Methylation of nucleolar and coiled-body phosphoprotein 1 is associated with the mechanism of tumorigenesis in hepatocellular carcinoma

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Received April 26, 2013; Accepted June 25, 2013

DOI: 10.3892/or.2013.2676

Abstract. Nucleolar and coiled-body phosphoprotein 1 (NOLC1) plays an essential role in the synthesis of rRNA and the biosynthesis of ribosomes. Previous studies suggest that NOLC1 is crucial for normal cell growth, and plays a role in the regulation of tumorigenesis of nasopharyngeal carcinoma (NPC) and demonstrate that both NOLC1 and tumor protein 53 work synergistically to activate the MDM2 promoter in NPC cells. Yet, the functioning of NOLC1 in liver cancer remains unknown. The aim of the present study was to understand how the NOLC1 gene is regulated in liver carcinogenesis. In this study, we showed that NOLC1 was silenced or downregulated in liver tumor tissues when compared with that in the matched non-cancer tissues. In addition, human hepatoma cells weakly expressed NOLC1, whereas cultured human normal liver cell lines expressed abundant levels. The hypermethylation status in the promoter CpG1 start region appeared to be correlated with the NOLC1 expression levels in liver cell lines or liver normal and tissue specimens. We found that four CpG dinucleotides were located at the CpG1 start region. Further molecular analysis of mutagenesis indicated that the four CpG dinucleotides play a role in the promoter activity of the NOLC1 gene. The expression of NOLC1 and DNA methylation of its promoter affected cell proliferation and apoptosis. The expression of NOLC1 in hepatoma cell lines was restored following exposure to the demethylation agent, 5-azacytidine. Low expression of NOLC1 in hepatoma cell lines and liver cancer tissues was associated with cyclin D3. In conclusion, our study demonstrated that DNA methylation is a key mechanism of silenced NOLC1 expression in human hepatocellular carcinoma cells, and NOLC1 gene hypermethylation of the four CpG dinucleotides is a potential biomarker for hepatocellular carcinoma.

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Key words: DNA methylation, nucleolar and coiled-body phosphoprotein 1, tumorigenesis, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide (1,2). As for many other tumors, development of HCC is due to a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes (TSGs) (3).

In the last three decades, cancer has been understood as a summary of altered genetic and epigenetic events. The epigenetic pathway is, in contrast to genetic events, a reversible alteration and is characterized by three main mechanisms: i) DNA hypermethylation leading to inactivation, ii) DNA hypomethylation causing genomic instability and iii) histone modifications affecting chromatin conformation (3).

These processes, particularly aberrant DNA methylation and histone modifications, are closely linked with each other by a protein complex of transcript activators and repressors and alter mRNA transcript expression of affected genes (4). Characteristically, DNA methylation does not change the genetic information, it simply alters the readability of the DNA and results in inactivation of genes by subsequent mRNA transcript repression (3). In humans and other mammals, CpG island methylation is an important physiological mechanism. The inactivated X-chromosome of females silenced alleles of imprinted genes or inserted viral genes and repeat elements are inactivated through promoter methylation (5).

The nucleolar and coiled-body phosphoprotein 1 (NOLC1, also called Nopp140 or NS5ATP13) is a family of proteins which is characterized by a conserved C-terminal SRP40 domain (6). NOLC1 is a phosphoprotein composed of N- and C-terminal domains and a unique central repeated domain consisting of alternating acidic and basic amino acid clusters and localized in the nucleolus (7). The highest steady state concentrations of vertebrate NOLC1 localize within the dense fibrillar component (DFC) of nucleoli (8,9). NOLC1 was first identified as a nuclear localization signal-binding protein and is thought to be a chaperone for shuttling between the nucleolus and the cytoplasm (9,10). NOLC1 plays an essential role in

the synthesis of rRNA and the biosynthesis of ribosomes. A previous study revealed that NOLC1 has transcription factor-like activity (11). By binding to the transcription factor C/EBP β (also known as AGP/EBP or NF-IL6), NOPP140 can indirectly activate the transcription of the α-1 acid glycoprotein gene (11). Overexpression of the partial or whole NOLC1 cDNA resulted in mislocalization of nucleolar proteins, improper formation of the nucleolus, and inhibition of rRNA gene transcription. These observations suggest that hNopp140 is crucial for normal cell growth (12).

In our previous study (6), we found that an altered DNA methylation pattern may play a definitive role in the regulation of NOLC1 gene expression in human liver cancers. In this report, we present evidence to support the notion that DNA methylation is a key mechanism of epigenetic regulation to suppress NOLC1 expression in HCC cells, and we identified the precise methylation sites in the NOLC1 gene. This study provides important insight into the epigenetic regulation in HCC.

Materials and methods

Cell culture and 5-aza-2'-deoxycytidine treatment. Human normal liver cell lines, L02 and Chang liver, and human hepatoma cell lines, HepG2 and Huh7, were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics [penicillin-streptomycin solution (PSN)], under identical conditions (37˚C in humidified 5% CO_2/95% air), respectively. For drug treatment, cell lines were treated with 5 μmol/l 5-aza-2'-deoxycytidine (Aza) (Sigma, St. Louis, MO, USA) for 3 days, changing Aza and medium every 24 h. Control cells were incubated with culture medium.

There were clear similarities between the results from the Chang liver and L02 cell. The results from Huh 7 were similar to those of the HepG2 cells. Therefore, the results from Chang liver and Huh7 cells are not presented.

Tissues and surgical specimens. HCC paraffin blocks and frozen tissues were obtained from the archives of the Department of Pathology, Beijing Ditan Hospital, Capital Medical University, Beijing, China, according to institutional review board-approved protocol. The use of human specimens in this research was approved by the ethics committee of our hospital according to the Declaration of Helsinki. We clearly confirm that we had all the necessary consent from any patient involved in the study, including consent to participate in the study and consent to publish.

RNA extraction and RT-PCR. RNA was extracted from cells or patient tissues using an RNA isolation reagent (TRIzol; Invitrogen Life Technologies, Carlsbad, CA, USA). To prevent DNA contamination, total RNA was treated with RNase-free DNase II (Invitrogen Life Technologies).

The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (forward primer, 5'-TCACCAGGGCTGCTTTTA-3' and reverse, 5'-TTCACACCCATGACGAACA-3') was used as an internal control in the PCR amplification. A two-step RT-PCR procedure was performed in all experiments. First, total RNA samples (2 μg/reaction) were reversely transcribed into cDNAs by RT II reverse transcriptase (Invitrogen Life Technologies). Then, the cDNAs were used as templates in PCR with NOLC1 gene-specific primers (5'-AGCTGGCCTGACGGTATG-3' and 5'-TTGGTCTGGCTGAGTACCG-3'). The primers for cyclin D3 were 5'-ATTCCTCTTTGCTTTGCTTTC-3' and 5'-CAGCAGCAAAGCTGTCAATC-3'. The primers that were used for amplification of MDM2 were 5'-CGGCACGAGCTAGGATCT and 5'-ACGGCAGCTCCATGAGTC-3'. The amplification reactions were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). The PCR was programmed as follows: 2 min at 95˚C and 30-32 cycles of 30 sec at 94˚C, 30 sec at 58-62˚C, and 30 sec at 72˚C, with an extension for 10 min at 72˚C. The PCR bands were visualized under UV light and photographed. Quantitative real-time PCR was used to measure mRNA levels of urea cycle genes for the HCC cell lines and NOLC1 mRNA levels for tissues using the StepOne Plus Real-Time PCR system with SYBR-Green Master Mix (Applied Biosystems).

