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Original article

CRKL knockdown promotes *in vitro* proliferation, migration and invasion, *in vivo* tumor malignancy and lymph node metastasis of murine hepatocarcinoma Hca-P cells

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ABSTRACT

Our previous study (Biomed Pharmacother 2015;69:11) demonstrated that the over-expression of CRKL, a chicken tumor virus number 10 regulator of kinase-like protein, suppresses *in vitro* proliferation, invasion and migration of murine hepatocarcinoma Hca-P cell, a murine HCC cell with lymph node metastatic (LNM) rate of ~25%. In current work, we investigated the effects of CRKL knockdown on the *in vitro* cell proliferation, migration and invasion, and on the *in vivo* tumor malignancy and LNM rate and level for Hca-P cells. Western blotting assay indicated that CRKL was down-regulated by ~90% in a monoclonal CrkL-shRNA-transfected Hca-P cells. Compared with Hca-P and unrelated-shRNA-transfected Hca-P cell, the *in vitro* proliferation, migration and invasion potentials were significantly enhanced following CRKL stable deregulation. CRKL knock-down significantly promoted the tumorigenicity malignancy, LNM rates and level of Hca-P-transplanted mice. Consistent with our previous work, it can be concluded CRKL plays an important role in hepatocarcinoma cell proliferation, invasion and migration as well hepatocarcinoma malignancy and metastasis. It functions as a potential tumor suppressor in hepatocarcinoma.

1. Introduction

Metastasis is a major biological characteristic of malignant neoplasms and is also one of the main causes of cancer related deaths [1,2]. Lymph node metastasis (LNM) is associated with a 50% reduction in a favorable prognosis [3]. The study on LNM in cancers contributes to early metastasis detection and track cancer progression [4]. Characterized with high recurrence, metastasis and poor prognosis [5], hepatocarcinoma is one of the leading causes of cancer-related deaths [6]. Initial LNM of epithelial carcinoma commonly results in poor survival and prognosis of cancer patients [7,8]. The study of LNM helps in better management of hepatocarcinoma.

The CT10 regulation of kinase (CRK) protein family functions as adapter proteins in intracellular signal transduction. The cellular homologues CrkI and CrkII, and the related Crk-like (CrkL) are ubiquitously expressed and conserved across eukaryotic organisms [9–13]. CRKL commonly localizes in multiple intracellular compartments mapped at 22q11.21 encoding a protein of 36 kDa [14]. CRKL deregulation is involved in the development and progression of a variety of cancers including gastric cancer (GC), glioblastoma multiforme (GBM), hepatocellular carcinoma (HCC), bladder cancer, lung cancer, colon cancer, ovarian cancer, leukemia, breast cancer, head and neck cancer, rhabdomyosarcoma and neuroblastoma [15–22]. Nevertheless, the role and action mechanism of CRKL in tumor progression are still unclear.

Our previous work indicated CRKL was linked to murine hepatocarcinoma [23]. Its overexpression in Hca-P, an initial (low) and specific lymph node metastasis (LNM) murine hepatocarcinoma cell line [2,4,23–28], suppresses the *in vitro* proliferation, migration and invasion abilities of Hca-P cells. In current work, we continuously validated the potential tumor suppressor role of

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CRKL playing in hepatocarcinoma. Using RNAi silencing technique, we successfully down-regulated CRKL in Hca-P by obtaining monoclonal CRKL-shRNA-Hca-P cells. Consistently, the stable knockdown of CRKL increased the *in vitro* proliferation, migration and invasion capacities of Hca-P cells. More importantly, the *in vivo* tumorigenicity malignancy, LNM rate and level of Hca-P-bearing mice were also significantly enhanced following the down-regulation of CRKL.

2. Materials and methods

2.1. Hca-P cell line, experimental animal and ethics statement

Mouse hepatocarcinoma Hca-P cell line with lymph node metastasis (LNM) rate ~25% was created by the Department of Pathology, Dalian Medical University and maintained in our laboratory. Hca-P cells was inoculated and purified in abdominal cavities of inbred Chinese 615-mice [aged 6 weeks, 20 ± 2 g; Certificate of quality number: SCXK (Liao) 2008-0002] [23]. The purified Hca-P cells were then cultured in RPMI 1640 (Gibco, USA) supplemented with 15% fetal bovine serum (PAA, Australia) at 37 °C with 5% CO_2.

Inbred Chinese 615 mice were provided by the SPF Animal Laboratory Center of Dalian Medical University. Mice were treated and sacrificed following the protocols approved by the Experimental Animal Ethical Committee of Dalian Medical University (Permit Number: L2012012).

