA and protein expressions of RACK1 and Cyclin D1 decreased significantly while the mRNA and protein BAX expressions increased substantially in the siRNA group (all $P < 0.05$). The results of CCK-8 assay revealed that the siRNA group exhibited significantly lower cell viability when compared with the blank group and the empty plasmid group (both $P < 0.05$); and the cell viability in the siRNA group decreased gradually with the increase of time. The results of flow cytometry and FITC-Annexin V/PI staining indicated that when compared with the blank group and the empty plasmid group, the proportion of cells in S phase was markedly lower and the apoptosis rate was significantly higher in the siRNA group (both $P < 0.05$). Our study suggests that inhibition of *RACK1* could suppress cell proliferation and induce apoptosis in HCC MHCC97-H cells.

Keywords *RACK1* · Hepatocellular carcinoma · Cell proliferation · Cell apoptosis · Gene silencing · MHCC97-H

Introduction

Hepatocellular carcinoma (HCC), the most common primary malignancy of the liver, is the sixth most common cancer and the third leading cause of cancer mortality worldwide [1]. According to statistics, about 780,000 new HCC cases and 145,000 deaths occurred in 2012 worldwide, among which the morbidity and mortality in China accounted for approximately 50% [2]. Although there are many predisposing factors for HCC, most of it is thought to be associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, liver cirrhosis and obesity [3–5]. Currently, liver transplant and surgical resection are recommended clinically for the treatment of HCC [6]. However, the above-mentioned methods are commonly accompanied with high postoperative recurrence rates and poor prognosis, and the 10-year mortality following operation for HCC reaches up to 87% [7]. With the development of gene technology, an increasing number of researchers focuses on the mechanisms underlying proliferation, migration and invasion capacity of HCC cells at a genetic level [8], aiming to reduce recurrence rates and improve long-term prognosis, survival rates and quality of life in HCC patients.

The receptor for activated C kinase 1 (RACK1), encoded by the *GNB2L1* gene [9], is the first member identified in the

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RACK family [10], also belonging to the Trp-Asp 40 (WD40) repeat family with seven highly conserved internal consensus WD40 repeats [10, 11]. RACK1 is homologous to the β subunit of G protein, which is widely expressed in important human and animal organs [8]. RACK1 has been implicated in the promotion of tumor invasion and metastasis, as well as other cellular functions such as cell adhesion and migration, through binding to two key signaling molecules, protein kinase C (PKC) or Src, with the WD40 repeats [12]. It was reported that RACK1, highly expressed in neuroblastoma cells, positively regulated cell migration and proliferation by regulating the activation of Src [13]. Another recent study found that in oral squamous cell carcinoma (OSCC) cells, *RACK1* silencing or *RACK1* knockdown could not only reduce cell proliferation, but also inhibit cell migration, invasion and adhesion [14]. It was also discovered that overexpressed *RACK1* may enhance the activity of c-Jun N-terminal protein kinase (JNK) and thereby promote HCC growth through directly binding to the MKK7 and enhancing MKK7 activity [15]. Nevertheless, there were only a few studies about the effects of *RACK1* silencing on HCC cells; thus, this study aims to investigate the effects of *RACK1* gene silencing on proliferation, cell cycle and apoptosis of HCC MHCC97-H cells.

Materials and Methods

Cell Culture

The human HCC cell line (MHCC97-H), was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were seeded into a Petri dish (100 mm) at a density of 1×10^5 cells/mL per well, and cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, CA, USA). After 48 h of culture, cells were digested with 0.25% trypsin and then transferred to a flask (75 mL) for subculture. The culture medium was refreshed every 3 days. Once cells reached 60% confluence, they were digested with 0.25% trypsin and centrifuged at 1000 rpm for 5 min. After the supernatant was discarded, the cells were rinsed twice in the DMEM, and centrifuged at 1000 rpm for 5 min, followed by resuspension in a serum-free DMEM. The number of cells was counted and cell suspension of 1×10^8 cells/mL was prepared.