Immunohistochemical staining. HCC biopsy specimens were subjected to routine immunohistochemical staining using a monoclonal antibody directed against NOLC1 (13), according to a previously described method (14). Immunoreactivity, defined as the number of positive tumor cells over the total tumor cells, was scored independently by two researchers. The number of NOLC1-positive and -negative HCC cells was counted under a light microscope at a magnification of x400. Only the cells displaying brown nucleoli on the section were considered NOLC1-positive. For each slide, 7-10 microscopic fields were randomly chosen. Positive scores were categorized into weak staining (only one nucleolus was stained), moderate staining (more than one nucleolus was stained), and strong staining (both the nucleus and nucleolus of the tumor cells were stained). The average percentage of NOLC1-positive HCC cells was then calculated for each group.

Western blot analysis. Lysates from the cultured cells were subjected to routine western blotting as described previously (15). The antibodies used were monoclonal anti-mouse antibodies against NOLC1 (13) and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and polyclonal rabbit antibodies against cyclin D3 and caspase-8 (Abcam, Cambridge, MA, USA). The results shown are representative of 2 independent experiments.

Bisulfite genomic sequencing. Genomic DNA was purified from cells with the Wizard Genomic DNA purification kit (Promega Corporation, Madison, WI, USA). DNA (2 μg) was bisulfite modified with the EZ DNA methylation direct kit (Zymo Research, Orange, CA, USA). Sequence-specific primers to amplify the CpG-rich regions of interest were designed using a computer program (MethPrimer; http://www.urogene.org/methprimer). The primers that were used for amplification were as follows: NOLC1 upstream CpG island-1 forward, 5'-TTGGGATGGTATTAGAAAGGGT-3' and reverse 5'-CTCAAAAACCCAACAAAAACAAT-3'; NOLC1 upstream CpG island-2 forward, 5'-GTTTTTGTTGGGTTTTTGAGT-3' and reverse 5'-CCTCAATAAAACAAAACTCTACCTC-3'. The PCR products were amplified, purified and cloned into a vector (pGEM-T Easy Vector; Promega Corporation).

Table I. Construct sequences of the shRNAs.

shRNA	Sequence
NOLC1 shRNA	5'-TGCTGTTGACAGTGAGCGCGACATCTAAGTCTGCAGTTAATAGTGAAGCCACAGAT GTATTAACTGCAGACTTAGATGTCTTGCCTACTGCCTCGGA-3'
NS-shRNA	5'-TGCTGTTGACAGTGAGCGAACCACTAAGCTTCTGTCTTAATAGTGAAGCCACAGAT GTATTAAGACAGAAGCTTAGTGGTCTGCCTACTGCCTCGGA-3'

NOLC1, nucleolar and coiled-body phosphoprotein 1.

Clones were selected through blue-white screening. Finally, the colonies harboring the insert were sequenced in a 96-well plate using the M13 reverse and/or forward primers.

Methylation-specific PCR. The bisulfite-treated DNA was amplified using primers that specifically amplify either the methylated or unmethylated sequence of the NOLC1 promoter containing four CpG dinucleotides, respectively. The PCR was performed for 40 cycles, with annealing temperatures of 58˚C for the methylated reaction and 52˚C for the unmethylated reaction. The human methylated and unmethylated DNA was used as a control to verify the specificity of the primers (Qiagen, Valencia, CA, USA).

Plasmid and transfection. Cells were subcultured and transfected as previously described (16,17). The cDNA encoding NOLC1, flanked by *Bam*HI and *Sal*I restriction sites, was cloned into the mammalian expression vector pCDNA4 (Stratagene Inc., La Jolla, CA, USA) to generate pCDNA-NOLC1, which expresses an N-terminal myc-tagged NOLC1 fusion protein. The promoter of NOLC1 was cloned into the pGL3 plasmid. Subconfluent cells were transiently transfected with pCDNA-NOLC1 DNA (4 μg/dish) mixed with Lipofectamine and Plus™ reagent (Invitrogen Life Technologies), according to the manufacturer's protocol. Cells were harvested ~48 h after transfection (18).

NOLC1 shRNA transfectants. The shRNA constructs described in Table I were purchased from Open Biosystems (Huntsville, AL, USA). When the HCC cultured cells had reached 70-80% confluence, the shRNA constructs were transfected into the HCC cells using the Arrest-In transfection reagent for RNAi (Open Biosystems) (12).

Luciferase assay. The cells were transfected with 0.6 μg of firefly luciferase reporter plasmid and 0.05 μg of control plasmid containing *Renilla* luciferase (pRL-TK; Promega Corporation). A promoterless basic vector (pGL3; Promega Corporation) was used as a negative control. To confirm the efficiency of transfection (Lipofectin; Invitrogen Life Technologies), a luciferase expression vector (pGL3-control; Promega Corporation) was used as a positive control. After 48 h, the cells were harvested for analysis. Luciferase enzyme assays and colorimetric β-galactosidase assays were performed according to the manufacturer's instructions (Promega Corporation). Luciferase activity was normalized to β-galactosidase activity to assess the transfection efficiency. When indicated, the firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega Corporation), according to the manufacturer's instructions. The *Renilla* luciferase activity of pRL-TK was used to normalize the firefly luciferase activity of the reporter construct. Each transfection experiment was repeated 3 times.

Cell growth and proliferation assay. Cell growth was determined by the colorimetric tetrazolium derived sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate assay (XTT), and DNA synthesis of cells was assessed by the bromodeoxyuridine (BrdU) incorporation assay (both from Roche Applied Science, Mannheim, Germany). For the cell growth and proliferation assays, the cells of each group at 48 h after treatment were re-seeded onto 96-well plates at a density of $3x10^3$ cells/well. Then XTT and incorporated BrdU were measured colorimetrically using a microtiter plate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm (19,20).

Determimation of apoptosis. Cells ($3x10^5$) were cultured onto 60-mm SWNHs-coated and non-coated dishes for 48 h. Then apoptotic cells were identified by using fluorescence-activated cell sorting (FACS) Annexin V-Fluos (BioLegend) following the protocol of the manufacturer. Cells having been cultured were washed at 4˚C for 30 min in PBS and then stained with Annexin-V staining solution at 4˚C for 3 h. Gels were washed 4 times in PBS at 4˚C and fixed at room temperature with 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 15 min. For counterstaining, 7-AAD (2 μg/ml) was added to the first washing step. The numbers of total, Annexin-V-positive, 7-AAD-positive, and double-positive cells were determined respectively using FACS technique. Apoptosis was verified by detection of activated caspases (21).

