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ORIGINAL ARTICLE

Basic Study

Effect of NDC80 in human hepatocellular carcinoma

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Abstract

AIM

To investigate the role of nuclear division cycle (NDC)80 in human hepatocellular carcinogenesis.

METHODS

NDC80 gene expression was analyzed by real-time reverse transcription polymerase chain reaction in 47 paired hepatocellular carcinoma (HCC) and adjacent tissues. The HCC cell line SMMC-7721 was transfected with lentivirus to silence endogenous *NDC80* gene expression, which was confirmed by real-time polymerase chain reaction and western blotting. The effects of NDC80 silencing on SMMC-7721 cell proliferation were evaluated by Cellomics ArrayScan VTI imaging. Cell cycle analysis and apoptosis were detected with flow cytometry. Colony formation was assessed by fluorescence microscopy.

RESULTS

NDC80 expression levels in HCC tissues were significantly higher than those in the adjacent tissues. Functional studies demonstrated that NDC80 silencing significantly reduced SMMC-7721 cell proliferation and colony formation. Knockdown of NDC80 resulted in increased apoptosis and cell cycle arrest at S-phase. NDC80 contributed to HCC progression by reducing apoptosis and overcoming cell cycle arrest.

CONCLUSION
Elevated expression of NDC80 may play a role in promoting the development of HCC.

Key words: NDC80; Cell proliferation; Apoptosis; Cell cycle; Hepatocellular carcinoma

Core tip: Nuclear division cycle (NDC)80 is a member of the NDC80 kinetochore complex and is highly expressed in cancer. NDC80 is a newly identified gene that is overexpressed in hepatocellular carcinoma (HCC). We analyzed the biological function of NDC80 in the proliferation and apoptosis of HCC cells, and provided new reference data and experimental support for HCC-targeting gene therapy.

Ju LL, Chen L, Li JH, Wang YF, Lu RJ, Bian ZL, Shao JG. Effect of NDC80 in human hepatocellular carcinoma. *World J Gastroenterol* 2017; 23(20): 3675-3683 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i20/3675.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i20.3675

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and the third most frequent cause of cancer mortality, following lung and stomach cancers[1,2]. Especially in China, which has a high incidence of hepatitis B, the magnitude of the problem should never be underestimated[3]. HCC has received considerable attention in recent years because of its rapidly increasing incidence[4,5]. Patients diagnosed with HCC have a poor prognosis because of the aggressive features of the disease. Only 10%-15% of HCC patients are suitable candidates for curative treatments, including surgical resection and liver transplantation. Surgical resection, ablation therapy, and liver transplantation are effective but only at an early stage of HCC development[5-8]. However, HCC is diagnosed at advanced stages in most patients when only limited therapeutic options are available. High metastasis and recurrence rates have become the major obstacles to improving long-term survival of HCC patients[8]. The molecular mechanisms of HCC development are not fully understood; thus, it is of importance to elucidate further the mechanisms of HCC and explore effective treatment. Gene therapy has emerged as a promising intervention against HCC. In this study, we investigated the role of a gene that has been previously associated with human HCC cells - nuclear division cycle (NDC)80 - in order to ascertain better its role in human hepatocellular carcinogenesis.

NDC80 (also called Hec1), a core component of the outer kinetochore and a mitotic regulator, is of particular interest because it clearly has an association with cancer progression. NDC80 forms a dumbbell-like heterotetramer with Nuf2, Spc24, and Spc25 to form the NDC80 complex[9-12]. NDC80 complexes are important to the spindle assembly checkpoint and participate in the regulation of mitosis[13-15]. NDC80 plays essential roles in chromosome segregation by mediating the spindle assembly checkpoint signaling and chromosome alignment. The spindle assembly checkpoint is a mechanism that ensures faithful chromosome segregation by monitoring the kinetochore-microtubule attachment[13,16]. Chromosome segregation dysfunction is one of the causes of chromosome instability. Chromosome instability is a common feature of tumor cells, and may be an important mechanism in tumor formation. In this study, we analyzed the biological function of NDC80 in the proliferation and apoptosis of HCC cells, and provided new reference data and experimental support for HCC-targeting gene therapy.

MATERIALS AND METHODS

Cell lines and clinical samples

We used HepG2, Huh-7, SMMC-7721 and Hep3B cell lines, which are commonly used in HCC research, purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum at 37 ℃ with 50 mL/L CO_2. Forty-seven pairs of HCC and matched adjacent tissues were provided by Nantong Third People's Hospital Affiliated to Nantong University. All patients had primary HCC and had not received any preoperative radiotherapy or chemotherapy. The diagnosis of all HCC patients was confirmed histopathologically. The pathological stage of HCC was determined according to the International Union Against Cancer Tumor-Node-Metastasis (TNM) Classification. The characteristics of the participants are summarized in Table 1. All tissues samples were frozen immediately after resection and stored in liquid nitrogen until use.