Construction of *RACK1* siRNA Vector

Two siRNA strands were designed by Invitrogen Inc. (Carlsbad, CA, USA), with sequences as shown below:

5′-CACCGAGATAAGACCATCATCATGTTTCAAGAGAACATGATGATGGTCTTATCTCTTTTTTG-3′; 3′-AGCTCAAAAAAGAGGATAAGACCATCATCATCATGTTCTCTTGAAACATGATGATGGTCTTATCT-5′, with the addition of two restriction enzyme sites (*Bam*H I and *Hin*dIII) to the end of sequences. Double-stranded DNAs were prepared by annealing. Then DNA fragments were ligated to the pGenesil-1 plasmid vector with the use of BamH I and HindIII restriction enzyme sites, and transformed into DH5α competent cells. After coating on an LP culture plate containing Kanamycin, the single colonies were selected and the plasmids were extracted for sequencing. The pGenesil-1-*RACK1* vector was obtained after verification and pGenesil-1 vector without siRNA sequences was selected as controls.

Cell Transfection and Grouping

One day prior to transfection, cells were seeded evenly into a 6-well plate at a density of 2×10^5 cells/well and cultured in an antibiotic-free DMEM. On the next day, when the cells were grown to 60% ~ 80% confluence (16 ~ 24 h after seeding), cell transfections were performed. The medium was changed with the antibiotic-free, serum-containing DMEM (2 mL/well) before transfection. Cells were divided into the blank group (without transfection), the siRNA group (transfected with pGenesil-1-*RACK1* plasmid) and the empty plasmid group (transfected with empty plasmid), with six wells in each group. The pGenesil-1-*RACK1* plasmids and the empty plasmids were extracted and diluted to a concentration of 0.1 ~ 3 μg/μL (pH 8.0). The plasmids (3 μg) were then dissolved in 1.5 mL of DMEM. In a separate tube, 36 μL of Lipofectamine 2000 (Invitrogen, Inc., Carlsbad, CA, USA) was added into the DMEM (1.5 ml), followed by a 5-min incubation at room temperature. The mixtures of mediums in both two tubes were incubated at room temperature for 20 min to form transfection complexes. In the siRNA and empty plasmid groups, cells were treated with transfection complexes. The phosphate-buffered saline (PBS) was added into cells of

Table 1 The primer sequences of RT-qPCR

Primer	Sequence
RACK1	F: 5′-TGTCTTGTGTCCGCTTCTCG-3'
	R: 5′-AGTCACCGTGTTCAGATAGCCT-3'
CyclinD1	F: 5′-CGATGCCAACCTCCTCAAC-3'
	R: 5′-GTCCATGGGGAAGATCGTC-3'
GAPDH	F: 5′-CATCTTCTTTTGCGTCGCCA-3'
	R: 5′-TCGCCCCACTTGATTTTGG-3'
BAX	F: 5′-CCACCAGCTCTGAACAGTTCATGA-3'
	R: 5′-TCAGCCCATCTTCTTCCAGATGGT-3'

GAPDH, glyceraldehyde phosphate dehydrogenase; BAX, Bcl-2 Assaciated X protein; F, forward; R, reverse

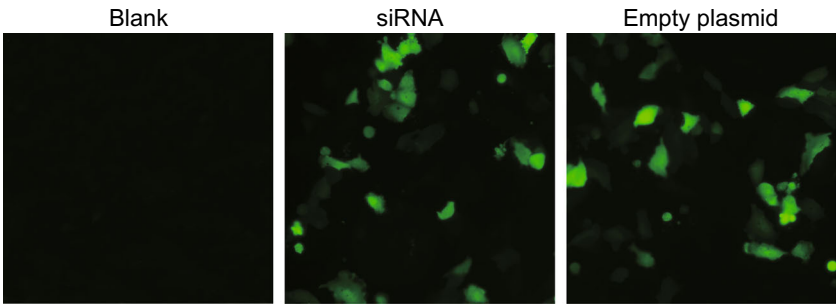

Fig. 1 The results of cell transfection among three groups (× 100)

the blank group. The fluorescence was observed under a fluorescence microscope 48 h after transfection. The positive cells were selected and cultured in fresh DMEM for extra 48 h. The cell suspension was prepared after trypsin digestion, and stored for subsequent experiments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from 1×10^7 cells of each group using the RNeasy Mini Kit (QIAGEN, GmbH, Germany), and then reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem, Foster, California, USA). The total reaction volume was 15 μL and reaction conditions were 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. The qRT-PCR was performed with the TaqMan Universal PCR Kit (Applied Biosystem, Foster, California, USA). The reaction volume of qRT-PCR was 20 μL and reaction conditions were pre-denaturalized at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. An ABI 7500 PCR machine (Applied Biosystem, Foster, California, USA) was used for qRT-PCR, and triplicate wells were set up in each experiment. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene, relative mRNA expressions of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

