Downregulation of *NIT2* inhibits colon cancer cell proliferation and induces cell cycle arrest through the caspase-3 and PARP pathways

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Abstract. Colorectal cancer, also known as colon cancer is the most devastating malignancy worldwide. Previous studies have reported that NIT2, a member of the nitrilase superfamily, is a potential tumor suppressor, although its function remains elusive in colon cancer. In the present study, we employed an RNA interference lentivirus system to silence endogenous *NIT2* expression in the colon cancer cell line, HCT116. The knockdown efficiency was determined by RT-qPCR and western blot analysis. The depletion of *NIT2* markedly inhibited colon cancer cell proliferation and colony formation and induced cell cycle arrest in the G0/G1 phase, as shown by MTT assay, colony fromation assay and flow cytometric analysis, respectively. Further investigation with an intracellular signaling array demonstrated that the depletion of *NIT2* triggered the apoptosis of colon cancer cells through the caspase-3 and poly(ADP-ribose) polymerase (PARP) pathways. Our findings suggest that *NIT2* may be an oncogene in human colon cancer and may thus serve as a promising therapeutic target for the treatment of colon cancer.

Introduction

Colorectal cancer, also known as colon cancer, is the most common type of gastrointestinal cancer worldwide. Despite advances in research and treatment modalities, colorectal cancer still causes 500,000 deaths each year (1), and approximately 150,000 US residents are diagnosed annually with colon cancer (2). It is one of the devastating cancers and one third of patients with colon cancer will eventually succumb to the disease (3). Over the past 3 decades, investigations into the molecular genetics of colon cancer have revealed the regulation of cellular metabolism, proliferation, differentiation and survival in colon cancer (4). However, much work remains to be done in order to fully understand and integrate the molecular changes associated with the pathophysiology of colorectal cancer.

Human nitrilase (NIT) proteins are members of the nitrilase superfamily that contain a conserved nitrilase domain and thiol enzymes involved in natural product biosynthesis and post-translational modification in plants (5), animals, fungi and certain prokaryotes (6,7). Based on the sequence analysis, the NIT superfamily has been divided into 13 branches (6,8). Branch 10 in the Brenner classification contains only 2 enzymes, designated as NIT1 and NIT2, found in mammalian tissues (8-10). The *NIT2* gene is ubiquitously expressed in multiple types of tissue and encodes the protein NIT2 mainly distributed in the cytosol; it has been reported that NIT2 is a potential tumor suppressor (11). However, to the best of our knowledge, no study to date has reported the role of *NIT2* in colon cancer, and the cellular function of the *NIT2* gene remains elusive.

The RNA interference (RNAi) technique is a powerful tool to carry out loss-of-function assays. It provides a new approach to investigating cancer gene therapy (12,13). In the present study, we employed a lentivirus-mediated RNAi system in order to achieve the highly stable silencing of *NIT2* in the colon cancer cell line, HCT116. We evaluated the biological function of *NIT2* and aimed to reveal its contribution to the progression of colon cancer. To the best of our knowledge, this is the first presentation providing evidence that the knockdown of endogenous *NIT2* expression suppresses the oncogenic properties of colon cells and induces apoptosis through the caspase-3 and poly(ADP-ribose) polymerase (PARP) pathways.

Materials and methods

Cell culture. The human embryonic kidney cell line 293T (HEK293T) and the human colon cancer cell line, HCT116, were obtained from the Shanghai Institute of Cell Biology, the Chinese Academy of Sciences, Shanghai, China. The HCT116 cells were maintained in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS)

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at 37˚C in a humidified atmosphere of 5% CO_2. The HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% FBS at 37˚C in a humidified atmosphere of 5% CO_2.

Construction of recombinant lentivirus. The following oligonucleotides were synthesized. The short hairpin RNA (shRNA) sequence (5'-ACATAATCAACTCCCTATTAACT CGAGTTAATAGGGAGTTGATTATGTTTTTT-3', sequence 1) against the human *NIT2* gene (NM_020202) was screened and validated to be a candidate shRNA. The negative control shRNA was 5'-TTCTCCGAACGTGTCACGT-3'. The stem-loop-stem oligos (shRNAs) were synthesized, annealed and ligated into the *Age*I/*Eco*RI-linearized pFH-L vector (Shanghai Hollybio, Shanghai, China). The lentiviral-based shRNA-expressing vectors were confirmed by DNA sequencing. The generated plasmids were named pFH-L-shNIT2 and pFH-L-shCon.