Real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from tissue samples and HCC cell lines using TRIzol (Takara Bio, Dalian, China). RNA purity and concentration were determined by NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). RNA was reversely transcribed using a Prime Script™ RT Reagent Kit (Takara Bio). The reactions were performed in a Bio-Rad Real-Time polymerase chain reaction (PCR) Detection System using a SYBR® Green Master Mix (Vazyme Biotech, Nanjing, China). The primers used were as follows: NDC80: forward, 5'-CCTCTCCATGCAGGAGTTAAGA-3',

Table 1 Clinical characteristics of hepatocellular carcinoma and paired adjacent tissues *n* (%)

Clinical variable	No. of patients
No. of patients	47
Age, yr, mean ± SD	56 ± 11
Sex	
Female	18 (38.3)
Male	29 (61.70)
Serum AFP, ng/mL	
≤ 400	36 (76.60)
> 400	11 (23.40)
HBV infection	
Positive	38 (80.9)
Negative	3 (19.1)
Largest tumor diameter, cm	5.7 ± 3.4
TNM stage	
Ⅰ	2 (4.25)
Ⅱ	30 (63.83)
Ⅲ	15 (31.92)
Lymph node metastatic	
Positive	20 (42.55)
Negative	27 (47.45)

reverse, 5′-GGTCTCGGGTCCTTGAT TTTCT-3′; GAPDH: forward, 5′- TGACTTCAACAGCGACACCCA-3′, reverse, 5′- CACCCTGTTGCTGTAGCCAAA-3′. After a pre-denaturation step at 95 ℃ for 5 min, 40 cycles of PCR were performed as follows: 10 s denaturation at 95 ℃ and 30 s annealing at 60 ℃. The fold amplification for each gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Lentiviral transfection of SMMC-7721 cells

The SMMC-7721 cell suspension was seeded onto six-well plates at 5 × 10^4 cells/well and incubated at 37 ℃ in 50 mL/L CO_2 until 30% confluence was reached. Two experimental groups were constructed: NDC80-siRNA, which was transfected with NDC80-siRNA green fluorescent protein (GFP) lentivirus; and control group, which was transfected with empty GFP lentivirus. An appropriate amount of lentivirus was added according to the multiplicity of infection (MOI). The cells were repeatedly cultured in normal culture medium after 12 h. GFP-tagged gene expression was observed under a fluorescence microscope at 3 d after transfection, and cells with a transfection efficiency > 80% were selected for subsequent analyses.

Western blotting

The cultured cells were lysed in RIPA lysis buffer containing phosphatase inhibitor cocktail (Beyotime Institute of Biotechnology, Shanghai, China), and the protein concentrations were determined. Protein extracts were separated on 10% SDS-PAGE and then electrophoretically transferred to PVDF membranes at 100 V for 90 min. The membranes were incubated with antibody against NDC80 (1:1000; Abcam, Cambridge, MA, United States). The target proteins were examined using an electrochemiluminescence system (Thermo Fisher Scientific) and visualized with X-ray films. GAPDH was used as the control.

Cell counts

NDC80-siRNA and control cells were removed using 0.25% trypsin-EDTA and resuspended in standard medium after achieving logarithmic growth. Cells were seeded in five wells at 1000 cells/well, followed by further incubation at 37 ℃ and 50 mL/L CO_2. A Cellomics ArrayScan VT1 (Thermo Fisher Scientific) was used to continuously measure GFP expression in each well over a 5-day period. In this study, statistical data were mapped and cell proliferation curves were drawn.

Colony-formation assay

NDC80-siRNA and control cells were digested in 0.25% trypsin to reconstitute the single cell suspension at 5 × 10^4 cells/mL. A hemocytometer was used to assess the cell count, and cell suspensions were transferred into six-well plates at 800 cells/well. After approximately 2 wk incubation, colonies containing > 50 cells were scored as surviving colonies. Colonies were visualized under a fluorescence microscope (MicroPublisher 3.3RTV; Olympus, Tokyo, Japan). The supernatants were discarded, and cells were washed with phosphate-buffered saline (PBS) and fixed with paraformaldehyde (Sangon, Shanghai, China) for 30 min. All wells were washed with PBS and stained with 500 μL Giemsa solution (ECM550; Chemicon, Temecula, CA, United States) for 20 min. The cells were washed several times with deionized distilled water and allowed to air dry at room temperature. Colonies were counted and images were captured by a digital camera under light microscopy. The assay was repeated three times.