Statistics. Significance was determined using the one-way ANOVA test for the mean of three different experiments. Significance was determined using the paired Student's t-test for the mean of 3 different experiments. Probabilities of ≤ 0.05 were considered to indicate statistically significant results.

Results

Low expression of NOLC1 in HCC cell lines and liver cancer tissues is associated with cyclin D3. To understand the potential mechanisms by which NOLC1 is regulated in HCC, we

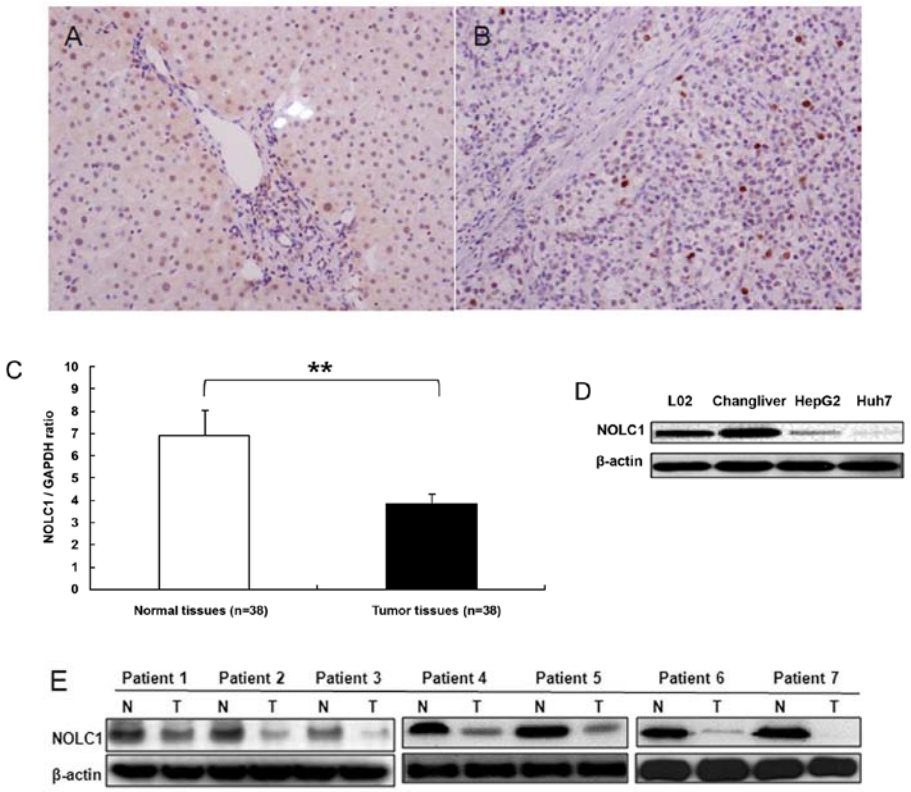


Figure 1. NOLC1 mRNA and protein expression in HCC cell lines and patient specimens. (A) The expression of NOLC1 in normal liver tissues. (B) The expression of NOLC1 in tissues of patients with HCC. (C) NOLC1 mRNA levels in normal and tumor tissues were detected by qRT-PCR in patients (n=38) with HCC. (D) For investigating the role of NOLC1 in the pathogenesis of the liver, we also observed the specific expression of NOLC1 protein in the human normal liver cell lines L02 and Chang liver, and in human hepatoma cell lines HepG2 and Huh7. (E) NOLC1 protein levels in normal (N) and tumor tissues (T) were detected by western blotting in patients with HCC. The data are presented as means ± SD from 3 independent experiments (**P<0.01). NOLC1, nucleolar and coiled-body phosphoprotein 1; HCC, hepatocellular carcinoma.

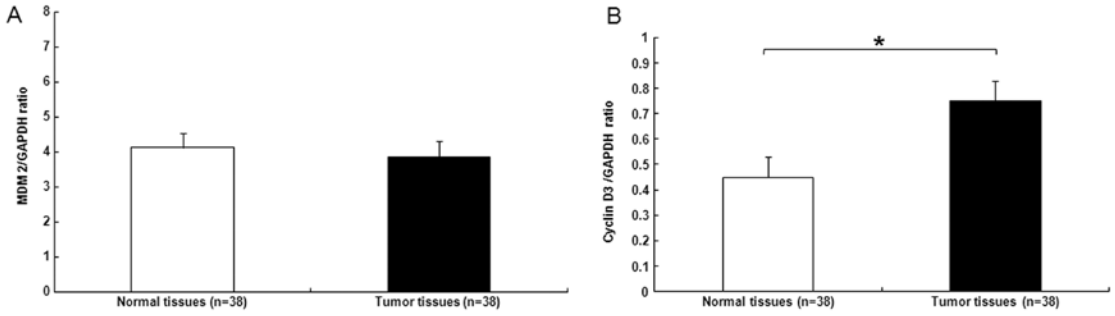
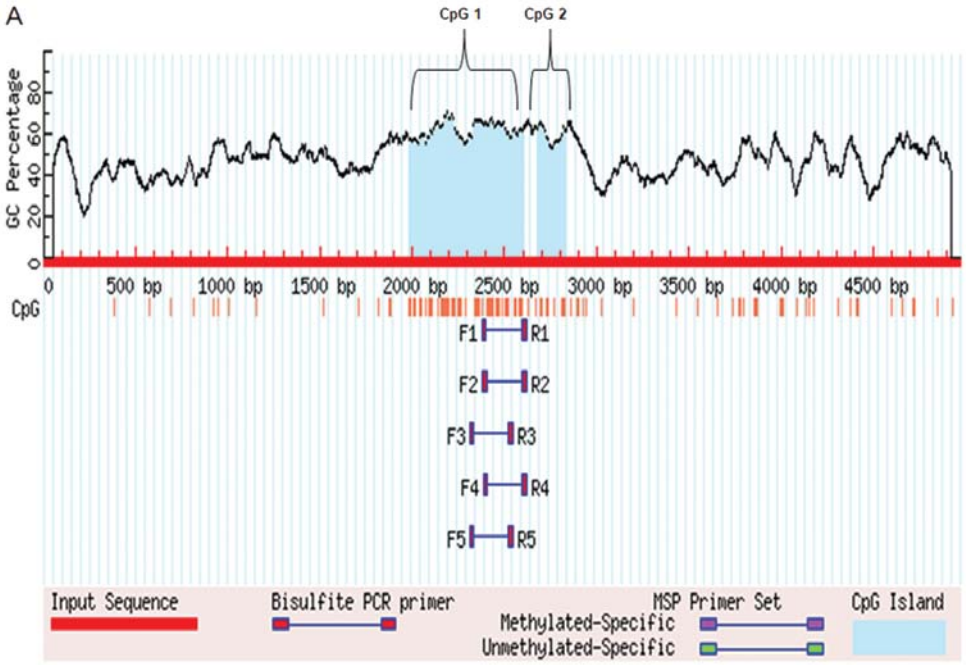