The HEK293T cells ($1.0x10^6$) were seeded into 10-cm dishes and cultured for 24 h to reach 70-80% confluence as previously described (14). Two hours prior to transfection, the medium was replaced with serum-free DMEM. The plasmids, including 10 μg of pFH-L-shNIT2 or pFH-L-shCon, 7.5 μg of the packaging vector pHelper-1.0 and 5 μg of the expression plasmid pHelper-2.0 were added to 0.95 ml of Opti-MEM and 50 μl of Lipofectamine 2000. The mixture was added to the cells followed by incubation for 8 h before replacing the medium with 10 ml of complete DMEM medium (supplemented with 10% PBS). Lentiviral particles were harvested 48 h after transfection. The HCT116 cells ($8.0x10^4$) were cultured in 6-well plates and inoculated with recombinant lentiviruses (Lv-shCon or Lv-shNIT2) at a multiplicity of infection (MOI) of 15. As the lentivirus carries green fluorescence protein (GFP), the infection efficiency was determined by counting the numbers of GFP-expressing cells under a fluorescence microscope (Olympus BX50; Olympus Corp., Tokyo, Japan) at 96 h after infection.

RNA extraction and reverse transcription-quantitative (real-time) PCR (RT-qPCR). The human colon cancer cells, HCT116, were pre-cultured and infected with the recombinant lentiviruses for 5 days as previously described (15). Total RNA was prepared using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA (5 mg) was used to synthesize the first-strand cDNA using 200 U/ml SuperScript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, CA, USA). The NIT2 mRNA expression was evaluated by quantitative PCR on a BioRad Connet Real-Time PCR platform (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR-Green PCR Core reagents. The qPCR reaction system contains 10 μl 2X SYBR Premix Ex Taq, plus 0.8 μl forward and reverse primers (2.5 μM), 5 μl cDNA template and 4.2 μl ddH_2O. β-actin was applied as the internal reference. The following primers were synthesized and applied: NIT2 forward, 5'-CGGGCTGTTGATAATCAGGT-3' and reverse, 5'-TTCAGCCAGCTTCTTCAGGT-3'; and β-actin forward, 5'-GTGGACATCCGCAAAGAC-3' and reverse, 5'-AAAGGGTGTAACGCAACTA-3' (14). The reaction procedure was initiated with denaturation at 95˚C for 1 min followed by 40 repeated cycles (denaturation at 95˚C for 5 sec and annealing extension at 60˚C for 20 sec). The results are presented as CT values, defined as the threshold PCR cycle number at which an amplified product is first detected. The average CT value was calculated for both NIT2 and β-actin, and the ΔCT value was determined as the mean of the triplicate CT values for NIT2 minus the mean of the triplicate CT values for β-actin.

Western blot analysis. The HCT116 cells were cultured and infected with the recombinant lentiviruses for 5 days. The cells were washed twice with ice-cold PBS and lysed in 2X SDS sample buffer [100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS and 10% glycine]. Equal amount of proteins (30 μg) were loaded and separated by electrophoresis (50 V, 3 h) on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) at 300 mA for 1.5 h. The membranes were blocked and then probed with primary antibodies, rabbit anti-NIT2 (1:8,000 dilution, #31280; Signalway Antibody LLC, College Park, MD, USA) or mouse anti-GAPDH (1:50,000 dilution, #10494-1-AP; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) overnight at 4˚C. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution, #SC-2054; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature and then visualized using Super ECL Detection reagent (Applygen Technologies, Inc., Beijing, China).