Cell cycle analysis

SMMC-7721 cells were plated in six-well plates under standard culture conditions. After treatment, cells were washed with PBS, fixed in 70% ice-cold ethanol and stored at -20 ℃ overnight. After being washed with PBS, the cells were incubated in 500 μL sample buffer containing 50 μg/mL propidium iodide (PI) and 0.25 mg/mL RNase A for 30 min at room temperature. Analysis of the apoptotic cells was performed by flow cytometry. The cells with sub-G1 DNA content were considered as apoptotic cells.

Apoptosis analysis

The cells were harvested with 0.25% trypsin and washed once with ice-cold PBS. After the cells were washed, the Annexin V-APC Apoptosis Detection Kit and PI (eBioscience, San Diego, CA, United States) were applied to assess apoptosis. Cells were centrifuged and resuspended in 500 μL binding buffer 10^6 cells/mL. And, 100 μL of the cell suspension was incubated with 10 μL PI and 5 μL Annexin V-FITC in a dark environment for 15 min at room temperature.

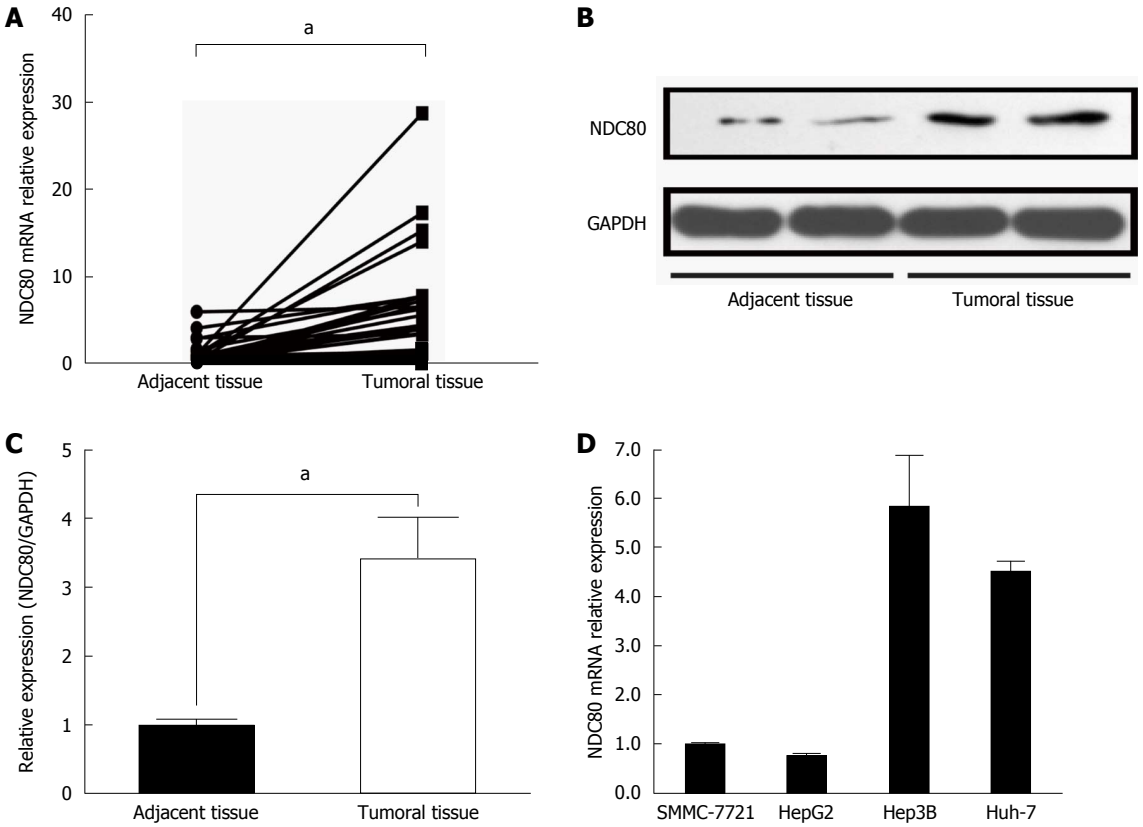

Figure 1 Reverse transcription polymerase chain reaction results for NDC80 mRNA expression. A: Expression levels of NDC80 mRNA in HCC (n = 47) and paired adjacent tissue samples (n = 47); B: NDC80 protein expression in HCC and paired adjacent tissue samples was determined by western blotting; C: Gray value analysis of western blot experiments, and data was normalized against GAPDH; D: NDC80 mRNA expression varied among SMMC-7721, HepG2, Hep3B and Hun-7 cell lines. GAPDH was used as an internal control. Statistical significance was assessed by paired t tests. Error bar indicates SD ($^aP < 0.001$ *vs* control).