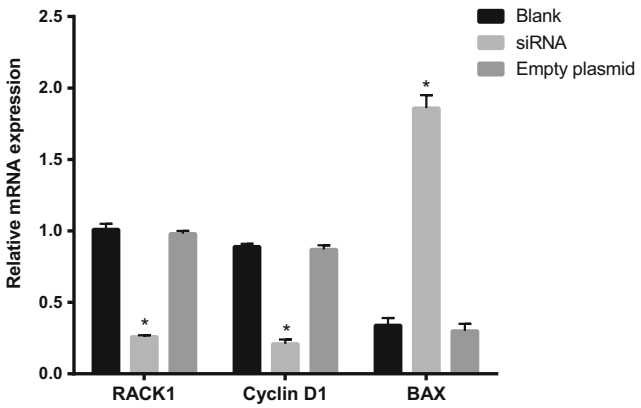

Fig. 2 qRT-PCR for determination of *RACK1*, *Cyclin D1* and *BAX* mRNA expressions. Note: *, $P < 0.05$, compared with the blank group and the empty plasmid group

Western Blotting

The transfected cells were washed with PBS and added into the cell lysis buffer containing protease inhibitors. After shaking at 4 °C for 5 min, the mixtures were centrifuged at 12000×g for 10 min at 4 °C. The supernatant was collected to detect protein concentrations using a protein assay kit and the protein concentration was adjusted to ensure equivalent loading of total protein. After addition of 6 × buffer, all samples were boiled and then stored at −20 °C. A sum of 50 μg proteins was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was subsequently transferred onto a nitrocellulose membrane. After blocking with milk, samples were incubated with addition of diluted mouse anti-human monoclonal antibodies (1:200) against RACK1 (MABT475), Cyclin D1 (CC12-100UGCN), BAX (AB2915) and GAPDH (SF-141) overnight. Washed 4 times for 10 min each with Tris-buffered saline Tween-20 (TBST), the membrane was incubated with addition of diluted IRDye™800DX-labeled goat anti-mouse IgG (1:10,000) at room temperature for 1 h. After washing again 4 times with TBST, samples were developed with a developer. All antibodies were purchased from Millipore Corp. (Billerica, MA, USA), and band densities were quantified using LabWorks Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA).

Cell Counting Kit-8 (CCK-8)

Cells in the logarithmic phase were selected and seeded into a 96-well plate at a density of 2×10^4/mL (100 μL /well). The cell viability was determined by CCK-8 at the time point of 24 h, 48 h and 72 h after cell culture, respectively. With the addition of 10 μL CCK-8 kits (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) per well, cells were incubated for 60 min. The plate was thoroughly shaken, and the optical density (OD) value was determined at the wavelength of 450 nm using a microplate reader (Thermo Fisher, USA).

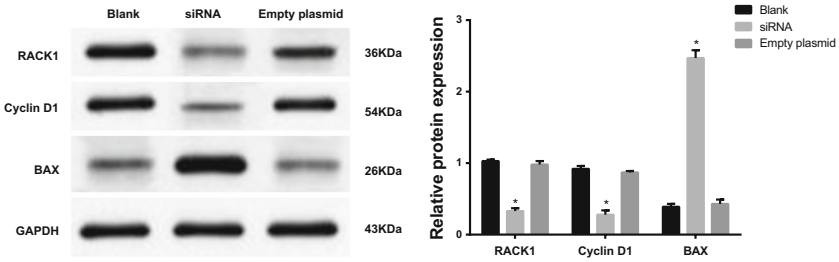

Fig. 3 Western blotting for determination of RACK1, Cyclin D1 and BAX protein expressions. Note: *, $P < 0.05$, compared with the blank group and the empty plasmid group

