

Original Article

Suppression of CK-19 expression by shRNA can inhibit the malignancy of hepatocellular carcinoma cells

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Abstract: *Objective:* Cytokeratin-19 (CK-19) is highly expressed in a novel subtype of hepatocellular carcinomas (HCC) displaying both hepatocellular and cholangiocellular differentiation which we firstly defined as dual-phenotype HCC (DPHCC). Compared with patients with classical HCC, the patients with DPHCC showed worse clinical prognosis. However, the role of CK-19 in development of DPHCC remains unknown. The main purpose of the present study was to investigate the possible effect of CK-19 on the malignant phenotype of DPHCC and its possible value as a potential therapeutic target. *Methods:* In this study, we prospectively examined the CK-19 expression in 404 clinical HCC tissues and evaluated its clinical significance. We also evaluated the biological function of CK-19 both in vitro and in vivo using knockdown HCC cells. *Results:* Our results showed that CK-19 expression was significantly associated with TNM stage ($p = 0.011$) and vascular invasion ($p = 0.035$). Patients with positive CK-19 expression had poorer overall-survival and disease-free survival, whereas those with negative CK-19 expression survived longer. Knockdown of CK-19 can reduce the MHCC-97H cell proliferation ($p = 0.006$) and the number of colonies formed in soft agar ($p = 0.0043$). The number of MHCC-97H cells invading the matrigel coated membrane was decreased in CK-19 knockdown cells ($p = 0.038$). In addition, the in vivo experiments in mice showed the tumor weight was significantly reduced in CK-19 knockdown group (0.257 ± 0.081 g) compared with negative control (0.443 ± 0.114 g) ($P < 0.01$). Our findings demonstrated the biological function in HCC cell lines of CK-19 expression, since suppression of CK-19 inhibits the growth of tumor cells both in vitro and vivo. *Conclusion:* Taken together, our results implied that CK-19 not only could be a candidate pathobiological biomarker for evaluating the malignant extent of DPHCC, also could consider being a potential candidate therapy target in DPHCC.

Keywords: Cytokeratin-19, dual-phenotype HCC, prospective study, suppression, malignancy

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, and it is the third leading cause of death in the world [1, 2]. Liver cancer is characterized by high malignancy, rapid progression and frequent recurrence after resection [2]. HCC is a typical tumor with high heterogeneity and often accompanied with strong ability of invasion and metastasis [3]. Since HCC has heterogeneity, it is no doubt difficult to further classify HCC based on traditional pathology features such as histological types and cell differentiation. Therefore, it is always a hot topic to find the index that reflects the invasive ability and values the prognosis of HCC.

According to WHO histological classification, traditionally liver cancers have been classifi-

ed into three types: hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC), and combined HCC-CC [4]. Due to the heterogeneity of tumor cells, actually there are many kinds of molecular subtypes or pathological subtypes. As a result, there are obvious differences in the molecular phenotype and biological behavior even in one kind of tumor which harbors similar histological appearance.

As we know, based on the criteria of histopathology, HCC mainly express hepatocyte-specific markers and originates from hepatocytes. ICC mainly expresses biliary-specific markers such as cytokeratin-19 (CK-19) and originates from cholangiocytes [5]. Interestingly, we previously reported that about 10% of HCC with typical morphological features can strongly coexpress both hepatocyte markers (such as Hep Par-1) and cholangiocyte markers (such as

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CK-19) within the same HCC cells [6]. We firstly named this novel subset of HCC as dual-phenotype HCC (DPHCC). According to our study, patients diagnosed as DPHCC had significantly lower 1-, 3-, and 5-year OS rates compared with the pure HCC group (71 vs. 88% at 1 year; 38 vs. 61% at 3 years; and 27 vs. 48% at 5 years, respectively, $P = 0.035$). Additionally, the 1-, 3- and 5-year RFS rates in patients with DPHCC also is lower (60 vs. 78% at 1 year; 38 vs. 48% at 3 years; and 27 vs. 39% at 5 years, respectively, $P = 0.002$) [6]. And we also found that CK-19 expression can be used as an independent prognostic factor for overall survival and recurrence-free survival. Almost in the same year, a large-scale study on two different cohorts of HCCs demonstrated that CK-19 was well correlated with clinicopathologic features of tumor aggressiveness, compared to other stemness-related proteins. CK-19 positive HCCs exhibited more frequent major vessel invasion, increased tumor size and poor prognosis [7].