Figure 2. MDM2 and cyclin D3 mRNA expression levels in normal and tumor tissues of patients with HCC. (A) MDM2 and (B) cyclin D3 mRNA expression levels were analyzed by RT-PCR in normal and tumor tissues of patients with HCC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of the RT-PCR for normalization. The data are presented as means ± SD (*P<0.05). HCC, hepatocellular carcinoma.

detected the NOLC1 expression levels at the transcription and translation levels in HCC cell lines and human HCC tissues using immunohistochemical staining and RT-PCR approach. The results showed that NOLC1 was moderately expressed in the cytoplasm and the nucleus (Fig. 1A). It was expression at a low level in HCC tissues and was localized in the nucleus (Fig. 1B). We then examined NOLC1 mRNA expression in 38 pairs of patient tissues. The NOLC1 expression was decreased in tumor tissues when compared with that in the matched noncancerous tissues after 32 cycles of PCR amplification (P<0.01) (Fig. 1C). As shown in Fig. 1D, strong expression of NOLC1 protein was detected in the normal liver cell lines, L02 and Chang liver, but NOLC1 protein was weakly expressed in hepatoma liver cell lines HepG2 and Huh7. To correlate the mRNA transcription with protein expression, Western blot analysis was performed to examine the NOLC1 protein expression in patient tissues. As shown in Fig. 1E, NOLC1 protein in the tumor samples was decreased at different degrees when compared with that in the adjacent noncancerous specimens, particularly in patients 1-7. Hwang *et al* (12) found that NOLC1 plays a role in the regulation of tumorigenesis of nasopharyngeal carcinoma (NPC) and demonstrated that both NOLC1 and tumor protein 53 synergistically activate the MDM2 promoter in NPC cells.



B

Methylation status of each CpG site

CpG position	57	72	82	93	122	128	155	171	173	181	219	221
Me-CpG	40/69 58.0%	33/69 47.8%	31/69 44.9%	26/69 37.7%	7/69 10.1%	10/69 14.5%	2/69 2.9%	3/69 4.3%	3/69 4.3%	3/69 4.3%	0/69 0.0%	0/69 0.0%
CpG position	231	241	246	249	255	267	278	294	298	308	326	331
Me-CpG	1/69 1.4%	1/69 1.4%	2/69 2.9%	4/69 5.8%	2/69 2.9%	0/69 0.0%	2/69 2.9%	1/69 1.4%	0/69 0.0%	0/69 0.0%	0/69 0.0%	0/69 0.0%
CpG position	333	341	363	418	425	429	431	433	436	444	457	Total
Me-CpG	4/69 5.8%	3/69 4.3%	0/69 0.0%	2/69 2.9%	2/69 2.9%	3/69 4.3%	1/69 1.4%	1/69 1.4%	1/68 1.5%	1/69 1.4%	3/69 4.3%	192/2414 8.0%

C

Methylation status of each CpG site

CpG position	22	29	32	38	47	49	68	72	75	82	85	87	99
Me-CpG	2/69 2.9%	0/69 0.0%	2/69 2.9%	0/69 0.0%	0/69 0.0%	1/69 1.4%	2/69 2.9%	1/69 1.4%	0/69 0.0%	0/69 0.0%	0/69 0.0%	1/69 1.4%	1/69 1.4%
CpG position	114	120	130	132	165	173	192	203	210	244	280	308	311
Me-CpG	1/69 1.4%	2/69 2.9%	1/69 1.4%	0/69 0.0%	0/69 0.0%	3/69 4.3%	2/69 2.9%	1/69 1.4%	0/69 0.0%	1/69 1.4%	2/69 2.9%	0/69 0.0%	1/69 1.4%
CpG position	319	342	348	352	383	421	424	430	441	475			Total
Me-CpG	2/69 2.9%	0/69 0.0%	0/69 0.0%	1/69 1.4%	2/69 2.9%	1/69 1.4%	0/69 0.0%	0/69 0.0%	2/69 2.9%	1/69 1.4%			33/2484 1.3%

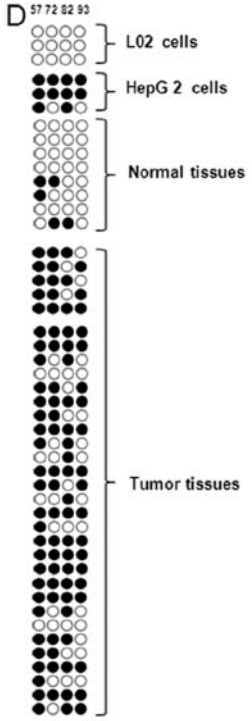


Figure 3. Regulation of NOLC1 expression by DNA methylation in HCC cases. (A) CpG islands of NOLC1 were predicted by software from PubMed. Methylation-specific PCR analysis of the NOLC1 promoter in liver cells and tissues [(B) CpG island 1; (C) CpG island 2]. (D) Genomic DNA extracted from liver cells and tissues was modified with sodium bisulfate, followed by PCR amplification, cloning and sequencing. The DNA methylation status was analyzed by software from http://quma.cdb.riken.jp/. The DNA methylation status at start site within CpG island 1 is shown. Methylated sites are indicated by filled dark circles; and unmethylated sites, empty white ones. NOLC1, nucleolar and coiled-body phosphoprotein 1; HCC, hepatocellular carcinoma.

The frequent downregulation of miR-138 regulates cyclin D3 and functions as a tumor-suppressor in HCC (22). We next examined MDM2 and cyclin D3 mRNA expression in patient tissues. MDM2 expression in tumor tissues was similar to that in the matched noncancerous tissues (Fig. 2A), while the tumor samples exhibited an increase in cyclin D3 expression when compared with that in the adjacent noncancerous specimens (Fig. 2B).

NOLC1 expression is regulated by DNA methylation in HCC tumor cells. Since many cancer cells exhibit aberrant epigenetic regulation, it is possible that NOLC1 expression is regulated by epigenetic modification. To confirm that DNA methylation regulates NOLC1 expression, detailed methylation analysis of the NOLC1 gene sequence was performed using genomic DNA extracted from cell lines and liver tissues. Sequence analysis (GenBank accession no. GI 470595142;