Flow Cytometry

The BD Annexin V-Enhanced Green Fluorescent Protein (EGFP) Kit (BD Bioscience, New Jersey, USA) was applied to detect the distribution of cell cycle. Cells were seeded into a 6-well plate at a density of 2×10^5 cells/well and cultured for 24 h. The supernatant was collected in a new tube (5 mL) and digested with an addition of 400 μL trypsin. When the cells turned into a round shape, the addition of supernatants terminated digestion. The mixture was centrifuged at 2000 rpm for 5 min, and then the supernatant was discarded. Cells were washed twice using PBS containing 5% bovine serum albumin (BSA). After the supernatant was discarded, cells were re-suspended with 300 μL PBS containing 5% BSA, followed by fixation with 700 μL absolute ethanol and reaction at −20 °C for 24 h. The mixture was centrifuged at 2000 rpm for 5 min, and the supernatant was discarded again. Fluorescein Isothiocyanate (FITC)-Annexin V staining was performed subsequently. Cells were washed with 1 mL PBS, and were re-suspended with 100 μL PBS, then incubated at 37 °C for 30 min with addition of 1 μL RNase A (10 mg/μL). Cells were stained with 300 μL Propidum Iodide (PI, 50 μg/mL) for 20 min prior to analysis. Cells were washed twice using PBS containing 2% BSA, and the supernatant was discarded. Cells were suspended with 500 μL Bingding Buffer. With addition of 5 μL Annexin V-EGFP and 5 μL PI, cells were stored at room temperature in darkness for 5 ~ 15 min. The distribution of the cell cycle was analyzed on a flow cytometer (FACSCalibur, BD Bioscience, New Jersey, USA).

The transfected cells were seeded into a 6-well plate and cultured in a 5% CO_2 incubator at 37 °C. When the cells were grown to 80% confluence, they were cultured in fresh DMEM. After cell digestion with ethylene diamine tetraacetic acid (EDTA)-free trypsin, 1×10^5 cells were collected. The cells were re-suspended with an addition of 500 μL Binding Buffer, followed by the addition of 5 μL of Annexin V-FITC and 5 μL of PI (KeyGen Biotech. Co., Ltd., Nanjing, China) in sequence. The reaction was carried out in darkness at room temperature for 5 ~ 15 min. Cell apoptosis was detected by a flow cytometer (FACSCalibur, BD Bioscience, New Jersey, USA). The early and late apoptotic cells were counted respectively, and the final apoptosis rates were calculated.

Statistical Analysis

SPSS19.0 software (Chicago, IL, USA) was used for the statistical analysis. Continuous variables were expressed as mean ± standard deviation (SD) ($\bar{x} \pm s$). Pair-wise comparisons were performed using the *t* test. Comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA) after a test for homogeneity of variance. Comparisons between mean values of groups were tested by the least significant difference (LSD)-*t* test. Two-tailed *P* values <0.05 were considered statistically significant.

Results

Transfection Efficiency of pGenesil-1-*RACK1* Plasmid in MHCC97-H Cells

According to the results of cell transfection shown in Fig. 1, the green fluorescent protein (GEP) was distinctly observed and relatively well-distributed in MHCC97-H cells of both the siRNA and empty plasmid groups, while no GEP was observed in that of the blank group. The transfection efficiency was approximately 73.5%.

Comparisons of mRNA and Protein Expressions of RACK1, Cyclin D1 and BAX among the Blank, siRNA and Empty Plasmid Groups

As shown in Fig. 2, the mRNA expressions of both RACK1 and Cyclin D1 were significantly decreased in the siRNA group when compared with these in the blank and empty plasmid groups (both $P < 0.05$). The siRNA group exhibited

Table 2 CCK-8 assay for determination of cell absorbance

	Blank	siRNA	Empty plasmid
24 h	3.42 ± 0.17	2.83 ± 0.13*	3.67 ± 0.18
48 h	3.92 ± 0.24	2.56 ± 0.11*	4.19 ± 0.23
72 h	4.18 ± 0.22	1.82 ± 0.08*	4.42 ± 0.20