CK-19 is one of the biliary progenitor cell markers, which is a member of the keratin family [8]. Although many publications have suggested that CK19 is associated with prognosis in HCCs [9-11], little is known why these specific tumors often with aggressively behavior. It has been reported that CK-19 knockdown significantly reduced HCC invasive ability in vitro. Additionally, CK-19 positive HCCs express more invasion/metastasis-related genes compared with the CK-19 negative HCCs [12]. However, there is still no report on the effect of CK-19 inhibition on the growth of HCC cells in vivo. So it cannot fully explain the role of CK-19 in cell biological function. And there is not enough evidence to suggest that it can be used as a potential therapeutic target.

The objective of the present study was to further explore the molecular mechanism of CK-19 in HCC, thus explaining the role of CK-19 in malignancy phenotype of DPHCC, and to provide experimental evidence for CK-19 as a potential therapeutic target.

Materials and methods

Patient information and tissue specimens

A total of 404 diagnosed HCC patients at Eastern Hepatobiliary Surgery Hospital between

December 1, 2011 and March 30, 2012 were included in this prospective study. No patients had received radiotherapy, chemotherapy or other related anti-tumor therapies prior to the surgery. The clinical features of the patients were summarized in (Table 1). All patients provided information consent according to a protocol approved by the Institutional Review Board of Second Military Medical University. All patients were contacted by phone to check on their health status. The last follow-up date was December 1, 2016. The overall survival (OS) and disease-free survival (DFS) times were computed from the operation date to metastasis or recurrence date or the date of death, or the last censor time.

Cell lines and materials

Since CK-19 was reported to be upregulated in the highly metastatic HCC cell line (MHCC97-L), in this study we choose this cell lines to evaluate the effect of knockdown of CK-19. Human MHCC97-H liver carcinoma cells were obtained from American Type Culture Collection (Manassas, VA, USA), and the cells were subsequently cultured in RPMI-1640 medium (GIBCO, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (GIBCO, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 50 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) and 50 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.), and were maintained at 37°C with 5% CO_2 in a sterile incubator.

Construction of CK-19 shRNA sequence and transfection of the MHCC-97H cells

Three CK-19 shRNA oligonucleotide sequences purchased from Shanghai Genechem Company Ltd., (Shanghai, China) were identified and matched with the CK-19 cDNA sequence obtained from GenBank through a BLAST search (<http://www.ncbi.nlm.nih.gov/nucleotide/>). The shRNA sense sequence was: (GGAAGATCACTACAACA-AT). CK-19 shRNA was transfected into MHCC97H cells (1×10^5 cells/ml) at concentrations of 100 nM mediated by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The group design consisted of a negative control group (NC group) and a transfection group (KD group).

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Table 1. Patients and clinicopathological characteristics