2.2. Establishment of CRKL knockdown monoclonal Hca-P cell line

Targeting the gene sequence of *CrkL* (GenBank: NM_007764) starting at 732, the siRNA sequence GAGAGTACCTTGTGCTTAT was designed as the interference sequence by using Whitehead and SiDirect softwares in accordance of the sequence specificity, thermal dynamic parameters and GC content [19]. An unrelated targeting sequence GTTCTCCGAACGTGTCACGT was also designed as a negative control shRNA (NC-shRNA). The corresponding shRNAs for CRKL and NC were then inserted into the pGPU6/GFP/Neo vectors according to our established methods [29,30], named as CRKL-shRNA-pGPU6/GFP/Neo (CRKL-shRNA) and NC-shRNA-pGPU6/GFP/Neo (NC-shRNA), respectively. 1×10^5 Hca-P cells/well were seeded into a 24-well plate in 500 μL of RPMI-1640 without antibiotic and cultured in a humidified environment at 37 °C with 5% CO_2 for 24 h. Transfection was performed on the following day when the cell reached 70% confluent with Lipofectamine™ 2000 (Life technologies, USA) according to the manufacturer's instructions. CRKL-shRNA-pGPU6/GFP/Neo and NC-shRNA-pGPU6/GFP/Neo vectors were then transfected into Hca-P cells. Stably transfected cells were screened against 400 μg/ml G418 for about 2 weeks, then the monoclonal CRKL-shRNA-Hca-P and NC-shRNA-Hca-P were obtained by limited dilution screening. CRKL knockdown in CRKL-shRNA-Hca-P cells were determined by Western blotting and compared with NC-shRNA-Hca-P and Hca-P cells, respectively.

2.3. SDS-PAGE and Western blotting

Pellets of Hca-P, NC-shRNA-Hca-P and CRKL-shRNA-Hca-P cells were obtained with centrifugation at 1000 rpm for 5 min. Pellet from each group cells was suspended and ultrasound sonicated in ice-cold 1 ml lysis buffer (KeyGEN, China) containing l μL 100 mM PMSF, 2 μL phosphate inhibitors and 0.2 μL protease inhibitor. The supernatants were collected by centrifugation at 12,000 rpm for 15 min at 4 °C. Protein concentration was determined using BCA quantization kit (Beyotime, China). 12% SDS-PAGE was performed for equal amounts of samples from three group cells. Protein bands were transferred onto nitrocellulose (NC) membrane (Pall Corporation, USA) and blocked by 5% skim milk (TBST) for 3 h at RT. The NC membrane was then incubated with primary antibodies shaking with 100 rpm at 4 °C overnight. The primary antibodies were CRKL (1:1000, Santa Cruz Biotechnology, USA) and β-actin (1:5000, TransGen Biotech, China). Being washed well with TBST 3× 10 min, incubated with peroxidase-conjugated goat Anti-Rabbit/Anti-Mouse IgG (1:1000, ZSGB-BIO, China) for 1 h at 37 °C, washed with TBST buffer 3× 10 min, the NC membrane bound protein bands were visualized by ECL (Adavansta, USA) and detected by Bio-Rad ChemiDoc™ MP imaging system (Bio-Rad, USA). The relative protein levels were calculated using β-actin reference.

2.4. Cell proliferation assay

The effect of CRKL down-regulation on Hca-P cell proliferation was evaluated using cell counting kit-8 (CCK-8 kit, Dojindo, Japan). The Hca-P, NC-shRNA-Hca-P and CRKL-shRNA-Hca-P cells were seeded into a 96-well plate at a density of 1500 cells in 100 μL RPMI-1640 containing 15% FBS (PAA, Australia) and incubated at 37 °C with 5% CO_2. 10 μL of CCK-8/well were added into each of the corresponding wells at the time intervals of 0, 24, 48, 72 and 96 h and continuously incubated at 37 °C with 5% CO_2 for 1.5 h. The absorbances at 450 nm were recorded using a microplate reader (Thermo, USA) for quantifying the relative cell numbers.

2.5. In vitro *cell migration assay*

Boyden transwell chamber assay was performed to measure the effect of CRKL knockdown on the migration capacity of Hca-P cells. 24-well transwell units with 8 μm I.D. polyester membrane plates (Corning, USA) were used. The filters surfaces were coated with 6 μL fibronectin (0.5 mg/ml, EMD Millipore, USA). 500 μL RPMI 1640 containing 20% PAA was added into each of the lower chamber compartments. Cells suspended in RPMI 1640 from each group were seeded into the upper chamber at density of 5×10^4 cells/200 μL per well and incubated in 5% CO_2 at 37 °C for 24 h. Non-migrated cells at the upper surface of filter were wiped out with cotton swabs. The cells migrated into the lower filter surface were fixed with 4% paraformaldehyde for 20 min, stained with 0.1% crystal violet for 20 min and washed with PBS. Then, the cells were counted by selecting 5 random fields per well under a light microscope at a magnification of 100×. Triplicate experiments were repeated for each assay.

2.6. Boyden transwell invasion assay

The 8 μm I.D. polyester membrane plates' surfaces of 24-well transwell units were coated with 50 μL ice-cold ECM gel (1:5 dilution with RPMI 1640, Sigma–Aldrich, USA), incubated at 37 °C for 8 h and then dried at RT for 30 min. Then, the lower surfaces of the filters were coated with 6 μL fibronectin (0.5 mg/ml, EMD, Millipore, USA). 5×10^4 cells were suspended in 100 μL RPMI 1640 and loaded into the upper chamber with 500 μL RPMI-1640 containing 20% FBS (PAA, Australia) in the lower chamber and incubated in 5% CO_2 at 37 °C for 36 h. The non-invaded cells were wiped and the invaded cells were fixed, stained, counted and processed as described in migration assay.

2.7. In vivo *tumorigenicity and LNM rate assays*

The effect of CRKL knockdown on the *in vivo* tumorigenicity malignancy and LNM rate and level of Hca-P cells were investigated using Hca-P transplanted mice. Twenty-one 615 mice