Analysis of the apoptotic cells was performed by flow cytometry.

Ethical considerations

All tissues samples from the patients were taken after informed consent. The study was reviewed and approved by the Nantong Third People's Hospital Affiliated to Nantong University Institutional Review Board.

Statistical analysis

GraphPad Prism version 6.0 was used for data analysis. Statistical significance was defined as $P < 0.05$. All data were presented as the mean ± SD. All the experiments were repeated at least three times.

RESULTS

NDC80 was overexpressed in HCC tissues and cell lines

To investigate whether NDC80 expression was altered in HCC tissues, we detected its expression level by qRT-PCR in 47 paired tumor and adjacent tissues. NDC80 mRNA expression levels in the tumor tissues were drastically increased compared with those in the adjacent tissues (Figure 1A). A similar trend in NDC80 protein levels was observed by western blot analysis (Figure 1B and C). To reveal the potential role of NDC80 in HCC, we also examined NDC80 expression in four HCC cell lines: SMMC-7721, HePG2, Hep3B and Huh-7 (Figure 1D). The cell line must be lentivirus-friendly (MOI < 10) and enjoy a vigorous proliferation. Hence, the SMMC-7721 cell line was selected for future investigation. The NDC80 complex is comprised of NDC80, Nuf2, Spc24 and Spc25, which together form a dumbbell-like heterotetramer. We then detected the expression levels of Nuf2, Spc24 and Spc25 mRNA by qRT-PCR. The expressions of Nuf2 and Spc24 were significantly enhanced in HCC tissues compared with paired adjacent tissues (Supplementary Figure 1A and B). However, the expression of Spc25 mRNA was not changed between HCC tissues and adjacent tissues (Supplementary Figure 1C).

NDC80 silencing inhibited SMMC-7721 cell proliferation

After NDC80-siRNA lentiviral transfection, RT-PCR analysis showed that NDC80-siRNA diminished the expression of the endogenous NDC80 mRNA by up to 80% (P = 0.0001) (Figure 2A). Correspondingly, the protein expression of NDC80 in NDC80-siRNA-treated cells was also suppressed (P = 0.0023) (Figure 2B and C). After transfection, cell proliferation was significantly inhibited in NDC80-siRNA-silenced cells relative to control cells, as shown by GFP-based Cellomics