*, $P < 0.05$, compared with the blank group and the empty plasmid group

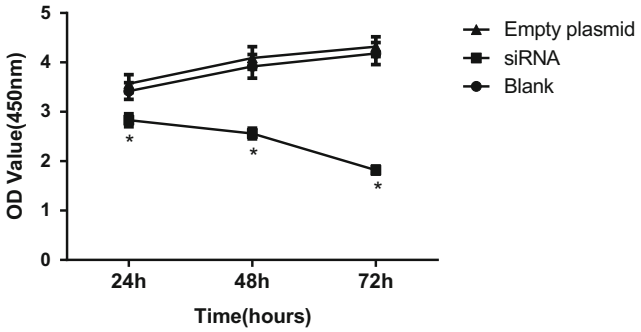

Fig. 4 CCK-8 assay for determination of the cell proliferation activity. Note: *, $P < 0.05$, compared with the blank group and the empty plasmid group; OD, optical density

a distinctly increased mRNA expression of BAX in comparison to the other two groups (both $P < 0.05$). Moreover, there was no significant difference of mRNA expressions of RACK1, Cyclin D1 and BAX between the blank group and the empty plasmid group (all $P > 0.05$). It was shown (Fig. 3) that RACK1 and Cyclin D1 protein expressions in the siRNA group were significantly lower than those in the blank and empty plasmid groups (both $P < 0.05$). The BAX protein expression in the siRNA group was evidently higher than that in the other two groups (both $P < 0.05$). The results showed no notable difference of RACK1 and Cyclin D1 protein expressions between the blank group and the empty plasmid group (both $P > 0.05$).

The Effect of *RACK1* Gene Silencing on the Proliferation of MHCC97-H Cells

The optical density (OD) values of the three groups were shown in Table 2 and Fig. 4. No marked difference of MHCC97-H cell proliferation was observed between the blank group and the empty plasmid group ($P > 0.05$). Compared with the blank and empty plasmid groups, the siRNA group exhibited significantly lower proliferation ability ($P < 0.05$). Moreover, OD values in the siRNA group indicated the proliferation ability in the siRNA group was gradually decreased in a time-dependent manner, thus postulating that the *RACK1* gene silencing might decrease the proliferation ability of MHCC97-H cells.

The Effect of *RACK1* Gene Silencing on the Cell Cycle of MHCC97-H Cells

As shown in Fig. 5, there was no obvious difference of cell cycles between the blank group and the empty plasmid group (all $P > 0.05$). Compared with the siRNA group, the percentages of cells in the G0/G1 phase and G2/M phase were not significantly different to those in the blank group and the empty plasmid group (all $P > 0.05$). However, the siRNA group presented a markedly lower percentage of cells in the S phase than the other two groups (both $P < 0.05$). These results implied that *RACK1* gene silencing could lead to delaying entry into S phase, which might further inhibit cell division.

