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Original article

Downregulation of CDK-8 inhibits colon cancer hepatic metastasis by regulating Wnt/β-catenin pathway

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ABSTRACT

Liver metastasis is a major cause of mortality from colon cancer. To investigate the role of cyclin-dependent kinase 8 (CDK8) in the progression of colon cancer hepatic metastasis. In this present study, human colon cancer HCT116 or HCT116-LUC-GFP cells were transfected with Lentiviral vector-mediated knockdown of CDK-8. After transfection, metastasis and invasion potential of colon cancer cell was investigated by wound healing and transwell invasion assays, respectively. A mice model of colon cancer liver metastases was established and observed with bioluminescence imaging. The protein expression of CDK-8, β-catenin, E2F1, MMP-7 and E-cadherin in liver tissues were detected by Western Blot. Our results revealed that lentiviral vector-mediated knockdown of CDK-8 inhibited metastasis and invasion of colon cancer cells *in vitro* and in vivo, respectively. Protein expression of CDK-8, β-catenin, MMP-7 and E-cadherin were inhibited, but protein expression of E2F1 was enhanced. In sum, our data provided compelling evidence that CDK-8 played a significant role in colon cancer hepatic metastasis by regulating the Wnt/β-catenin signal pathway and might sever as a potential therapeutic target for colon cancer patients.

1. Introduction

Colon cancer is the second most commonly diagnosed cancer in females and the third in males [1]. Despite advanced understanding the initiation and progress of colon cancer, the incidence of colon cancer is continually increasing. Hepatic metastasis from colon cancer patients are very common even after the surgery [2,3]. Therefore, it is urgent to find new treatments to prevent liver metastasis from colon cancer are vital to improve survival and life quality of these patients.

Activation of the Wnt/β-catenin pathway occurs in the majority of colon cancers and defines as a key event in the development of colon cancer [4–6]. Growing evidence revealed that cyclin-dependent kinase 8(CDK-8), a CDK component of the Mediator complex, could imply in the transcriptional regulation of key pathways of colon cancers, including Wnt/β-catenin [7]. CDK-8 acts as a colon cancer oncogene that is necessary to regulate β-catenin dependent transcription and oncogenesis [8,9]. Taken together, these observations suggested that therapeutic interventions that target CDK-8 of colon cancer may be of clinical value. RNA interference (RNAi), which has been proven to be a powerful tool for suppressing gene expression, may provide a promising way forward in making this pathway a particularly attractive target therapy [10–13]. In this study, we explored the role of CDK-8 in colon cancer and found that knockdown of CDK-8 inhibited cell metastasis and invasion *in vitro* and *in vivo*. In addition, we explored the underlying mechanism of CDK-8 functions in colon cancer by regulating Wnt/β-catenin pathway. Taken together, our data demonstrated that CDK-8 was indeed an oncogene in colon cancer, and the study also shed light on the molecular mechanisms of its pro-metastasis function through the Wnt/β-catenin signaling pathway.

2. Materials and methods

2.1. Ethics statement

The study was carried out in compliance with the guidance suggestion of Animal Care Committee of Guangzhou Medical University [Permit Number: SYXK (Guangdong) 2010-0104]. The study protocol was approved by the ethics committee of Guangzhou

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medical college. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Cell culture and transfection

Human colon cancer HCT116 cells that transduced with lentiviral vectors transfer of luciferase and green fluorescent protein (GFP), the human colon cancer HCT116-LUC-GFP cells were developed, which has described in our previous study [14], were grown in Dulbecco's Modified Eagle Medium (DMEM, Gbico, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100units/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air.

Cells were transfected with lentiviral vectors encoding short hairpin RNA targeting human CDK-8 for CDK-8 knockdown (shCDK-8) or a scrambled shRNA as negative control (shNC) (Sigma–Aldrich). Multiplicity of infection was 10. Cells were cultured for 72 h after transfection. Cells were grown to 80% confluency in 60-mm dish.

2.3. Transwell invasion assays

To assess the invasion ability of cells, transwell invasion assay was performed as our previous study [15]. 1×10^5 cells in serum-free medium were plated into top chamber, which was pre-coated with BioCoat Matrigel Invasion Chambers (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Following incubation in a saturated humidity incubator at 37 °C for 24 h, the membranes were fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet for 10 min. Cells passing through the membranes were counted under a light microscope. The average values were calculated and compared.

2.4. Wound healing assays

Log-phase cells were collected after trypsinization and inoculated into a 6-well plate at 5×10^5 cells per well. The plates were cultured for 24 h to ~100% confluency in a monolayer. A scratch wound was made using a yellow pipette tip vertically across each well and washed three times with serum-free DMEM to remove the cell debris. Images of the wells were taken under an inverted microscope. The distance from one side of the scratch to the other was measured at different intervals using Image Pro-Plus 6.0 software (Media Cybernetics, USA).