Colony formation assay. The human colon cancer cells, HCT116, were cultured in 6-well plates and treated with the recombinant lentiviruses. After 96 h of incubation, the infected cells were washed, re-cultured in the prepared 6-well plates at a density of 400 cells/well, and allowed to form natural colonies. After 7 days of incubation, the treated cells were subjected to Crystal violet staining. Subsequently, the cells were washed and fixed with paraformaldehyde. The fixed cells were washed twice with PBS solution, treated with Crystal violet for 10 min, washed 3 times with ddH_2O and then photographed using a digital camera (D7000; Nikon Corp., Tokyo, Japan). The number of colonies (>50 cells/colony) was counted.

MTT assay for cell viability. The HCT116 cells were cultured in 6-well plates and inoculated with the recombinant lentiviruses. After 96 h of infection, the cells were washed and re-cultured in 96-well plates at $2.5x10^3$ cells/well. MTT solution was added to the wells followed by incubation at 37˚C for 4 h at different time points after lentivirus infection (1, 2, 3, 4 and 5 days). The converted dye was then solubilized in acidic isopropanol (10% SDS, 5% isopropanol and 0.01 M HCl) followed by incubation at 37˚C for 10 min. The optical density was measured using a microplate reader (Epoch; BioTek, Winooski, VT, USA) at the wavelength of 595 nm. The experiment was repeated at least 3 times.

Flow cytometric analysis. The HCT116 cells were cultured in 6-well plates and inoculated with the recombinant lentiviruses at a MOI of 20. After 3 days of infection, the cells were inoculated into 6-cm dishes at a density of $1x10^5$ cells/dish. After 40 h of incubation, the cells in each well were harvested and the cell cycle was determined by propidium iodide (PI) staining before the cell density reached 80% confluency. Tests were performed in triplicate for each sample, and analyses were performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA) in accordance with the manufacturer's guidelines.