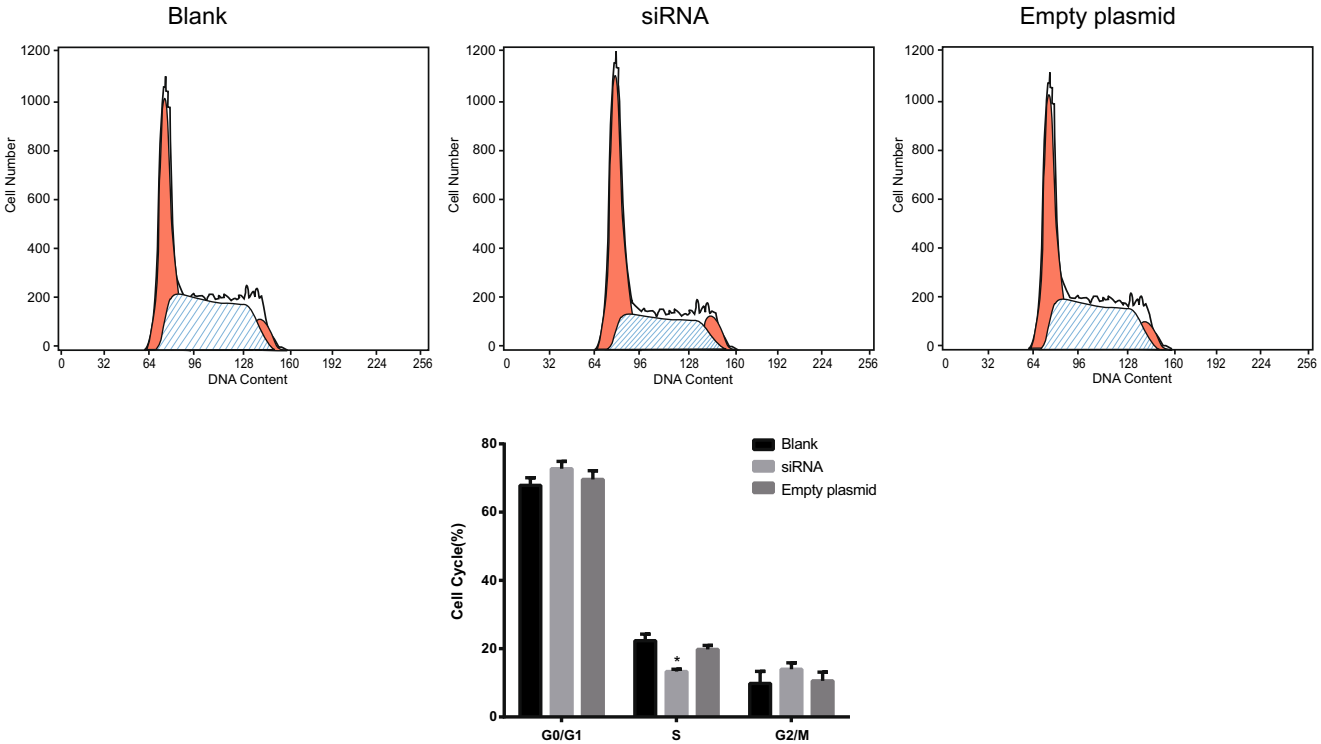

Fig. 5 Annexin V staining for determination of cell cycles. Note: *, $P < 0.05$, compared with the blank group and the empty plasmid group

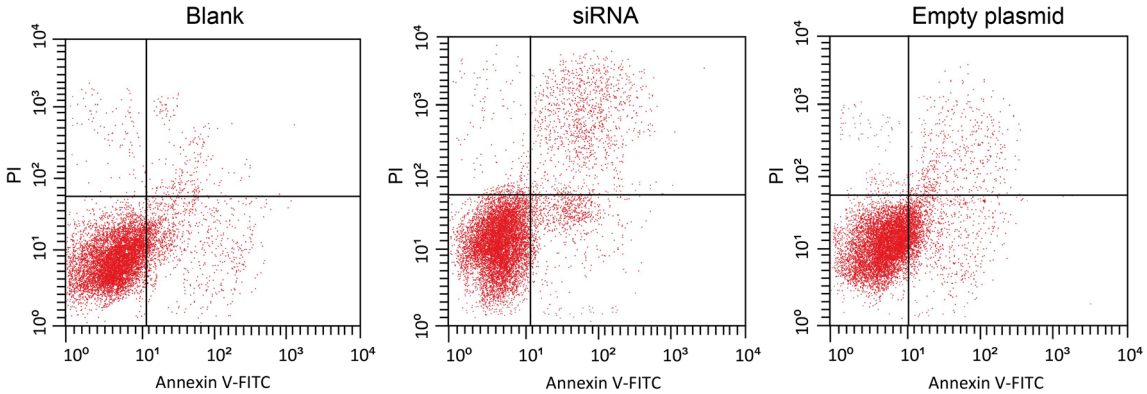

Fig. 6 AnnexinV-FITC/PI staining for determination of cell apoptosis

The Effect of *RACK1* Gene Silencing on the Apoptosis of MHCC97-H Cells

As shown in Fig. 6 and Table 3, there was no significant difference of cell apoptosis between the blank group and the empty plasmid group (all $P > 0.05$). The siRNA group showed a notably higher apoptosis rate than both blank and empty plasmid groups. In addition, the early apoptosis rate and the late apoptosis rate were markedly increased in the siRNA group when compared with these in the other two groups (both $P < 0.05$), thus implying that the *RACK1* gene silencing might induce cell apoptosis.