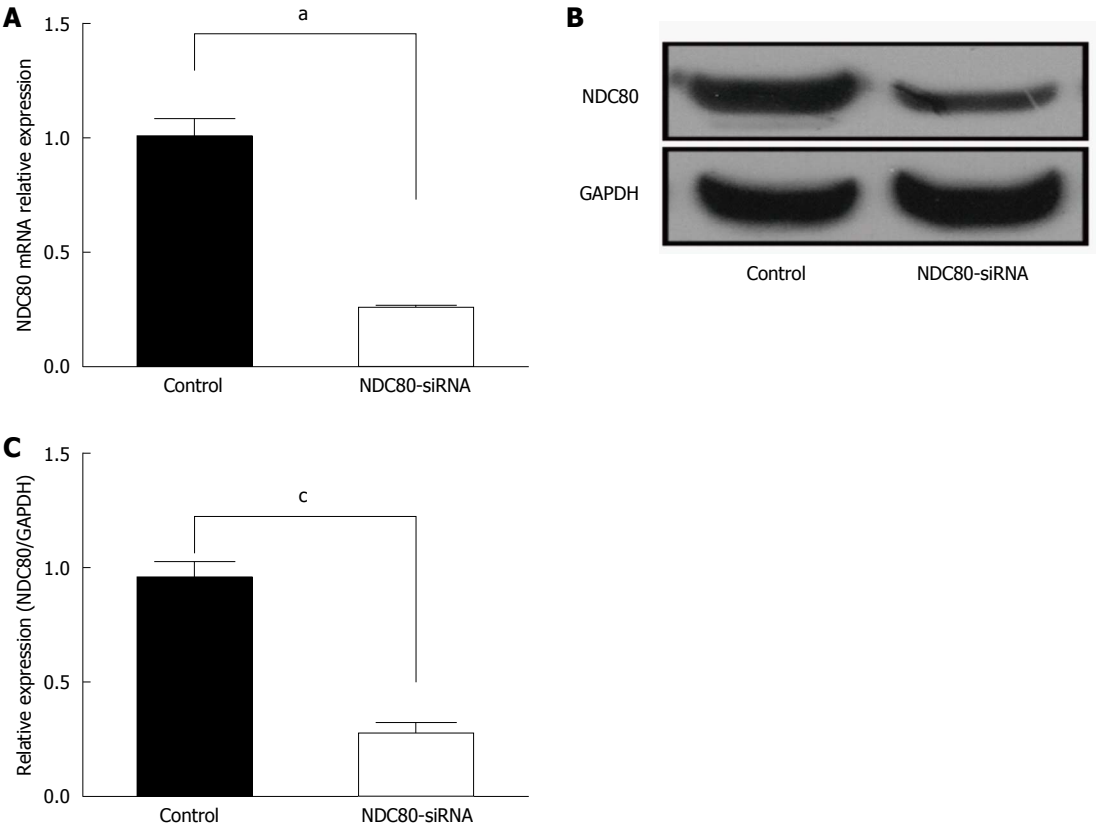

Figure 2 Interference efficiency 72 h after transfection. A: After lentiviral transfection, relative NDC80 mRNA expression was significantly inhibited in the SMMC-7721 NDC80-siRNA silenced cells as compared to SMMC-7721 negative control cells by RT-PCR; B: Western blotting of NDC80-depletion efficiency in SMMC-7721 cells; C: Gray value analysis of western blotting, and data were normalized against GAPDH. GAPDH was used as an internal control. Statistical significance was assessed by two-tailed Student's *t* test. Error bar indicates SD ($^cP < 0.01$ *vs* control; $^aP < 0.001$ *vs* control).

ArrayScan VTI imaging (Figure 3A). Cell numbers were monitored for 5 consecutive days. The number of cells and the fold-change in proliferation were markedly reduced in the NDC80-siRNA-silenced cells (Figure 3B). Accordingly, the results suggested that the silencing of NDC80 was associated with cell proliferation.

NDC80 silencing reduced SMMC-7721 cell colony formation

Silencing of NDC80 reduced the anchorage-independent growth of SMMC-7721 cells in soft agar (Figure 4A). The number of cell clones was significantly decreased in SMMC-7721 cells infected with NDC80-siRNA ($P = 0.0005$) (Figure 4B). The colony formation experiment confirmed that the silencing of NDC80 reduced the proliferative potential of SMMC-7721 cells.

NDC80 silencing induced cell cycle progression and the cell apoptosis process

To elucidate further the growth-suppressing effect of NDC80-siRNA on SMMC-7721 cells, cell cycle distribution was analyzed by flow cytometry. In comparison with the control group, NDC80-siRNA significantly increased the fraction of S-phase cells, but decreased G1- and G2/M-phase cells in NDC80-siRNA group (Figure 5A). The result demonstrated that the silencing of NDC80 might induce cell cycle arrest at S-phase and that the effect of NDC80 on the cell cycle was time-dependent. Whether the silencing of NDC80 was related to the level of apoptosis in SMMC-7721 cells was further investigated. The apoptotic rate was assessed by flow cytometry using the Annexin V-APC Apoptosis Detection Kit. The proportion of apoptotic cells was significantly higher in NDC80-silenced cells than in the control cells (Figure 5B). These data suggested that the silencing of NDC80 interrupted cell cycle progression and affected cell survival.

DISCUSSION

Chromosomal instability has long been suggested to be a driving force for tumor development and progression. Accurate chromosome segregation requires that sister kinetochores of each mitotic chromosome interact with microtubules connected to opposite spindle poles, so that separated sister chromatids migrate toward opposite directions at anaphase onset[17-19]. NDC80 is a core component of the outer kinetochore, the function of which is intricately involved in the establishment of appropriate microtubule attachments. NDC80 protein maintains chromosome stability, and spindle checkpoint dysfunction, abnormal chromosome separation and cell cycle disorder may occur in cells with NDC80 overexpression, which perhaps leads to