| Variable | CK19 | | P |
|-----------------------|--------------------|-------------------|-------|
| | Negative (n = 380) | Positive (n = 24) | |
| Sex | | | 0.268 |
| Male | 331 (94.57%) | 19 (5.43%) | |
| Female | 49 (90.74%) | 5 (9.26%) | |
| Age | | | 0.106 |
| ≤50 | 207 (95.83%) | 9 (4.17%) | |
| >50 | 173 (92.02%) | 15 (7.98%) | |
| HBsAg | | | 0.603 |
| Negative | 45 (95.74%) | 2 (4.26%) | |
| Positive | 335 (93.84%) | 22 (6.16%) | |
| Serum AFP | | | 0.395 |
| ≤20 ng/ml | 127 (95.49%) | 6 (4.51%) | |
| >20 ng/ml | 253 (93.36%) | 18 (6.64%) | |
| Liver cirrhosis | | | 0.741 |
| No | 84 (93.33%) | 6 (6.67%) | |
| Yes | 296 (94.27%) | 18 (5.73%) | |
| TNM | | | 0.011 |
| I | 261 (96.31%) | 10 (3.69%) | |
| II | 59 (93.65%) | 4 (6.35%) | |
| III | 48 (85.71%) | 8 (14.29%) | |
| IV | 12 (85.71%) | 2 (14.29%) | |
| Child-pugh class | | | 0.944 |
| A | 363 (94.04%) | 23 (5.96%) | |
| B | 17 (94.44%) | 1 (5.56%) | |
| Tumor size | | | 0.393 |
| ≤3 cm | 110 (95.65%) | 5 (4.35%) | |
| >3 cm | 270 (93.43%) | 19 (6.57%) | |
| Tumor number | | | 0.119 |
| Single | 304 (95.00%) | 16 (5.00%) | |
| Multiple | 76 (90.48%) | 8 (9.52%) | |
| Tumor differentiation | | | 0.137 |
| Well | 53 (100.00%) | 0 (0.00%) | |
| Moderate | 308 (93.05%) | 23 (6.95%) | |
| Poor | 19 (95.00%) | 1 (5.00%) | |
| Vascular invasion | | | 0.035 |
| No | 349 (94.84%) | 19 (5.16%) | |
| Yes | 31 (86.11%) | 5 (13.89%) | |

Cell proliferation assay

Tumor cell viability was determined by MTT (Sigma-Aldrich, USA). Briefly, cells were plated in 96-well culture plates (2000 cells per well). After an attachment period of 24 h, the cells were cultured in the presence of shRNA oligonucleotides or negative control oligonucleotides for additional 5 days. At the time of 1 day, 2 day, 3 day, 4 day and 5 day, the cells

were incubated with MTT for 4 h at 37°C. After incubation the supernatant was aspirated and formazan products were solubilized with dimethyl sulphoxide. The staining intensity was measured by determining the absorbance at 492 nm. In this work, cell viability is expressed as the percentage of viable cells relative to the controls. We completed each experiment at least three times, each with triplicate samples.

Soft agar colony formation assay

The MHCC-97H cells transfected with shRNA or negative control oligonucleotides were suspended in concentration of 1×10^3 cells/ml. Medium mixed with 5% soft agar (Hyclone, Logan, MA, USA) at a ratio of 1:9 was added to the culture dishes. Then keep at room temperature for solidification. Additionally, 1.5 ml cell suspension was mixed with an equivalent volume of 5% soft agar in a 5% CO₂ incubator at 37°C for 2 weeks. Colony formation conditions and rates were observed using the following formula: Colony formation rate (%) = (colony number/incubated cell number) × 100.

In vitro cellular invasion test

Transwell chamber models (Corning Coster, Cambridge, MA) were employed to perform an in vitro cell invasion test. The MHCC-97H cells were transfected with shRNA or negative control oligonucleotides for 24 h. Then 5×10^4 pretreated cells in serum-free DMEM were added to the upper chamber (8 μ pore size) coated with Matrigel inserted in a well of a 24-well plate. DMEM containing 10% FBS was added to the lower chamber of each well. At 48 h post-incubation, the cells on the upper chamber were wiped off and the cells that had reached the underside of the membrane were fixed with paraformaldehyde (4%) and stained with Giemsa (10%) for 10 min. The cells that located on the underside of the filter (5 fields/filter) were counted.