Discussion

Although liver transplant and surgical resection have been recommended clinically for the treatment of HCC, these therapeutic methods are commonly accompanied with high postoperative recurrence rates, and thus HCC remains a life-threatening disease in the majority of patients [7]. In order to comprehensively understand the mechanisms underlying the occurrence and development of HCC, while consequently provide guiding significance to the optimized HCC treatment as well as improve prognosis of HCC patients, it is of crucial importance to identify key genes associated with the proliferation, differentiation and migration of HCC cells. In this study, we explored the effects of *RACK1* gene silencing on proliferation, cell cycle and apoptosis of HCC MHCC97-H cells, and our results found that *RACK1* gene silencing could reduce proliferation and induce apoptosis of MHCC97-H cells.

Results of this study show that the expression of the cell cycle-related factor cyclin D1 was significantly decreased, and the expression of the apoptosis-related factor BAX was increased in the siRNA group when compared with the blank and empty plasmid groups, which might be explained by the interactions between RACK1 and Akt. RACK1 is a scaffold protein for many enzymes with the ability to interact with a wide variety of signaling proteins [16]. In certain signaling pathways, *RACK1* gene silencing could significantly suppress the constitutive phosphorylation of Akt and MARK [17]. Additionally, hypo-activation of Akt can also result in the release of cytochrome c from mitochondria, and an increase in the Bax/Bcl-2 ratio [18]. A previous study also demonstrated that siRNA-induced *RACK1* gene silencing could inhibit the proliferation, as well as invade and promote the apoptosis of human glioma U87 and CHG-5 cells [19].

Further results showed that the percentage of cells in the S phase was significantly decreased while the apoptosis rate was notably increased in the siRNA group when compared with those in the blank group and the empty plasmid group. Similarly, a previous study found that RACK1 could regulate cell proliferation by suppressing cell cycle regulators in G1 and delaying entry into S phase [19]. Rajput et al. also concluded that RACK1 inhibited the transcription of the cell cycle-related factor Cyclin D1 via inactivating Akt [18]. Cyclin D1 functioned as a positive regulator of the G1-S cell-cycle transition through binding and activating its kinase partner's cdk4/6 [21]. Besides,Vidya et al. found that RACK1 binding to the signaling molecule Scr could prolong the G0/G1 phase of the cell cycle, inhibited the G1-S cell-cycle transition, and thus reduced the number of cells in S phase [20].

Table 3 The results of cell apoptosis among three groups

	Early apoptosis rate (%)	Late apoptosis rate (%)	Total apoptosis rate (%)
Blank	7.88 ± 0.12	1.79 ± 0.18	9.73 ± 0.19
siRNA	16.56 ± 0.16*	5.38 ± 0.12*	21.94 ± 0.14*
Empty plasmid	8.04 ± 0.18	1.85 ± 0.14	9.89 ± 0.12

*, $P < 0.05$, compared with the blank group and the empty plasmid group

In addition, the study also indicated that compared with the blank group and the empty plasmid group, the siRNA group had significantly lower cell viability which further decreased over time. Epidermal growth factor receptor (EGFR) was closely related to cell viability, and inhibition of EGFR could cause a decrease of cell viability [22]. The inhibition of Akt revealed a similar concentration-dependent decline in cell viability as the EGRF inhibitor [23], thus postulating that *RACK1* silencing could suppress the phosphorylation of Akt, followed by the decrease of cell viability [17]. A recent report also demonstrated that blocking the phosphorylation of Akt by MK2206 could reduce the cell viability in two human cholangiocarcinoma cell lines, CCLP-1 and SG231 [24].

In summary, this present study demonstrated that *RACK1* gene silencing could inhibit HCC cell growth and proliferation, and induce HCC cell apoptosis. Our results provide an undisclosed insight into the pathogenesis of HCC and a new therapeutic target to improve postoperative survival rates and quality of life for HCC patients. Nevertheless, this study only investigated the in vitro effects of *RACK1* gene silencing on HCC cells. Further in vivo studies would be required for validation of our results.

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Compliance with Ethical Standards

Conflict of Interests None.

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