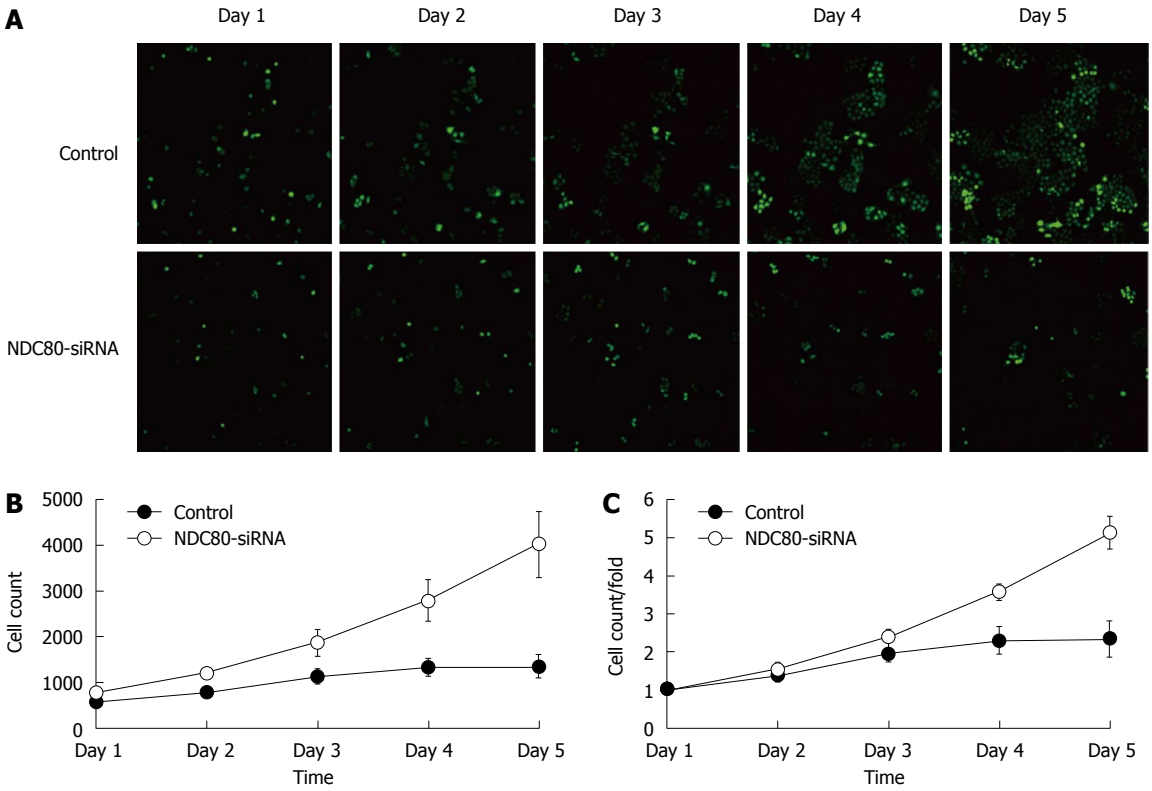

Figure 3 Cell proliferation analysis by green fluorescent protein-based imaging and MTT assay. A: After lentiviral transfection of SMMC-7721 cells, cell proliferation was significantly inhibited in NDC80-siRNA-silenced cells as compared to the control cells according to green fluorescent protein-based Cellomics ArrayScan VTI imaging; B: After lentiviral transfection of SMMC-7721 cells, MTT assays were performed at the days indicated to show the proliferation of SMMC-7721 cells. The MTT value ratio was significantly reduced in the NDC80-siRNA-silenced cells as compared to the control cells.

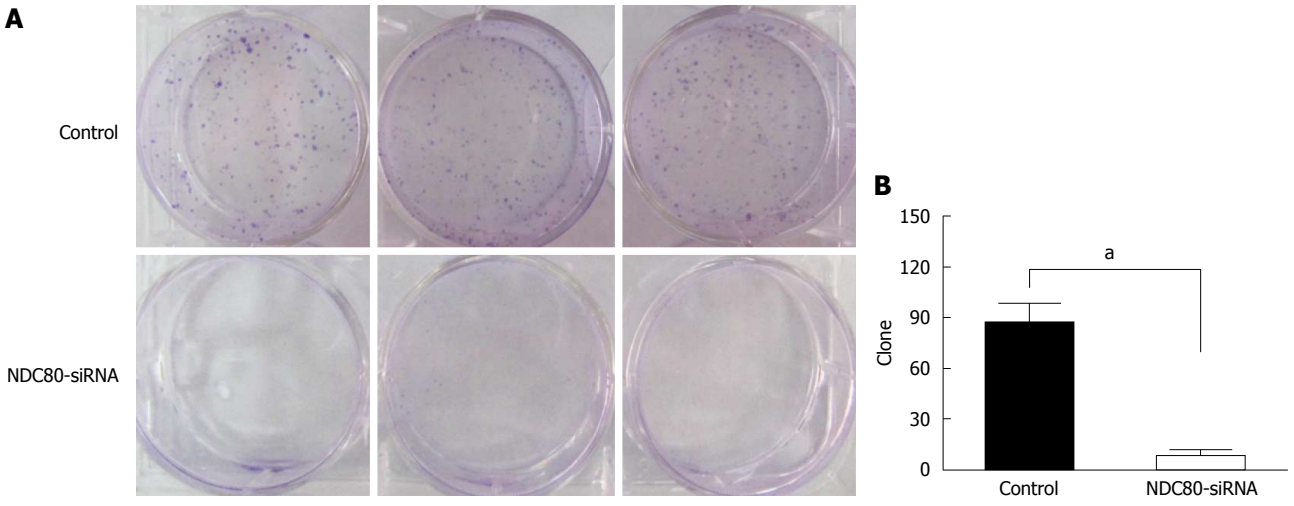

Figure 4 Effects of the silencing of NDC80 on SMMC-7721 cell colony formation. A: After lentiviral transfection of SMMC-7721 cells, the NDC80-siRNA-silenced cells displayed a significantly reduced number of cell colonies compared to control cells. Colonies were stained with crystal violet. The whole plate fields were photographed and presented. The number of cell colonies of triplicate values in a representative experiment was counted; B: Statistical significance was assessed by two-tailed Student's *t* test. Error bar indicates SD ($^{a}P < 0.001$ *vs* control).

tumor initiation[18,20-22].