2.5. Animals and assay for Experimental Liver Metastasis

Four-to-six week old male nude mice (BALB/C nu/nu) were purchased from the Sun Yat-Sen University's Laboratory Animal Center (Guangzhou, People's Republic of China). The mice were maintained in the laboratory of Guangzhou Medical University for animal experimentation in a Specific Pathogen Free (SPF) environment, at a temperature of 22–25 °C, in laminar air-flow conditions with a 12-h light–dark cycle. All animals had free access to standard laboratory mouse food and water.

HCT116-LUC-GFP cells transfected with shCDK-8 or shNC were harvested and suspended in PBS at a final concentration of 5×10^7 cells/ml. After anesthetization, the mice were incised about 10 mm on the left subcostal, the peritoneum was opened for about 8 mm and the spleen was exposed over the peritoneum. The cell suspension of 5×10^6/100 ml was injected into the spleen using a 29G needle. The spleen was returned to the abdominal cavity and the abdomen was closed.

2.6. Bioluminescence imaging to monitor tumor development

Bioluminescence imaging was conducted using bioluminescence technology (Night OWL^{II} LB983 NC100, Berthold, Germany). Prior to acquiring images, 2.5 mg of D-luciferin (Xenogen Corporation, Hopkinton, MA) was administered by intra-peritoneal injection to all mice. We imaged the mice weekly in this same manner to monitor tumor growth after intra-spleen injections.

2.7. Protein expression of CDK-8, β-catenin, E2F1, MMP-7 and E-cadherin in metastatic liver lesions were detected by Western Blot

Protein lysates were prepared with lysis buffer (Beyotime, Shanghai, China), equal quantities of protein were s subjected to 10% SDS-PAGE, transferred onto PVDF membranes for two hours at 4 °C, run at a current of 125 mA. After blocking, the membrane was incubated overnight with rabbit anti-human CDK-8 monoclonal antibody, rabbit anti-human β-catenin monoclonal antibody, rabbit anti-human E2F1 monoclonal antibody, rabbit anti-human E-cadherin monoclonal antibody and rabbit anti-human MMP-7 (1:1000, BioWorld, Atlanta, GA, USA), followed by incubation with HRP-linked secondary antibodies for 2 h. To control sample loading, the blotting membranes were stripped and re-probed with an anti-a-tubulin antibody (Sigma–Aldrich). Immunocomplexes were visualized using ECL system (Millipore, Billerica, WI, USA) following the manufacturer's protocol.

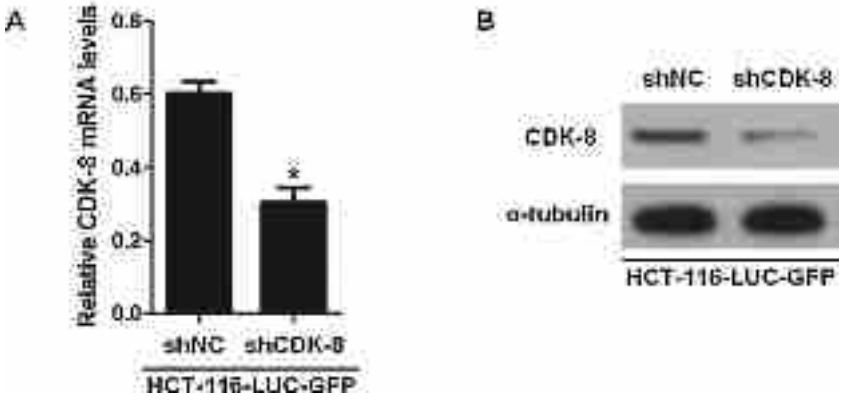

Fig. 1. Expression of CDK-8 in human colon cancer HCT-116 cell line. A, Real-time PCR analysis of CDK-8 mRNA expression in human colon cancer HCT-116 cells after transfected with sh-CDK-8 or sh-NC. B, protein expression of CDK-8 in human colon cancer HCT-116 cells after transfected with sh-CDK-8 or sh-NC were detected by Western Blot. Experiments were repeated at least three times (A and B). Each bar represents the mean of three independent experiments. $^*P < 0.05$.

2.8. Statistical analyses

All data were expressed as mean ± standard deviation (SD), and all statistical analyses were performed using SPSS version 18.0 (SPSS Inc.; Chicago, IL, USA). A *P*-value <0.05 was considered statistically significant.

4. Result

4.1. CDK-8 was downregulated in HCT116-LUC-GFP cells after transfection with shCDK-8

To investigate the role of CDK-8 in colon cancer, HCT116-LUC-GFP cells were transfected with shCDK-8 or shNC, respectively. The expression levels of CDK-8 were detected by qRT-PCR and western blot. The results showed that mRNA level and nuclear protein levels of CDK-8 were both significantly decreased after transfected with shCDK-8, compared with the control cells (Fig. 1).