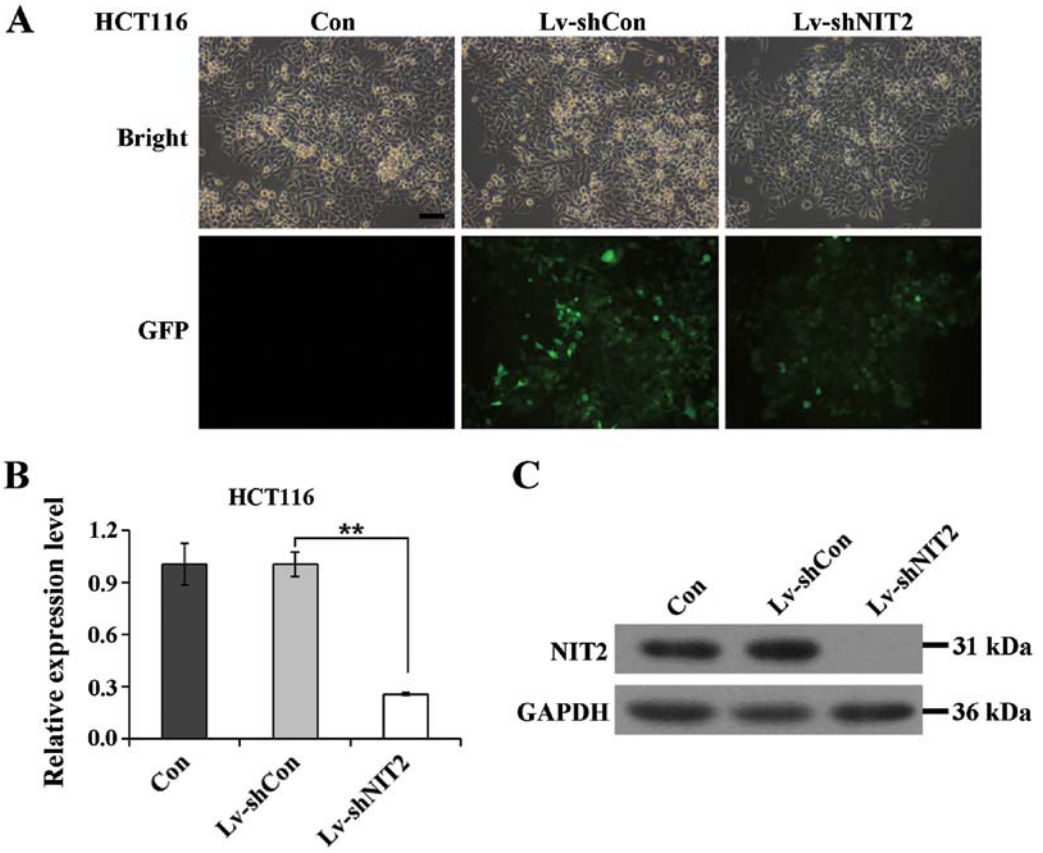

Figure 1. Knockdown efficiency of *NIT2* by lentivirus infection in the colon cancer cells, HCT116. (A) Microscopic images of colon cancer cells infected with lentivirus at a multiplicity of infection (MOI) of 15 (scale bar, 50 μm). Visible green fluorescence protein (GFP) expression proved that >80% of the cells were successfully infected. (B) qPCR analysis of *NIT2* knockdown efficiency in HCT116 cells. The mRNA expression of *NIT2* was significantly suppressed when the cells were infected with Lv-shNIT2. (C) Western blot analysis of *NIT2* knockdown efficiency. **P<0.01.

Intracellular signaling array. Intracellular signaling array was carried out as previously described (16). The HCT116 cell lysates were analyzed using the PathScan Intracellular Signaling Array kit (Cat. no. 7323; Cell Signaling Technology) for the simultaneous detection of 18 important and well-characterized phosphorylated signaling molecules. The lysate of HCT116 was diluted to 200 mg/ml, and 75 ml of lysate was added to nitrocellulose-coated glass slides pre-coated with the primary antibodies. The plate was incubated overnight at 4˚C, followed by exposure to the detection antibody cocktail for 1 h at room temperature. HRP-conjugated secondary antibody was then added and the plate was incubated for 30 min at room temperature. Substrate was then added and chemiluminescent signals were detected to screen and compare the differences between cell lysates infected with Lv-shCon and Lv-shNIT2.

Statistical analysis. All statistical analyses were performed using SPSS version 13.0 software. The differences between groups were compared using the Student's test, and data are expressed as the means ± SD of 3 independent experiments. A value of P<0.05 was considered to indicate a statistical significant difference.

Results

Efficacy of lentivirus-mediated RNAi targeting of NIT2. In the present study, we constructed both a control lentivirus (Lv-shCon) and a specific *NIT2*-targeting lentivirus (Lv-shNIT2). The HCT116 cells were cultured and infected with Lv-shCon or Lv-shNIT2. Non-infected cells were deemed as the negative control (Con). To demonstrate the infection efficiency, we employed a GFP tag which was embedded in the lentivirus to provide visualized confirmation. As shown in Fig. 1A, >80% of the colon cancer cells were GFP-positive, indicating that the transfection rate was satisfactory. To further investigate the efficiency of the *NIT2* knockdown, we examined the expression levels of *NIT2* in the colon cancer cells following lentivirus infection. The non-silencing lentivirus encoded with the irrelevant sequence had a negligible effect on *NIT2* expression, but the *NIT2*-silencing lentivirus markedly downregulated the mRNA expression of *NIT2* by 74.6% (Fig. 1B). We further investigated the protein expression level following infection with the constructed lentiviruses. According to the NCBI gene database, the human *NIT2* gene encodes a deduced protein comprising of 276 amino acids, with a calculated molecular mass of 30.6 kDa. As is shown in Fig. 1C, the protein expression of NIT2 in the HCT116 cells was noticeably depleted. Taken together, the lentiviruses we constructed conferred us an efficient vector system to knock down *NIT2* expression in colon cancer cells.