Expression of the mitotic regulator NDC80 is increased in various human malignancies, including HCC. Considering the significant overexpression of NDC80 in multiple human malignancies[23-27], studies on the mechanisms of NDC80 overexpression are significant. Liu *et al*[28] also reported that the expression level of NDC80 was remarkably up-regulated in HBV-related HCC tissues. In this study, we investigated the significance of the increased NDC80 expression in carcinogenesis in general and HCC in particular, by both loss and gain of function analysis. This study was conducted to ascertain the role of NDC80 in human hepatocellular carcinogenesis.

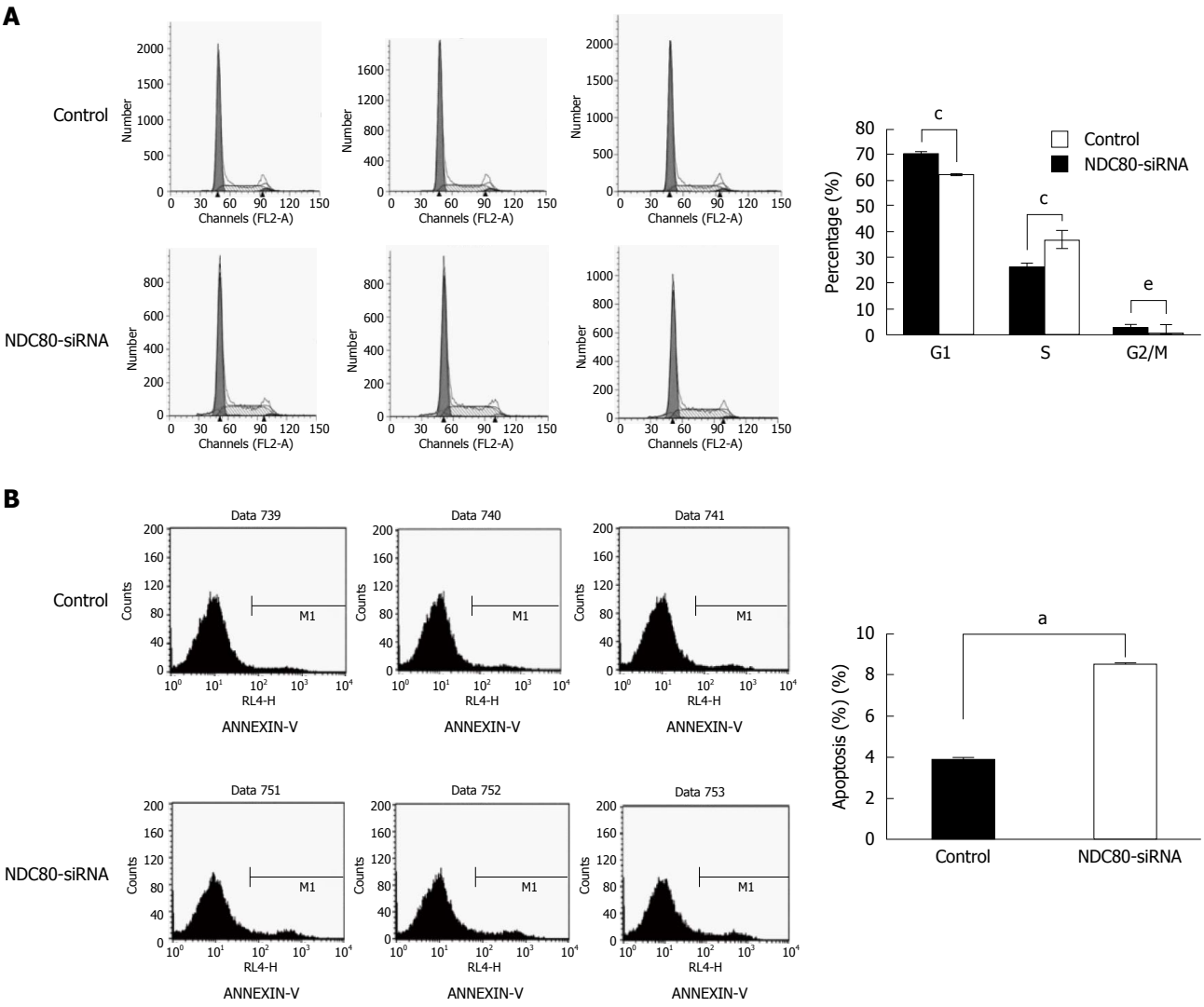

Figure 5 Effects of the silencing of NDC80 on SMMC-7721 cell cycle distribution and apoptosis. A: After lentiviral transfection of SMMC-7721 cells, cell cycle assessment showed that knockdown of NDC80 in SMMC-7721 cells induced accumulation in S-phase. The percentages of cells in different phases are shown as the mean ± SD of three independent experiments; B: Apoptotic rates were analyzed by Annexin V-FITC/PI assay. Apoptosis was significantly increased in NDC80-siRNA-silenced cells as compared to control cells. Statistical significance was assessed by two-tailed Student's *t* test. Error bar indicates SD ($^{e}P < 0.05$; $^{c}P < 0.01$; $^{a}P < 0.0001$, *vs* control).

Lentiviral packaging has become an ideal genetic engineering technology after years of optimization and improvement[29,30]. In this study, lentivirus-mediated siRNA provided an attractive approach to suppress NDC80 gene expression. We monitored the lentiviral infection efficiency by fluorescence microscopy, and confirmed the target gene knockdown by western blotting and real-time RT-PCR, which provided a basis for the continued observation of the role of NDC80 in SMMC-7721 cells. Real-time RT-PCR analysis showed that the NDC80 expression levels in the tumor tissues were significantly increased compared with those in adjacent tissues.

We found that cell proliferation and cell colony formation were significantly inhibited in NDC80-silenced HCC cells. Moreover, apoptosis was significantly increased in NDC80-silenced HCC cells. We performed a cell-cycle assay to illustrate the mechanism by which NDC80 promotes cell proliferation. We found that attenuation of NDC80 expression in carcinoma cells delayed cell-cycle progression through S-phase, signifying arrest at this phase. This suggests that abnormal NDC80 expression leads to severe disruption of cell-cycle progression. The present study also demonstrated that NDC80 knockdown induced HCC apoptosis. The apoptosis caused by NDC80 knockdown might be due to incomplete mitosis caused by severe mitotic spindle checkpoint dysfunction. Hence, NDC80 plays a significant role in maintaining the growth of HCC cells.