4.2. CDK-8 promoted cell migration and invasion colon cancer cell migration and invasion

To assess the effect of CDK-8 on cell migration and invasion, the wound healing and transwell invasion assay were performed in the HCT116-LUC-GFP cells after the corresponding transfection experiments. The wound healing assay results indicated that the cells transfected with shCDK-8 showed markedly lower migration ability compared to control cells transfected with shNC (Fig. 1A). Reduced invasion ability of HCT116-LUC-GFP cells was observed after transfection with shCDK-8 (Fig. 2B). Together, the data suggested a role of CDK-8 in promoting colon cancer cell migration and invasion.

4.3. In vivo bioluminescence

The human colon cancer HCT116-LUC-GFP cells were transduced with the firefly luciferase gene in order to measure cancer cell bioluminescence *in vivo*. The ventral imaging position was found to be more sensitive to detect hepatic metastases. After injection of the cancer cells, the mice were imaged weekly by bioluminescence technique after intra-peritoneal injection with D-luciferin substrate. The ventral imaging position was found to be more sensitive to detect hepatic metastases. Our results showed that metastasis ability of HCT116-LUC-GFP cells was decreased after transfection with shCDK-8 *in vivo* (Fig. 3).

4.4. CDK-8, β-catenin, E-cadherin, E2F1, MMP-7 expression in metastatic liver lesions of mice model

Increasing evidences indicate that CDK-8 function mainly through regulation of Wnt/β-catenin pathway, the downstream genes of this pathway that functions in colon cancer pathogenesis were further analyzed. We examined CDK-8, β-catenin, E-cadherin, E2F1, MMP-7 protein expression in metastatic liver lesions by Western Blot.

The expression of CDK-8, β-catenin, E-cadherin and MMP-7 were decreased, while E2F1 expression was increased in metastatic liver lesions of si-CDK-8 groups compared to control groups (Fig. 4). Altogether, our results indicated that CDK-8 functionally modulates cell metastasis regulators, CDK-8, β-catenin, E-cadherin, E2F1 and MMP-7, thus relevant to cell metastasis.

5. Discussion

Colon cancer is one of the most common cancers and becoming the fourth leading cause of cancer death worldwide [16]. In the past few decades, the incidence and mortality of colon cancer in China have shown a gradual upward trend [17,18]. The prognosis of

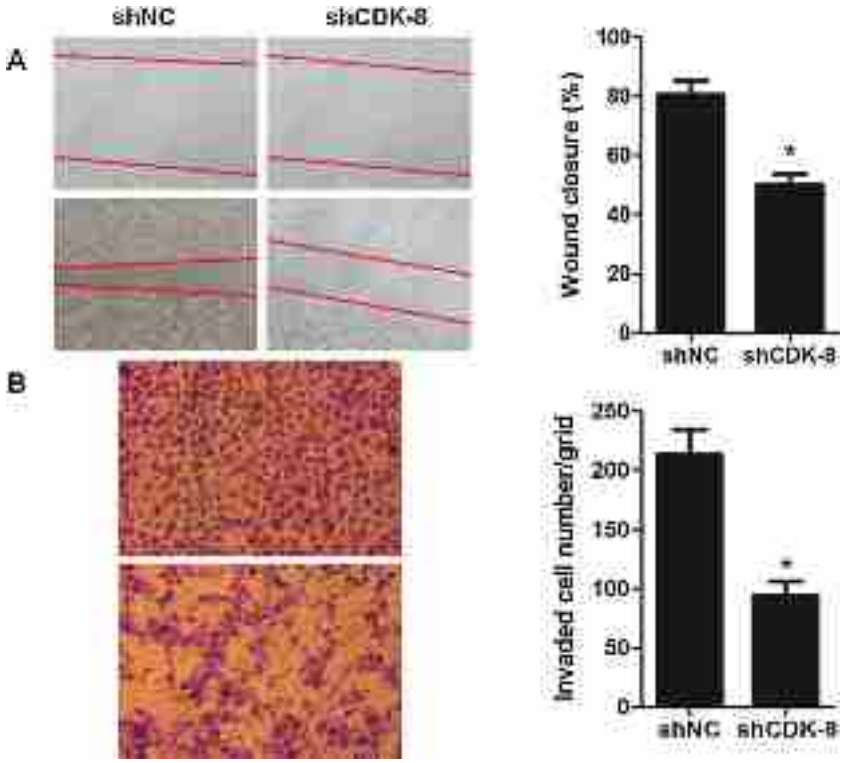

Fig. 2. CDK-8 downregulation inhibited cell migration and invasion of colon cancer. A, Impact of intervening CDK-8 expression on colon cancer cell migration by wound-healing assay *in vitro*. B, Impact of intervening CDK-8 expression on colon cancer cell invasion by transwell assay *in vitro*. $^{*}P < 0.05$.