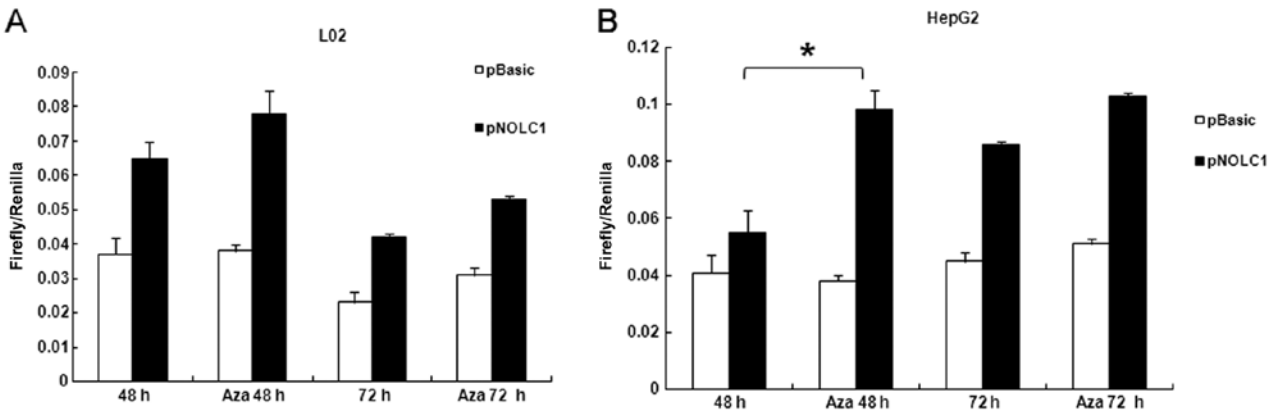


Figure 4. Luciferase reporter assay of NOLC1 minimal promoter activity. (A) L02 and (B) HepG2 cells were transfected with reporter gene plasmid (pGL3-NOLC1), together with the pRL-TK *Renilla* luciferase plasmid (as an internal control). Twenty-four hours after transfection, the cells were treated with or without Aza for another 24 or 48 h. The cells were harvested, and luciferase activity was determined using the Dual Luciferase kit. The data are presented as means ± SD from 3 independent experiments (*P<0.05). NOLC1, nucleolar and coiled-body phosphoprotein 1; Aza, 5-aza-2'-deoxycytidine.

http://www.ncbi.nlm.nih.gov/mapview) (23) indicated that the NOLC1 promoter has 2 typical CpG islands (CpG1 and CpG2) upstream of it. We predicted the CpG islands of the NOLC1 promoter: the length of CpG island 1 (CpG1) was 616 bp (-1987 and -2602), and the length of CpG island 2 (CpG2) was 148 bp (-2678 and -2825) (Fig. 3A). To determine whether NOLC1 promoter methylation is associated with the control of NOLC1 expression in liver cell lines and clinical specimens, we performed methylation-specific PCR and compared the promoter methylation status in the hepatoma cell lines with that in the normal liver cell lines, and in tumor tissues with that in paired noncancerous tissues. Methylation of the 2 CpG dinucleotides in the promoter region was detectable. As shown in Fig. 3, the results revealed that 4 dinucleotides at the start of CpG1 were strongly methylated (Fig. 3A and D), particularly in the hepatoma cell lines and tumor tissues when compared with that in normal liver cell lines and adjacent noncancerous tissues (Fig. 3D). The methylation frequencies were 58.07% in CpG1-57, 47.8% in CpG1-72, 44.9% in CpG1-82 and 37.7% in CpG1-93 (Fig. 3B). But CpG2 was not methylated (Fig. 3C). The methylation status in the promoter CpG1 start region appeared to be correlated with NOLC1 expression levels in the liver cell lines and tissues specimen.

Effect of the CpG1 island on NOLC1 promoter activity. To investigate the possible effect of methylation of the CpG1 on promoter activity and determine the functional significance of the CpG1, we generated a reporter gene construct using a NOLC1 promoter sequence containing CpG1. The reporter construct (pNOLC1) together with pRL-TK *Renilla* luciferase expression vector were transiently transfected in L02 and HepG2 cells, followed by Aza treatment. The promoter activity was determined by luciferase assay. Firefly and *Renilla* luciferase activities were measured at the points indicated. *Renilla* luciferase activity was used to normalize firefly luciferase activity of the reporter constructs. As shown in Fig. 4, Aza treatment caused a significant increase in promoter activity in both cell lines, particular in the HepG2 cells (Fig. 4B). Luciferase activity of pGL3-basic, which has no promoter element, was not affected by Aza treatment. The CpG1 dinucleotides in the plasmid were not methylated at transfection (data not shown). Thus, the data suggest that the 4 CG dinucleotides at the CpG1 island start site appear to be critical for NOLC1 promoter activity.

Function of NOLC1 in cell biology. It is uncertain how NOLC1 affects cellular function. Cells were synchronized at the G1/S boundary by double thymidine block, and then released into mitosis. After 24 h, BrdU was added into the medium at the indicated time points to evaluate DNA synthesis. As shown in Fig. 5A, incorporation of BrdU into the control, and accumulation of mitotic L02 cells was significantly promoted by shRNA interfere NOLC1 (P<0.05). In contrast, overexpression of NOLC1 in HepG2 cells were significantly delayed at 36 and 48 h, particularly in cells treated with Aza (P<0.05) (Fig. 5B).

As determined by XTT assays, NOLC1 silencing of L02 cells resulted in a significant increased in cell growth when compared to that in the control and other cells (P<0.05) (Fig. 5C). However, Aza treatment in HepG2 cells significantly inhibited cell growth when compared with than the control group (P<0.01) (Fig. 5D).

The effect of NOLC1 expression on apoptosis was determined in L02 and HepG2 cells by flow cytometry. Downregulation of NOLC1 in L02 cells significantly suppressed apoptosis (P<0.01) (Fig. 6A). Furthermore, HepG2 cells treated with Aza exhibited increased apoptosis (P<0.01) (Fig. 6B), which may be associated with overexpression of caspase-8 and low levels of cyclin D3 (Fig. 7B).

Discussion

In high incidence areas, such as Asia and Africa, HCC is strongly associated with chronic viral hepatitis B and C and liver cirrhosis; 70-80% of HCC occurs in cirrhotic liver. Nutritional factors, toxins and metabolic diseases also contribute to hepatocarcinogenesis (1,2).

Aberrant epigenetic states may predispose to genetic changes, but genetic changes may also initiate aberrant epigenetic events. Epigenetic and genetic mechanisms may