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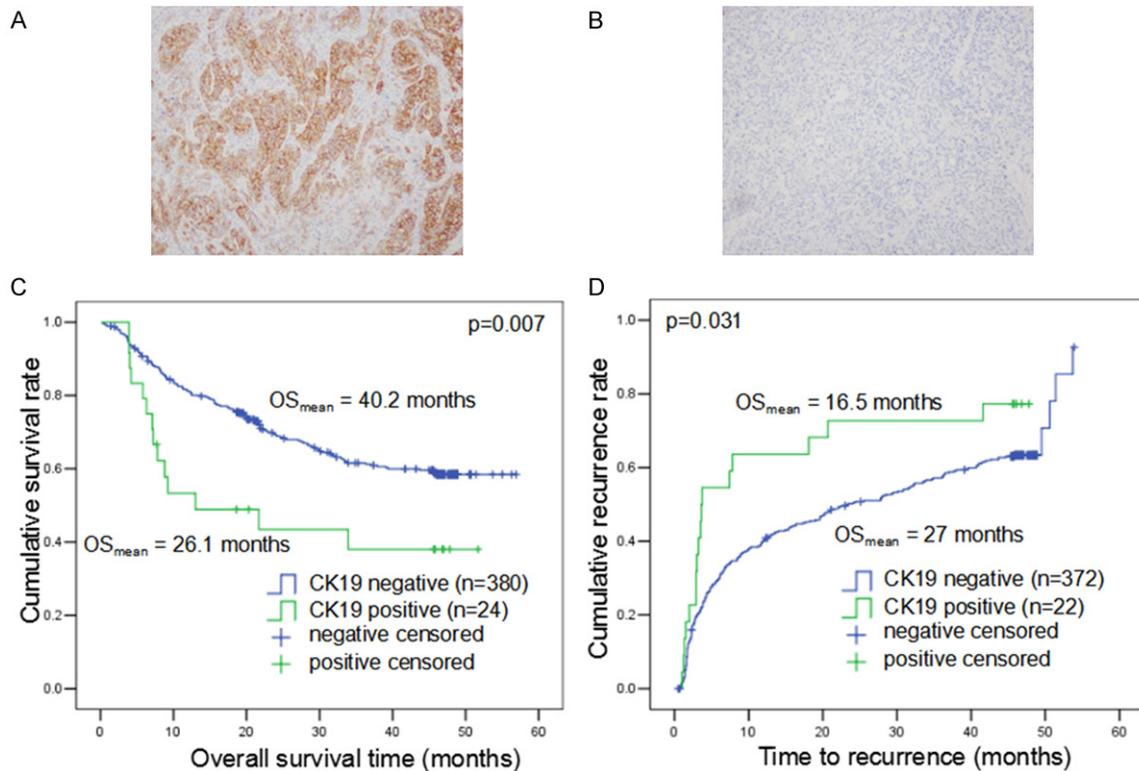


Figure 1. Positive CK-19 expression is correlated with poor overall survival and less time to recurrence. A. CK-19 positive tumor. B. CK-19 negative tumor. C. Patients with positive CK-19 expression had a significantly shorter overall survival. D. Patients with positive CK-19 expression had a significantly less time to recurrence.

Western blot analysis

Protein was extracted from the lysate and equal amounts of total proteins were separated by 10% SDS/PAGE. Then the proteins were transferred to polyvinylidene difluoride membranes (Millipore) using a Bio-Rad SemiDry apparatus. The membranes were blocked for 2 h with 5% nonfat milk in TBS containing 0.1% Tween 20 (TBST). Then the membranes were incubated with the primary antibodies (mouse anti-CK19 and rabbit anti-GAPDH) over night at 4°C. After wash of primary antibodies, the HRP-conjugated secondary antibodies were added and incubated 1 h at room temperature. After washing three times in TBST, the membrane was incubated with Chemiluminescent Detection Reagent for 5 min and exposed to X-ray film.

Real-time quantitative reverse transcription PCR

Transfection effect was validated by qPCR. Total RNA from cells was isolated using TRIzol

reagent (Invitrogen Life Technologies, USA). The first-strand complementary DNA was synthesized using Reverse Transcription System Kit (TAKARA, Japan). For PCR, SYBR green dye (TAKARA, Japan) with a 7900HT Detection System (Applied Biosystems) was used and normalized to GAPDH.