Depletion of NIT2 markedly inhibits the proliferation of colon cancer cells. To better understand the role of *NIT2* in colon cancer tumorigenesis, we examined the variation tendency of

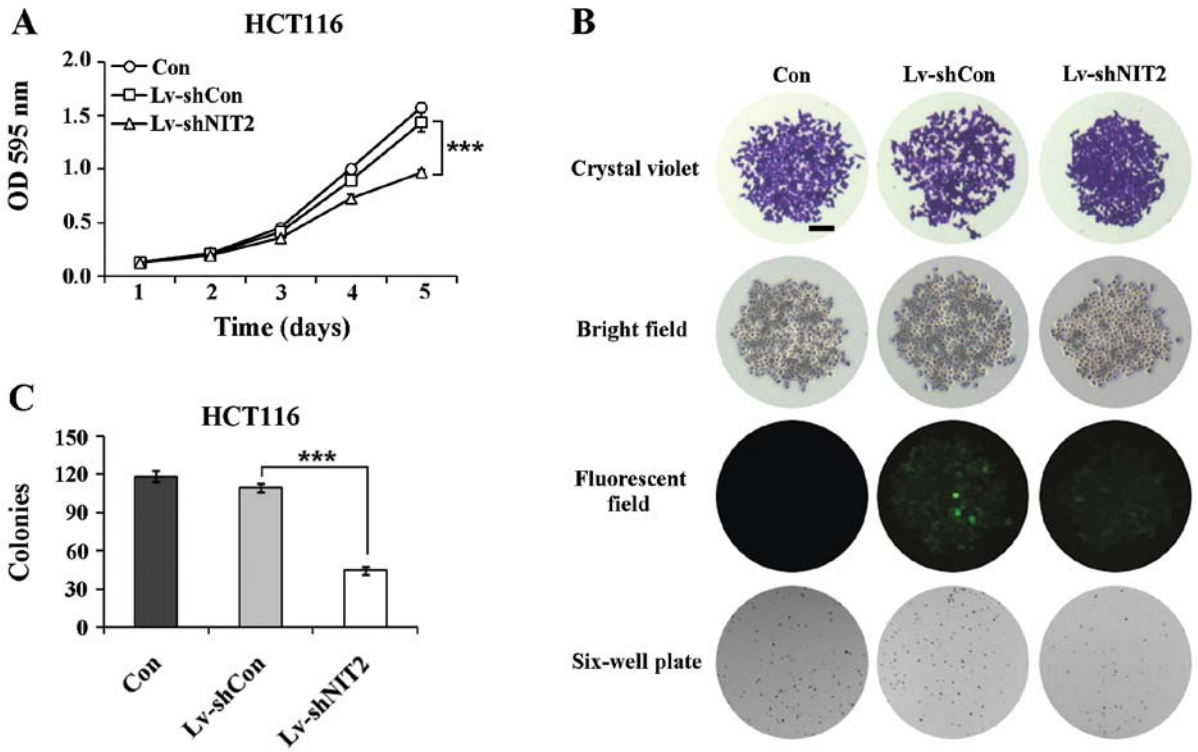

Figure 2. Depletion of *NIT2* suppresses the proliferation and colony formation capacity of HCT116 cells. (A) The monolayer growth rates of HCT116 cells from triplicate groups (Con, Lv-Con and Lv-shNIT2) were determined by MTT assay. (B) Representative microscopic images of colonies were stained by crystal violet (scale bar, 125 μm). The full vision of the 6-well plate under a microscope showed a significant inhibition of colony formation in the Lv-shNIT2 group. (C) Statistical analysis of the number of colonies with >50 single cells stained with crystal violet. ***P<0.001.