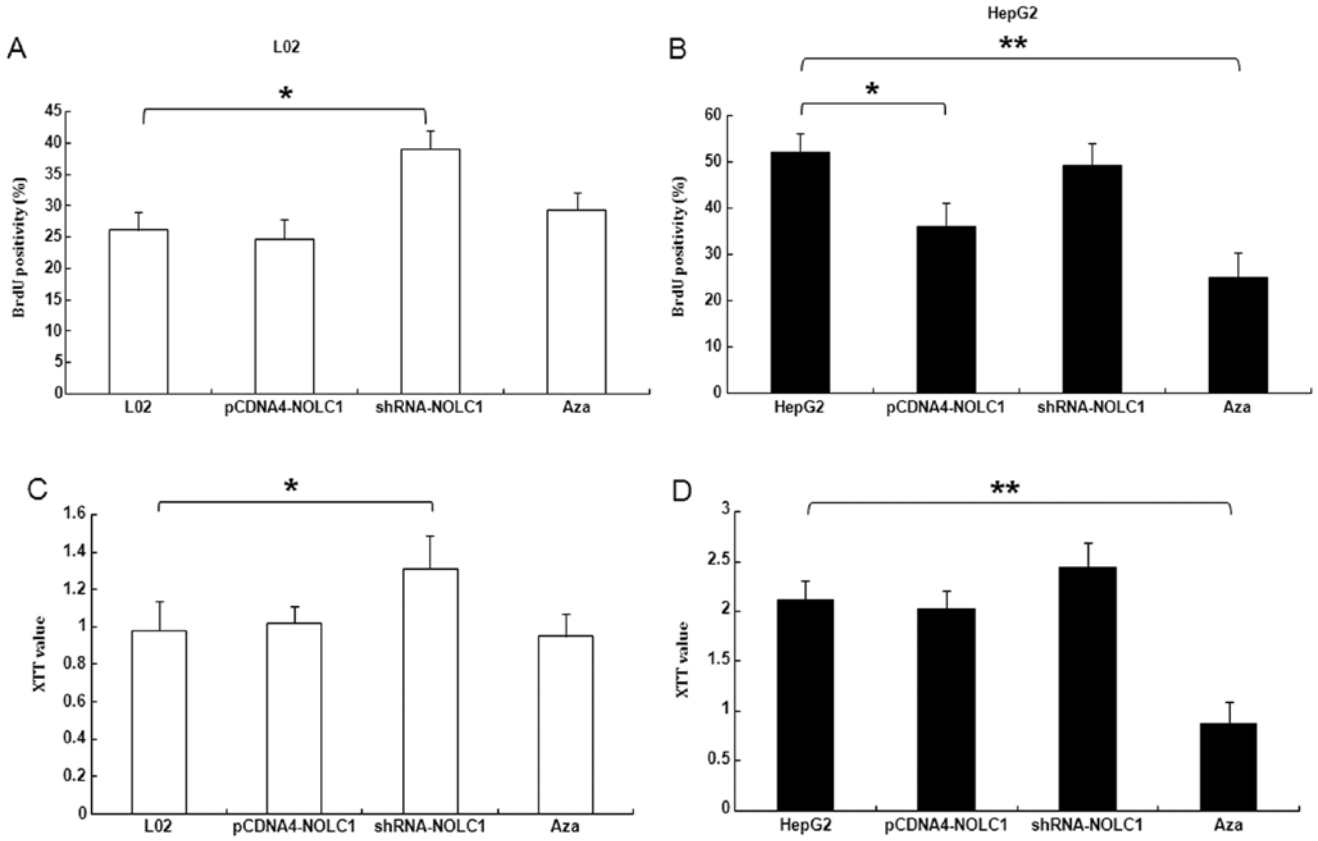


Figure 5. Expression of NOLC1 and DNA methylation of its promoter affect cell proliferation. (A and C) L02 and (B and D) HepG2 cells were transfected with plasmid pCDNA4-NOLC1 or shRNA-NOLC1. Twenty-four hours after transfection, the cells were treated with or without Aza for another 24 h. The effects of expression of NOLC1 and DNA methylation of its promoter on cell growth and viability were determined by (A and B) BrdU and (C and D) XTT assays. All data are represented as means ± SEM (*P<0.05, **P<0.01). NOLC1, nucleolar and coiled-body phosphoprotein 1; Aza, 5-aza-2'-deoxycytidine; BrdU, bromodeoxyuridine.

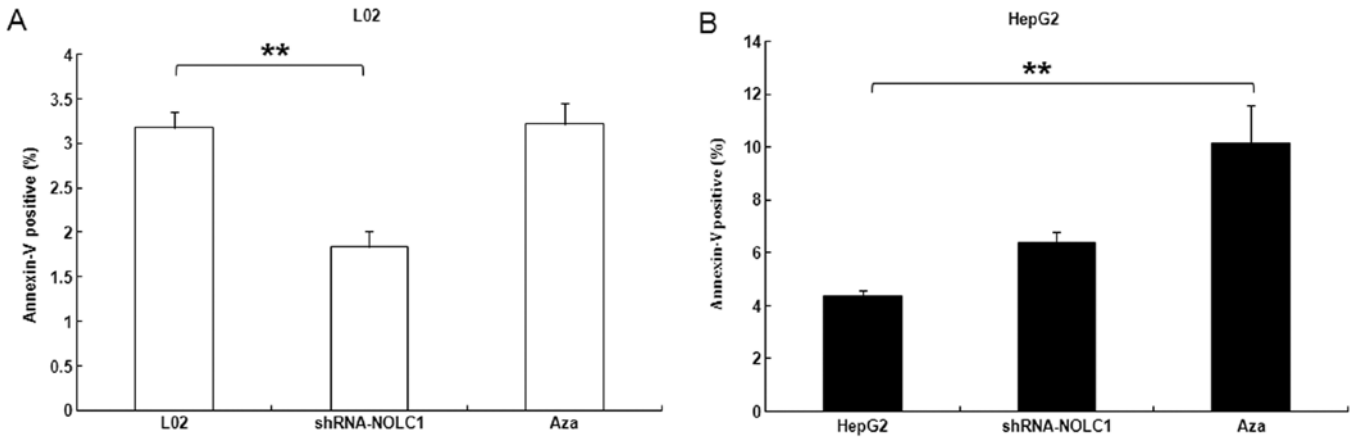


Figure 6. Expression of NOLC1 and DNA methylation of its promoter affect cell apoptosis. (A) L02 and (B) HepG2 cells were transfected with the shRNA-NOLC1 plasmid. Twenty-four hours after culture, the cells were treated with or without Aza for another 24 h. The effects of expression of NOLC1 and DNA methylation of its promoter on cell apoptosis were determined by fluorescence-activated cell sorting (FACS) using Annexin V-Fluos following the protocol of the manufacturer. All data are represented as means ± SEM (**P<0.01). NOLC1, nucleolar and coiled-body phosphoprotein 1; Aza, 5-aza-2'-deoxycytidine.

thus work together to silence key cellular genes and destabilize the genome, leading to oncogenic transformation and the observed complexity and heterogeneity in human cancers, including HCC (24-26). The development of HCC results from a multistep process beginning with the accumulation of genetic and epigenetic alterations in regulatory genes (3). In general, cancer cells have global hypomethylation, but they have hypermethylation in some specific genes. DNA hypermethylation in promoter regions is associated with the silencing of tumor-suppressor genes because of direct or indirect prevention to accessing transcription factors in the promoter region (27). Recent studies (3,28,29) have demonstrated that CpG island

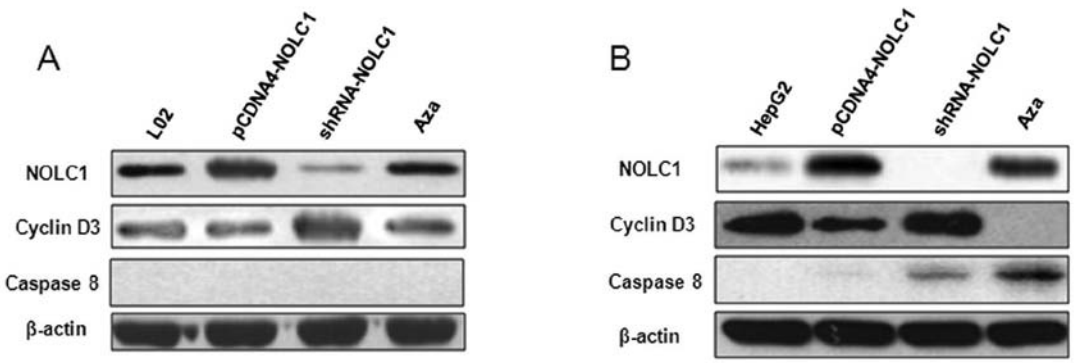


Figure 7. Functional characterization of NOLC1 involves cyclin D3 and caspase-8. Downstream executioners of apoptosis induced by NOLC1 silencing were identified as caspase-3 and -7 and activated cleavage products are shown. No activation cleavage was detectable in the controls. Overall, these results indicate that NOLC1 constitutively suppress apoptosis in HepG2 cells via pathway(s) involving caspase-3 and -7. (A) L02 and (B) HepG2 cells were transfected with plasmid pCDNA4-NOLC1 or shRNA-NOLC1. Twenty-four hours after transfection, the cells were treated with or without Aza for another 24 h. The effects of expression of NOLC1 and DNA methylation of its promoter on cell biology were studied by western blotting. NOLC1, nucleolar and coiled-body phosphoprotein 1.

hypermethylation, via silencing of key cancer-related genes, plays a major causal role in cancer, including HCC (23).

