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Targeted Disruption of Nemo-Like Kinase Inhibits Tumor Cell Growth by Simultaneous Suppression of Cyclin D1 and CDK2 in Human Hepatocellular Carcinoma

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ABSTRACT

The Wnt/β-catenin signaling pathway regulates various aspects of development and plays important role in human carcinogenesis. Nemo-like kinase (NLK), which is mediator of Wnt/β-catenin signaling pathway, phosphorylates T-cell factor/lymphoid enhancer factor (TCF/LEF) factor and inhibits interaction of β-catenin/TCF complex. Although, NLK is known to be a tumor suppressor in Wnt/β-catenin signaling pathway of colon cancer, the other events occurring downstream of NLK pathways in other types of cancer remain unclear. In the present study, we identified that expression of NLK was significantly up-regulated in the HCCs compared to corresponding normal tissues in five selected tissue samples. Immunohistochemical analysis showed significant over-expression of NLK in the HCCs. Targeted-disruption of NLK suppressed cell growth and arrested cell cycle transition. Suppression of NLK elicited anti-mitogenic properties of the Hep3B cells by simultaneous inhibition of cyclinD1 and CDK2. The results of this study suggest that NLK is aberrantly regulated in HCC, which might contribute to the mitogenic potential of tumor cells during the initiation and progression of hepatocellular carcinoma; this process appears to involve the induction of CDK2 and cyclin D1 and might provide a novel target for therapeutic intervention in patients with liver cancer. J. Cell. Biochem. 110: 687–696, 2010.

KEY WORDS: NLK; CELL CYCLE ARREST; CDK2; CYCLIN D1; HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide; it has the third highest mortality among all cancers. HCC is one of the main causes of cancer related death in Asia and Africa. Hepatitis B virus (HBV), hepatitis C virus (HCV), and aflatoxin B1 are well-known major causes of HCC. However, the underlying mechanisms involved in the development and progression of HCC remain poorly understood [Bruix et al., 2004]. Recent studies have reported that genetic alterations of tumor associated genes such as p53, β-catenin, and AXIN1 are involved in hepatocarcinogenesis [de La Coste et al., 1998; Satoh et al., 2000; Pang et al., 2003]; however, the frequencies of mutations, in these genes, appear to be very low in patients with HCC. Furthermore, it is unclear how these genetic changes precisely cause the clinical characteristics observed in individual patients with HCC. Therefore, the major molecular events underlying HCC remain to be identified.

The Nemo-like kinase (NLK) is a member of the extracellular-signal regulated kinases/microtubule-associated protein kinases (Erks/MAPKs) and cyclin-directed kinases (Cdks), and transforming growth-factor-b-activated kinase 1 (TAK1) of the MAPK kinase kinase (MAPKK) superfamily is known to as a potential activator of NLK in Wnt signaling pathway [Meneghini et al., 1999; Shin et al., 1999]. *Nmo*, in *Drosophila* and LIT-1, in *Caenorhabditis elegans*, have been found to be homologous to vertebrate NLK in genetic studies; they act as regulators of Wnt signaling during the development of the wings in the fly and in the cell division of *C. elegans* [Choi and Benzer, 1994; Kaletta et al., 1997; Verheyen et al., 2001]. Therefore, NLK/Nmo/LIT-1 has been identified as a very important regulator of cell growth, patterning, and death in a variety of organisms.

The Wnt signaling pathway regulates developmental signal transduction and is known to be involved in the process of human

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carcinogenesis through dysregulation of the β-catenin/T-cell factor/lymphoid enhancer factor (TCF/LEF) complex, which are essential components of the Wnt signaling pathway [Bienz and Clevers, 2000; Fearnhead et al., 2001]. Wnt signaling stabilizes β-catenin, which then forms a complex with TCF/LEF transcription factors and activates gene transcription. Somatic mutations in most of the components of the Wnt pathway, such as APC, β-catenin, and Axin, have been found in many human cancers [Polakis, 1999; Peifer and Polakis, 2000]. Stabilized β-catenin protein accumulates in the cell nucleus and forms complex with and activates TCF/LEF transcription factors. NLK plays an important role in the development of organisms as an inhibitor of the β-catenin/TCF complex in the Wnt signaling pathway of mammalian cells [Ishitani et al., 1999, 2003a,b]. NLK phosphorylates TCF/LEF factors and then suppresses the transcription activity of the β-catenin-TCF complex from binding DNA.