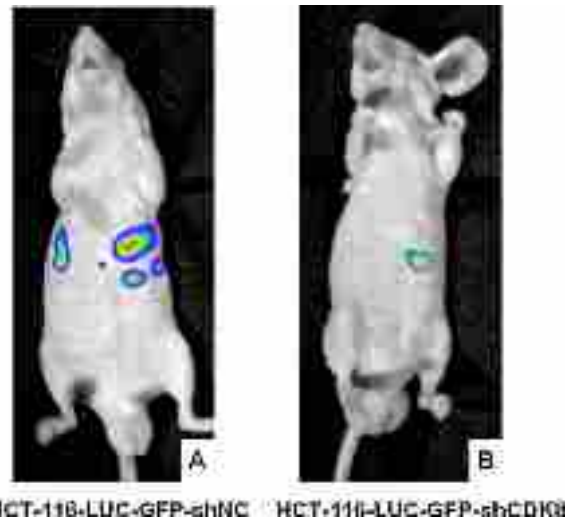

Fig. 3. Downregulation of CDK-8 inhibits liver metastasis. HCT-116-LUC-GFP-shNC (A) and HCT-116-LUC-GFP-shCDK-8 (B) cells were injected into the spleens of BAbl/c mice. Bioluminescence imaging to monitor liver metastasis of HCT-116-Luc-GFP cells *in vivo* 28 days after splenic injection.

Fig. 4. Expression of CDK-8, β-catenin, E-cadherin, E2F1 and MMP-7 in liver metastatic tumors were detected by Western Blot. Western blotting analysis of expression of CDK-8, β-catenin, E-cadherin, E2F1 and MMP-7 in liver metastatic tumors. α-Tubulin served as the loading control.

early-stage colon cancer is usually favorable, however, nearly two-thirds of colon cancer cases have metastasis diseases when diagnosed [19]. Liver is the most common site for metastasis from colon cancer and is the main reason for clinical treatment failure, which is considered to be the main cause of death from advanced-stage colon cancer [20–22]. As a result, the prevention and treatment of colon cancer with liver metastasis is a clinically important topic that requires further investigation of the biological mechanisms and find out more efficient treatment regimens. Gene therapy techniques performed in recent years have become highly promising strategies for treatment of colon cancer [23–25].

Cyclin-dependent kinase (CDK)-8 is a cyclin-dependent kinase member of the mediator complex, is located at 13q12.13, which was involved in the regulation of mRNA transcription and was identified as a colon cancer oncogene [8,26]. In this study, our finding revealed that downregulation of CDK-8 could inhibit migration and invasion of colon cancer cell *in vitro*. In animal models of colon cancer, metastasis ability to liver was decreased after downregulation of CDK-8. We further explored the underlying mechanism of CDK-8 functions in colon cancer. In over 90% of colon cancers, the Wnt/β-catenin pathway is implicated [27]. β-catenin acts as the central component in the Wnt signal transduction system. In addition, the activation of CDK-8 is essential for β-catenin-dependent transcription [8,28]. It is reported that E2F1 deregulation suppresses β-catenin activity, however, E2F1 activity is also repressed by CDK-8 [28]. β-catenin participates in the transcriptional activation of target genes, such as MMP-7 and E-cadherin. Degradation of extracellular matrix (ECM) and the loss of intercellular adhesion are prerequisites for tumor cells to become fully metastasis and invasive [29]. Matrix metalloproteases (MMPs) as important factors which are capable of degrading of ECM, taking part in invasion and metastasis of tumor cells, especially MMP-7. Expression of E-cadherin is pivotal in maintaining the integrity of intercellular adhesion through binding to various catenins, including α, β, g-catenin, and the loss of E-cadherin is associated with an increased tendency for tumor metastasis [29,30]. On the basis of these data, we investigated whether downregulated of CDK-8 expression and then the expression of E2F1 will be increased, further suppresses β-catenin expression, and achieve the aim to inhibit the metastasis of cancer cell. To this end, in this study, metastasis ability to liver of colon cancer cells was decreased after downregulation of CDK-8. Result of western blot showed that expression of CDK-8, β-catenin, E-cadherin and MMP-7 were decreased, while E2F1 expression was increased in metastatic liver lesions of si-CDK-8 groups.

In conclusion, the current study revealed that CDK-8 is an oncogene in colon cancer and the molecular mechanisms of its pro-metastasis function through the Wnt/β-catenin signaling pathway and this implies CDK-8 to be a potential target for treatment of colon cancer.

Conflict of interest

The authors do not have any conflict of interest about this paper.

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