Tumor xenograft in nude mice

Male BALB/C nude mice (5- to 6-week-old) were obtained from Shanghai Institute of Materia Medica (Chinese Academy of Sciences, Shanghai, China). The *in vivo* experiments were performed according to protocols approved by the institutional animal care and use committee at the Second Military Medical University. MHCC-97H hepatocellular cancer xenograft model was established as described previously. Briefly, MHCC-97H cells ($1-2 \times 10^6$) transfected with shRNA or negative control oligonucleotides were injected subcutaneously into the right side of axillary of nude mice. And the 24 mice were randomly assigned to two experimental groups (12 mice/group): negative con-

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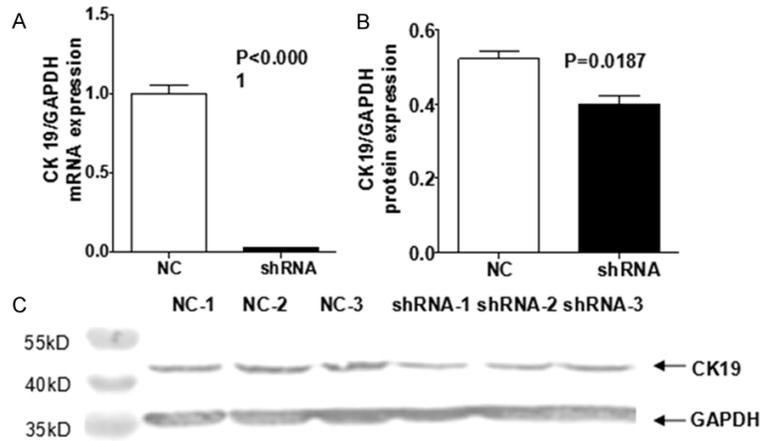


Figure 2. The expression of CK-19 was inhibited by shRNA transfection. A. CK-19 expression in MHCC-97H cells was detected by real-time PCR. B. CK-19 expression in MHCC-97H cells was detected by western blot. C. Relative protein expression of CK-19 in MHCC-97H cells.

control (NC) group and (KD) group. The length and width of tumor xenografts were measured once a day by calipers using the formula: tumor volume = (length × width²)/2. On the day 15, all the mice were sacrificed to obtain fresh tumor tissues. The tumor size and weight were measured.

Stastic analysis

Stastic analysis was performed with SPSS 15.0 software. In vitro cell proliferation, apoptosis, migration, invasion assays and tumor volumes were analyzed by Student's t-test. $P < 0.05$ were considered statistically significant.

Results

Relationship between CK-19 and HCC patients' survival

The significance of CK-19 in prognosis was further validated in HCC patients. The immunohistochemical results of 404 HCC tissues are summarized in (Table 1). The result showed that 24 of 404 (6.32%) HCC tissues have positive CK-19 expression. We found that positive CK-19 expression was significantly associated with TNM stage ($P = 0.011$) and vascular invasion ($p = 0.035$). Kaplan-Meier analysis and log-rank test were used to analyze the relationship between CK-19 expression and overall survival. The Results showed that patients with positive CK-19 expression had poorer OS than those with negative expression (Figure 1C, long-rank test: $p = 0.007$). The postopera-

tive median OS of patients with negative staining of CK-19 was 40.2 months, while that of patients with positive staining of CK-19 was only 26.1 months. Significant difference was also observed in the DFS curve between negative and positive group (27 months VS. 16.5 months, Figure 1D, Long-rank test: $p = .035$).

Inhibition of CK-19 by shRNA in MHCC-97H cell lines

Among hepatocellular carcinoma cells, CK-19 was expressed higher in MHCC-97H cell lines. So in the following ex-

periment, we used MHCC-97H as the main experimental cell lines. MHCC-97H cells were transfected with CK-19 shRNA or control shRNA for 48 h. The efficiency of transfection was confirmed by Western assay. It showed that CK-19 protein levels decreased substantially in MHCC-97H cell lines following shRNA transfection (Figure 1).