These findings strongly suggest that NDC80 contributes to the pathogenesis of HCC through its proliferative and anti-apoptotic effects. Our data suggest that NDC80 overexpression may be an early event in hepatocellular carcinogenesis. Therefore, NDC80 may represent a new target for HCC gene

therapy. The results of this study provide a new theoretical basis for gene therapy in HCC.

In conclusion, the present study evaluates the association between NDC80 expression and HCC. NDC80 is highly expressed in HCC tissues relative to adjacent tissues. NDC80 significantly promotes HCC cell proliferation and colony formation and significantly inhibits apoptosis by arresting cells at the S phase. Therefore, we determined that NDC80 plays critical roles in tumor growth and HCC development. Although detailed mechanisms remain to be elucidated, the critical role of NDC80 in HCC development may provide evidence for development of novel therapeutics against NDC80 for the early detection and treatment of HCC.

ACKNOWLEDGMENTS

We would like to thank Nantong Third People's Hospital Affiliated to Nantong University for providing the HCC tissue samples.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and the third leading cause of cancer mortality. High metastasis and recurrence rates have become the major obstacles to improving long-term survival of HCC. The molecular mechanisms of HCC development are not fully understood; thus, it is of importance to elucidate the mechanisms of HCC and explore effective treatment.

Research frontiers

Nuclear division cycle (NDC)80 plays essential roles in chromosome segregation by mediating the spindle assembly checkpoint signaling and chromosome alignment. Chromosome instability is a common feature of tumor cells, and may be an important mechanism in tumor formation.

Innovations and breakthroughs

This is the first study which shows that NDC80 contributes to the pathogenesis of HCC through its proliferative and anti-apoptotic effects. The authors hypothesized that the critical role of NDC80 in HCC development could provide evidence for development of novel therapeutics against NDC80 for the early detection and treatment of HCC.

Applications

NDC80 may represent a new target for HCC gene therapy. The results of this study provide a new theoretical basis for gene therapy in HCC.

Peer-review

The authors demonstrated that NDC80 expression levels in the tumor tissues were significantly increased compared with those in the adjacent tissues. NDC80 significantly promotes HCC cell proliferation and colony formation and significantly inhibits apoptosis by affecting cell cycle S-phase arrest. However, the detailed mechanisms about the critical role of NDC80 in HCC development have not been elucidated.

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