Our study of NOLC1 gene methylation provides a novel insight into the epigenetic regulation in HCC. NOLC1 plays a role as an oncogene in NPC tumorigenesis. Although an increasing numbers of reports have shown that NOLC1 is a multiple functional protein (30), Hwang *et al* (12) demonstrated that NOLC1 plays a role in enhancing NPC tumorigenesis. In our study, NOLC1 expression was suppressed in hepatoma cell lines and tumor tissues. Moreover, NOLC1 expression in hepatoma cell lines was restored by treatment with the demethylating agent Aza and was associated with cyclin D3. We further demonstrated that the methylation status of the CpG-rich region (CpG1) in the promoter region was correlated with NOLC1 gene expression in hepatoma cell lines. Consistently, the NOLC1 promoter activity was noted in the reporter assay in the hepatoma cell lines tested. Taken together, these observations indicate that DNA methylation regulates NOLC1 expression in liver cancer cells.

Most studies investigating the mechanism that regulates gene expression by CpG methylation focus on CpG islands in the promoter. In the present study, we found that the NOLC1 promoter region CpG1. Hypermethylation of CpG islands in promoter sequences is associated with silencing of tumor-suppressor genes and tumor-related genes by subsequent downregulation of mRNA transcript expression. Epigenetic silenced genes are involved in important molecular pathways of carcinogenesis e.g., cell cycle regulation, apoptosis, DNA repair or cell adhesion (3). As known, the imbalance between cell proliferation and death is considered to be an early and important event in the process of carcinogenesis; thus it is desirable to develop new strategies to induce apoptosis and inhibition of proliferation in tumor cells. The results of this study demonstrated that NOLC1 inhibited the proliferation of liver cell lines, and promoted hepatoma cell apoptosis. When compared with other types of malignant tumors, in hepatocellular carcinomas, aberrant methylation of several TSGs and tumor-related genes such as RASSF1A, hMLH1 or SOCS1 was frequently observed (31). In our study, NOLC1 may play a role in suppressing HCC tumorigenesis.

Further studies are required to explore the mechanisms involved in the suppression of NOLC1 gene expression by DNA methylation. The answer to why NOLC1 plays a role in suppressing HCC tumorigenesis, but enhancing NPC tumorigenesis warrants investigation. Gao *et al* (9) found that NF-κB and CREB positively regulated the NOLC1 promoter. NOLC1 was found to play a role in the regulation of tumorigenesis of NPC and both NOLC1 and tumor protein 53 were demonstrated to synergistically activate the MDM2 promoter in NPC cells (9). We hypothesized that the key signaling pathway of NOLC1 is different between NPC and HCC.

From a cell biology point of view, our finding concerning NOLC1 and its methylation raises an important conclusion. NOLC1 expression affects the proliferation of liver cells. To attempt to confirm this hypothesis, we overexpressed the NOLC1 gene in normal liver cell lines and examined its effect on the cell phenotype. The normal liver cells with NOLC1 expression promote proliferation, as our experiment demonstrated (Fig. 5A and C). Restoration of NOLC1 inhibited the proliferation of hepatoma cell lines and was associated with cyclin D3 (Figs. 5B and D and 7B). The underlying mechanisms for these changes remain to be determined. Cyclin D3 as a target of miR-138 in HCC provides new insights into the mechanisms underlying tumorigenesis (22). Cyclin D3 is expressed in nearly all proliferating cells and could promote initiation of the cell cycle (32-42). Further study of the relationship between cyclin D3 and the function of NOLC1 or its methylation is needed to determine the relevant mechanisms.

Nevertheless, methylation of the NOLC1 gene can, at least, serve as a surrogate marker to reflect the DNA methylation status in HCC cells; therefore, it can serve as a biomarker for HCC diagnosis. It is potentially more important to use NOLC1 methylation as an early biomarker for HCC. In addition, the demethylation agent for NOLC1 can be used as a potential target for HCC therapy.

In summary, we found that the low expression of NOLC1 and high levels of aberrant DNA methylation levels of its promoter in cancer cell lines and tissues are associated with cyclin D3. The important methylation sites were identified at the CpG1 start region of the NOLC1 gene. Our findings

provide new means for developing better diagnostic tests and more effective therapies for HCC.

Acknowledgements

This study was supported by grants from the Healthy Talent Leadership Programs of Beijing (no. 2009-1-09), the National Importance Foundation of Infectious Diseases during the Five-Year Plan Period of China (nos. 2012ZX10002003, 2013ZX10002005 and 2012ZX10004904), and the National Natural Science Foundation of China (nos. 30600524, 81071990, 81172383 and 81201758).