Knockdown of CK-19 inhibited proliferation of MHCC-97H cells

To further investigate the role of CK-19 in HCC cells proliferation, after an attachment period of 24 h MHCC-97H cell lines were transfected with CK-19 shRNA or negative control oligonucleotides for additional 5 days. The MTT test showed that the number of reactive cells in CK-19 sh-RNA group was almost the same from day 1 to day 5 (Figure 2). At the day 5, the living cells in negative control group was 2 times as much as that of the shRNA group ($P = 0.006$). In soft agar colony formation assay, more colonies were observed in CK-19 knockdown cells than in negative control cells ($p = 0.0043$).

Knockdown of CK-19 suppressed MHCC-97H cells invasion

Invasion assay in vitro were carried out to evaluate the effects of CK-19 on MHCC-97H cell mobility. In the invasion assay we found that MHCC-97H cells in CK-19 shRNA group and negative control group both can pass through the invasion chamber, suggesting the potential

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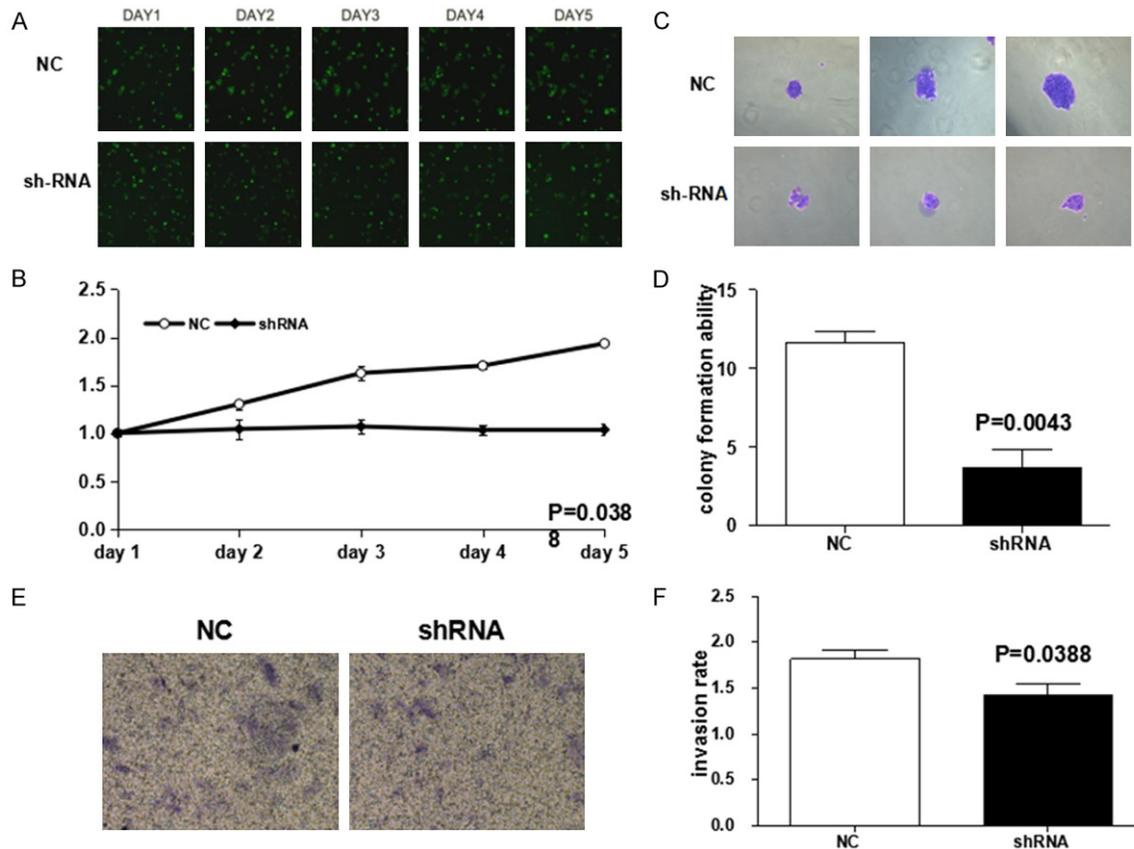