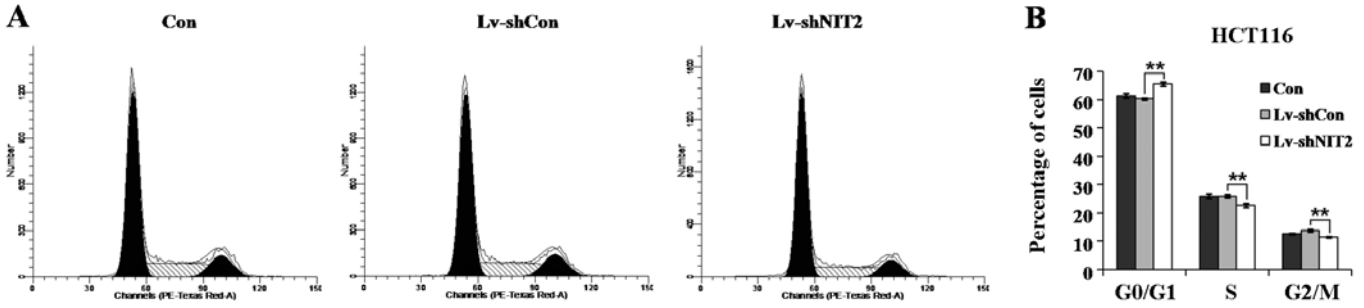

Figure 3. Downregulation of *NIT2* induces G0/G1 phase cell cycle arrest in the HCT116 cells. (A) Cell cycle distribution of HCT116 cells was analyzed by flow cytometry. (B) The population of cells in the G0/G1 phase was markedly increased, accompanied by a noticeable reduction in the number of cells in the S and G2/M phase. **P<0.01.

cell proliferation following lentivirus infection. We used MTT assay due to its testing sensitivity and dynamic range and examined the cell proliferation rate after 5 days of incubation. As illustrated by the line chart in Fig. 2A, the non-silenced cells showed no obvious difference compared with the control cells (Lv-shCon vs. Con); however, a significant inhibition of cell proliferation was observed in the *NIT2*-silenced cells (Lv-shNIT2 vs. Lv-shCon; P<0.001).

We subsequently cross evaluated the colony formation capacity of the HCT116 cells. As shown in Fig. 2B and C, the downregulation of *NIT2* induced a significant decrease in the colony formation ability of the cells. The colonies were markedly smaller and the colony numbers were statistically fewer compared with the control cells (P<0.001), whereas there were no noticeable differences between the non-silenced cells and the control cells. Collectively, the knockdown of *NIT2* by RNAi markedly suppressed the proliferation and colony formation ability of the colon cancer cells.

Cell cycle arrest is induced by the downregulation of NIT2. To examine whether the knockdown of *NIT2* suppresses the growth of colon cancer cells through the direct regulation of the cell cycle, the effects of the suppression of *NIT2* on the cell cycle distribution were examined. We explored the alterations in cell cycle distribution when the HCT116 cells were infected with the *NIT2*-silencing lentivirus. As illustrated in Fig. 3, the distribution of cells in the cell cycle (G0/G1, S and G2/M phase) varied significantly between the 3 groups (Con,

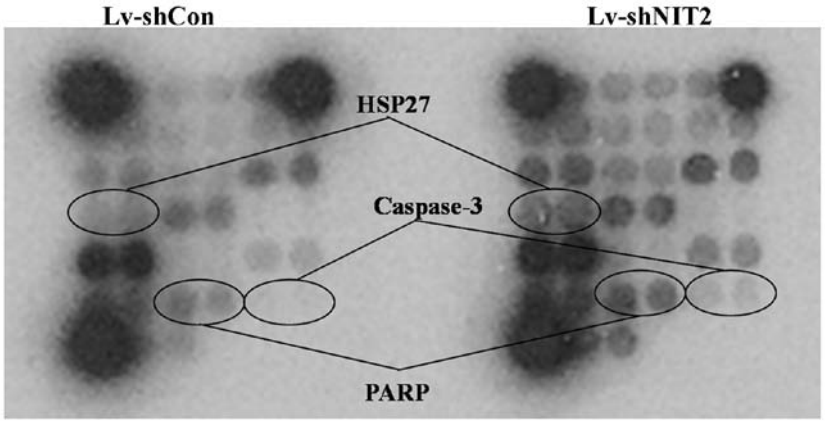

Figure 4. Chemiluminescent array shows the phosphorylation/cleavage status of 18 important signaling proteins in HCT116 cells. Modulation of *NIT2* was associated with several important apoptotic signaling pathways in the HCT116 cells. The levels of cleaved PARP at Asp214 and caspase-3 at Asp175 were significantly elevated, accompanied by an increase of phosphorylated HSP27 (phosphor-site at Ser78).

Lv-shCon and Lv-shNIT2). In contrast to the control group, the cells infected with Lv-shNIT2 were mostly distributed in the G0/G1 phase (65.61%) and fewer cells were distributed in the S phase (22.78%) and the G2/M phase (11.61%), indicating that the cell cycle was arrested in the G0/G1 phase. Our results demonstrated that treatment with Lv-shNIT2 markedly induced cell cycle arrest ($P<0.01$); however, the cells infected with the non-silencing lentivirus did not show any significant differences compared with the non-infected cells (Lv-Con vs. Con). These findings are in agreement with those observed for cell growth, which suggests that *NIT2* modulates colon cancer cell growth by controling the cell cycle.