References

1. Beasley RP, Hwang LY, Lin CC and Chien CS: Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. Lancet 2: 1129-1133, 1981.
2. Parkin DM, Bray F, Ferlay J and Pisani P: Estimating the world cancer burden: Globocan 2000. Int J Cancer 94: 153-156, 2001.
3. Tischoff I and Tannapfe A: DNA methylation in hepatocellular carcinoma. World J Gastroenterol 14: 1741-1748, 2008.
4. Baylin SB and Herman JG: DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 16: 168-174, 2000.
5. Riggs AD and Pfeifer GP: X-chromosome inactivation and cell memory. Trends Genet 8: 169-174, 1992.
6. Yang Q, Cheng J, Liu Y, Hong Y, Wang JJ and Zhang SL: Cloning and identification of *NS5ATP2* gene and its spliced variant transactivated by hepatitis C virus non-structural protein 5A. World J Gastroenterol 10: 1735-1739, 2004.
7. Meier UT: Comparison of the rat nucleolar protein nopp140 with its yeast homolog SRP40. Differential phosphorylation in vertebrates and yeast. J Biol Chem 271: 19376-19384, 1996.
8. Meier UT and Blobel G: Nopp140 shuttles on tracks between nucleolus and cytoplasm. Cell 70: 127-138, 1992.
9. Gao XS, Wang Q, Li W, Yang B, Song H, Ju W, Liu SA and Cheng J: Identification of nucleolar and coiled-body phosphoprotein 1 (NOLC1) minimal promoter regulated by NF-κB and CREB. BMB Rep 44: 70-75, 2011.
10. Meier UT and Blobel G: A nuclear localization signal binding protein in the nucleolus. J Cell Biol 111: 2235-2245, 1990.
11. Miau LH, Chang CJ, Tsai WH and Lee SC: Identification and characterization of a nucleolar phosphoprotein. Nopp140, as a transcription factor. Mol Cell Biol 17: 230-239, 1997.
12. Hwang YC, Lu TY, Huang DY, Kuo YS, Kao CF, Yeh NH, Wu HC and Lin CT: NOLC1, an enhancer of nasopharyngeal carcinoma progression, is essential for TP53 to regulate MDM2 expression. Am J Pathol 175: 342-354, 2009.
13. Pai CY, Chen HK, Sheu HL and Yeh NH: Cell-cycle-dependent alterations of a highly phosphorylated nucleolar protein p130 are associated with nucleologenesis. J Cell Sci 108: 1911-1920, 1995.
14. Lin CT, Lin CR, Tan GK, Chen W, Dee AN and Chan WY: The mechanism of Epstein-Barr virus infection in nasopharyngeal carcinoma cells. Am J Pathol 150: 1745-1756, 1997.
15. Wu HC and Lin CT: Association of heterotrimeric GTP binding regulatory protein (Go) with mitosis. Lab Invest 71: 175-181, 1994.
16. Jiang M and Milner J: Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. Genes Dev 17: 832-837, 2003.
17. Jiang M, Rubbi CP and Milner J: Gel-based application of siRNA to human epithelial cancer cells induces RNAi-dependent apoptosis. Oligonucleotides 14: 239-248, 2004.
18. Kim YK, Lee WK, Jin YN, Lee KJ, Jeon HS and Yu YG: Doxorubicin binds to un-phosphorylated form of hNopp140 and reduces protein kinase CK2-dependent phosphorylation of hNopp140. J Biochem Mol Biol 39: 774-781, 2006.
19. Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, Li M, Yagyu R and Nakamura Y: SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells. Nat Cell Biol 6: 731-740, 2004.
20. Ford J, Jiang M and Milner J: Cancer-specific functions of SIRT1 enable human epithelial cancer cell growth and survival. Cancer Res 65: 10457-10463, 2005.
21. Li H, Bergeron L, Cryns V, Pasternack MS, Zhu H, Shi L, Greenberg A and Yuan J: Activation of caspase-2 in apoptosis. J Biol Chem 272: 21010-21017, 1997.
22. Wang W, Zhao LJ, Tan YX, Ren H and Qi ZT: MiR-138 induces cell cycle arrest by targeting cyclin D3 in hepatocellular carcinoma. Carcinogenesis 33: 1113-1120, 2012.
23. Liu HY, Dong HJ, Robertson K and Liu C: DNA methylation suppresses expression of the urea cycle enzyme carbamoyl phosphate synthetase 1 (CPS1) in human hepatocellular carcinoma. Am J Pathol 178: 652-661, 2011.
24. Herath NI, Leggett BA and MacDonald GA: Review of genetic and epigenetic alterations in hepatocarcinogenesis. J Gastroenterol Hepatol 21: 15-21, 2006.
25. Li HP, Leu YW and Chang YS: Epigenetic changes in virus-associated human cancers. Cell Res 15: 262-271, 2005.
26. Herceg Z: Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. Mutagenesis 22: 91-103, 2007.
27. Adrien LR, Schlecht NF, Kawachi N, Smith RV, Brandwein-Gensler M, Massimi A, Chen S, Prystowsky MB, Childs G and Belbin TJ: Classification of DNA methylation patterns in tumor cell genomes using a CpG island microarray. Cytogenet Genome Res 114: 16-23, 2006.
28. Lund AH and van Lohuizen M: Epigenetics and cancer. Genes Dev 18: 2315-2335, 2004.
29. Sparmann A and van Lohuizen M: Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6: 846-856, 2006.
30. Lo SJ, Lee CC and Lai HJ: The nucleolus: reviewing oldies to have new understandings. Cell Res 16: 530-538, 2006.
31. Esteller M, Corn PG, Baylin SB and Herman JG: A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-3229, 2001.
32. Lin J, Jinno S and Okayama H: Cdk6-cyclin D3 complex evades inhibition by inhibitor proteins and uniquely controls cell's proliferation competence. Oncogene 20: 2000-2009, 2001.
33. Rao CN, Liu YY, Peavey CL and Woodley DT: Novel extracellular matrix-associated serine proteinase inhibitors from human skin fibroblasts. Arch Biochem Biophys 317: 311-314, 1995.
34. Sprecher CA, Kisiel W, Mathewes S and Foster DC: Molecular cloning, expression, and partial characterization of a second human tissue-factor-pathway inhibitor. Proc Natl Acad Sci USA 91: 3353-3357, 1994.
35. Rao CN, Cook B, Liu Y, Chilukuri K, Stack MS, Foster DC, Kisiel W and Woodley DT: HT-1080 fibrosarcoma cell matrix degradation and invasion are inhibited by the matrix-associated serine protease inhibitor TFPI-2/33 kDa MSPI. Int J Cancer 76: 749-756, 1998.
36. Rao CN, Mohanam S, Puppala A and Rao JS: Regulation of ProMMP-1 and ProMMP-3 activation by tissue factor pathway inhibitor-2/matrix-associated serine protease inhibitor. Biochem Biophys Res Commun 255: 94-98, 1999.
37. Wong CM, Ng YL, Lee JM, Wong CC, Cheung OF, Chan CY, Tung EK, Ching YP and Ng IO: Tissue factor pathway inhibitor-2 as a frequently silenced tumor suppressor gene in hepatocellular carcinoma. Hepatology 45: 1129-1138, 2007.
38. Kim H, Jen J, Vogelstein B and Hamilton SR: Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. Am J Pathol 145: 148-156, 1994.
39. Oliveira C, Seruca R, Seixas M and Sobrinho-Simões M: The clinicopathological features of gastric carcinomas with microsatellite instability may be mediated by mutations of different 'target genes': a study of the *TGFβ RII*, *IGFII R*, and *BAX* genes. Am J Pathol 153: 1211-1219, 1998.
40. Park JH, Cho SB, Lee WS, Park CH, Joo YE, Kim HS, Choi SK, Rew JS, Lee JH and Kim SJ: Methylation pattern of DNA repair genes and microsatellite instability in hepatocellular carcinoma. Korean J Gastroenterol 48: 327-336, 2006 (In Korean).
41. Matsukura S, Soejima H, Nakagawachi T, Yakushiji H, Ogawa A, Fukuhara M, Miyazaki K, Nakabeppu Y, Sekiguchi M and Mukai T: CpG methylation of MGMT and hMLH1 promoter in hepatocellular carcinoma associated with hepatitis viral infection. Br J Cancer 88: 521-529, 2003.
42. Wang L, Bani-Hani A, Montoya DP, Roche PC, Thibodeau SN, Burgart LJ and Roberts LR: hMLH1 and hMSH2 expression in human hepatocellular carcinoma. Int J Oncol 19: 567-570, 2001.