Figure 3. CK-19 knockdown reduced cell proliferation and invasion. A. MHCC-97H cell lines were transfected with CK-19 shRNA or negative control oligonucleotides for additional 5 days. B. Growth curve of MHCC-97H cells transfected with CK-19 shRNA or negative control oligonucleotides. C, D. Colony formation of MHCC-97H cells in soft agar was assessed after transfected with CK-19 shRNA. The number of colonies formed in soft agar was less in CK-19 knockdown cells than in NC cells. E. The number of cells passed through membrane was less in CK-19 knockdown cells than in NC cells. F. The bar chart demonstrated the ratio of cells passed through membrane in shRNA and negative control groups.

of invasion of MHCC-97H cell lines. However, CK-19 shRNA significantly reduced the number of MHCC-97H cells invading the matrigel coated membrane, with 0.8-fold decrease compared with the untreated controls ($P < 0.05$, **Figure 3**).

In vivo effects of CK-19 on tumor growth

In order to determine whether the effect of CK-19 in MHCC-97H cell line has relevant clinical implications, we used a xenograft tumor model to test the *in vivo* effect of CK-19 on the tumor growth. In the period of observation a tumor growth-curve of tumor volume according time in every groups was obtained. Obvious tumor growth was observed on day 7 and day 9 after the injection in negative control group and CK-19 knockdown group respectively. Compared with negative control group, CK-19

knockdown group showed inhibition of tumor growth. The mice were sacrificed 2 weeks after establishing models. As shown in (**Figure 4**), the size of the tumors of the CK-19 shRNA group ($330 \pm 103 \text{ mm}^3$) were smaller than those of the negative control group ($640 \pm 173 \text{ mm}^3$). Additionally, there was a striking difference in the weight of tumor between CK-19 knock-down group (with average tumor weight of $0.257 \pm 0.081 \text{ g}$), and negative control group (with average tumor weight of $0.443 \pm 0.114 \text{ g}$) ($P < 0.01$). The rate of tumor growth inhibition index by the CK-19 knockdown was 42%.

Discussion

As a unique novel pathological subtype, DPHCC has the features of classical histopathological features of HCC, but can reveal both hepatocyte and cholangiocyte phenotypes within the

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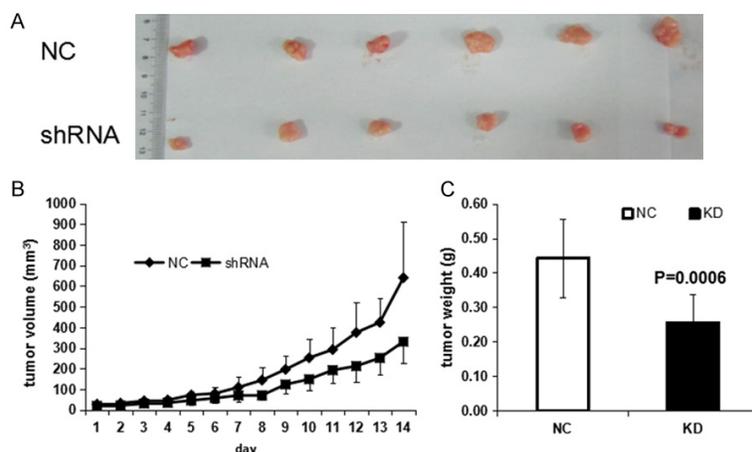


Figure 4. CK-19 knockdown in MHCC-97H cell inhibited growth of subcutaneous xenografts. A. Size of subcutaneous xenografts in negative control group was larger than those in shRNA group. B. Tumor growth-curve of tumor volume according time in CK-19 shRNA group and in negative control group. C. Average tumor weight of each group on 2 weeks after tumor injection.

same cells. Compared with typical HCC, DPHCC showed significant malignant biological behaviors on the cell differentiation, metastasis recurrence and long-term prognosis [12]. Therefore, it is an important research topic that how to diagnose the heterogeneity of HCC and urgently required to find new therapeutic strategies that effectively control the growth of HCC cells.