Cell cycle arrest and inhibition of proliferation induced by the depletion of NIT2 are associated with caspase-3 and PARP signaling. The PathScan Intracellular Signaling Array kit was used to monitor the expression of 18 signaling molecules that are phosphorylated or cleaved in response to signal transduction pathway activation. Caspase-3 is a critical executor of apoptosis. Caspase-3 is activated by endoproteolytic cleavage at Asp175 and exerts its pro-apoptotic activity through the cleavage of multiple cellular targets (17-20). PARP, an enzyme that is involved in DNA repair, is one of the main substrates of activated caspase-3 (21,22). Increased levels of cleaved caspase-3 and cleaved PARP are reliable indicators of apoptosis (23). Heat shock protein 27 (HSP27), phosphorylated at Ser78 within the p38 MAPK pathway, is a mediator of cell stress that confers resistance to adverse environmental changes (24). In the present study, we demonstrated that when the colon cancer cells, HCT116, were treated with *NIT2*-silencing lentivirus, the cleavage of PARP and caspase-3 were noticeably increased, accompanied with an intracellular increase of phosphorylated HSP27 (Fig. 4).

Discussion

In mammals there are 2 metabolism routes for glutamine. One is catalyzed by glutaminases K and L, followed by the conversion of glutamate to α-ketoglutarate by transamination or by the glutamate dehydrogenase reaction, whereas the glutaminase II pathway employs ω-amidase catalyzed hydrolysis reaction from α-ketoglutamate (KGM) to α-ketoglutarate (25-34). The glutaminase II pathway is one of the clinical interests, particularly in certain inborn errors of metabolism and cancers (35-38). This has been strengthened by the finding of NIT2, which was demonstrated to be a putative tumor suppressor (11) and identical to ω-amidase (28,29,39). NIT2/ω-amidase plays a crucial role in glutamine metabolism. However, its role in human colon cancer remains unclear.

In the present study, we examined the biological role of *NIT2* in cell growth through an RNAi lentivirus system using the colon cancer cell line, HCT116, *in vitro*. We demonstrated that the downregulation of *NIT2* suppressed cell proliferation and colony formation. Moreover, cell cycle arrest was observed by FACS analysis and apoptotic signaling markers were detected by an intracellular signaling array. The significant alterations in cell proliferation and cell cycle progression prove that NIT2 plays a pivotal role in the growth of colon cancer cells, which is in alignment with an increase in HSP27 phosphorylation at residue Ser78 and the cleavage of caspase-3 at residue Asp175 and PARP at residue Asp214.

Semba *et al* (40) reported that aberrant NIT1 expression induced caspase-dependent apoptosis. In addition, an apoptosis-inducing effect of NIT1 in plant wound and herbicide-induced cell death has been proposed (41). In contrast to NIT1, much less is known about the biological function of NIT2 in humans. To the best of our knowledge, this is the first study to prove that the depletion of *NIT2* induces apoptosis through the caspase-3 and PARP pathways. Caspase-3 is a member of the cysteine-aspartic acid protease family (42). It interacts with caspase-8 and caspase-9 proteins and can trigger the sequential activation of cell apoptosis. Caspase-3 zymogen has virtually no activity until it is cleaved at Asp175 by an inhibitor caspase (43). Together with its main substrate PARP, both of them in the activated format can be deemed as reliable indicators of apoptosis (23).

Looking back to the observation of cell cycle arrest, all these data demonstrate that the depletion of *NIT2* may not only inhibit colon cancer cell growth, but may also promote the apoptosis of cancer cells. The biochemical significance of *NIT2* has been demonstrated in the present study, indicating

that the depletion of *NIT2* in colon cancer cells leads to a significant reduction in cell growth. Therefore, *NIT2* may be a promising therapeutic target in colon cancer and may also be an alternative option for the treatment of colon cancer.

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