CK-19 is often expressed in cholangiocytes, hepatic progenitor cells (HPCs) and early hepatoblasts [2]. Although CK-19 is an immunopathological marker for diagnosis of ICC, our previous study showed that the positive rate of CK-19 in HCC was 10% [6]. Thus we believe that DPHCC is a special subtype of HCC, which may be derived from the bidirectional differentiation of HPC. Previous researches of HCC have shown that CK-19 positive leads to poor prognosis. Herein, we depicted a similar phenomenon by prospective study. In addition, although much evidence has demonstrated that CK-19 expression correlates with clinical features, the biological functions of CK-19 in HCC cell lines have not been fully investigated [13, 14].

Therefore, in present study MHCC-97H cell lines with high CK-19 expression was used to establish CK-19 knock-down cell models. From these results, we found that CK-19 knockdown in HCC cells significantly reduced proliferation and colony formation in vitro. By xenograft

models we confirmed that knockdown of CK-19 obviously inhibited tumor growth in vivo. Both the tumor volume and tumor weight of the HCC xenografts in nude mice were remarkably inhibited by CK-19 Sh-RNA transfection. To our knowledge, our results firstly demonstrated the role of CK-19 in the proliferation of HCC cell lines by xenograft experiment.

By transwell experiment we found that reduced cell migration was observed in the CK-19 knockdown cells. Since CK-19 knockdown significantly reduced HCC invasive ability in vitro, our findings further

demonstrated the functional consequences of CK-19 expression which is consistent with several previous studies. [12]. Moreover, the role of the cytoskeleton and related proteins in the tumor formation has already been demonstrated [15, 16].

Our present data clearly showed that CK-19 plays an important role in the proliferation and invasiveness of HCC cell lines. However CK19 has not been found as an oncogene in HCCs so far since it is also expressed in non-malignant cells [17]. It is also has been reported that CK-19-associated gene expression profiles are in common with the genes in other more malignant HCC subtypes reported previously [12]. The abnormal expression of NOTCH1, JAG1, DTX1 as well as the TGF- β signal pathway was induced by the inhibition of CK-19 in vitro [18, 19]. The Notch signal pathway was thought to be involved in various malignant tumors, including HCC [21]. Furthermore, it has been reported that CK-19 positive HCCs often express more invasion/metastasis-related gene compared with the CK-19-negative HCCs [12].

At present, widely used in the treatment of liver cancer drugs are Sorafenib, with VEGF, PDGFR, and EGFR as the target [21]. So far we still lack of objective evidence to guide the therapy by detection of target molecular in liver cancer. Hence CK-19 may provide a potential therapeutic target for DPHCC. Our studies demonstrate the CK-19 can inhibit the proliferation

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and invasion of HCC in cellular and animal level. We and other research provided the evidence of high expression of CK-19 in HCC malignant biological behavior from the clinical, cellular and animal level respectively, further strengthened its role as a molecular target for the treatment [6, 7, 12]. CK-19 has been recognized as an index to evaluate the prognostic, but it is necessary to further explore the CK-19 specific inhibitors for the target therapeutic experiment.

In conclusion, we have revealed that knock-down of CK-19 expression by shRNA could inhibit the malignancy both in vivo and in vitro in MHCC-97H cell lines. Our study provided more evidence for the critical role of CK-19 in the pathogenesis of DPHCC, especially in tumor growth and metastasis. CK-19 not only could be a biomarker for dividing HCC patients into different subgroups, but also a potential molecular target for HCC treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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