The results of recent studies have shown that inhibition of the β-catenin/TCF complex by induction of dominant-negative TCF suppresses cell proliferation by G1 arrest of cells, and the colony growth rate of soft agar in human colorectal carcinoma cells, including DLD-1 [Naishiro et al., 2001; van de Wetering et al., 2002]. Similarly, over-expression of NLK by a wild-type vector has been shown to decrease growth activity and induce apoptosis in the human colon cancer cell line, DLD-1 [Yasuda et al., 2003]. Based on these studies, the β-catenin/TCF complex appears to play an important role in transcriptional processing causing cell proliferation in colorectal carcinoma. In addition, Wnt/β-catenin signaling has been reported to be associated with the development and progression of hepatocellular cancer [Polakis, 1999; Peifer and Polakis, 2000]. However, the suppression associated with NLK, in hepatocarcinogenesis, has not been investigated and the underlying mechanism of NLK is unknown.

Therefore, in the present study, to investigate the biologic role of NLK in HCC development and progression, the expression of NLK in tumors and normal paired tissue samples was assessed. In addition, NLK expression was disrupted by siRNA-mediated protein knockdown methods to explore the possible role it plays in hepatocarcinogenesis as well as in the regulatory mechanisms of the cell-cycle, in the particular G1/S transition, in Hep3B cells.

MATERIALS AND METHODS

TISSUE SAMPLES AND TISSUE MICROARRAY

Five frozen HCCs and their corresponding normal background liver tissue samples from five patients with HCC (all Korean patients) were evaluated in this study. The background liver showed chronic hepatitis in all cases and HBV was detected in all cases. Approval was obtained from the institutional review board of the Catholic University of Korea (CUMC09U029), College of Medicine. Informed consent was provided by every patient according to the Declaration of Helsinki. Frozen tissues were ground to a very fine powder in liquid nitrogen, and then were preserved for molecular testing. For tissue microarray (TMA) construction, a total of 50 liver samples (30 HCCs and 20 normal liver tissues) of formalin fixed, paraffin-embedded liver samples were obtained from the archives of the Department of Pathology at our institution. Two replicate core samples of neoplastic tissue and normal liver tissue were punched out of each donor-tumor block and placed into recipient paraffin blocks using a 0.6 mm diameter stylet. The liver samples were fully anonymized prior to inclusion in the study.

CELL CULTURE

Human liver cancer cell line HepG2, Hep3B, PLC/PRF/5, CHANG, SNU-182, SNU-387, SNU-423, and SNU-449 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Then, Human liver cancer cell line SNU-354 and SNU-368 were purchased from Korean Cell Line Bank (KCLB, Korea). The cells were maintained in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum (Sigma, St Louis, MO) and 1 mg/ml of penicillin/streptomycin (Invitrogen, Grand Island, NY).

IMMUNOHISTOCHEMISTRY ANALYSIS OF NLK

To investigate the level of NLK protein in HCC, we performed immunohistochemical staining with monoclonal antibodies against NLK (1:50, Abcam, Cambridge, UK) on TMA samples of HCC. Prior to the immunostaining, the TMA slides were deparaffinized and hydrated through graded ethanol to deionized the water. Endogenous peroxidase activity was blocked by 5 min incubation in 3% hydrogen peroxide–methanol buffer. Antigens were retrieved by boiling the slides in a steamer with sodium citrate buffer (pH 6.0) for 20 min. After incubation with monoclonal antibodies against NLK overnight at 4°C, detection was carried out using biotinylated goat anti-mouse antibodies (1:200; Sigma), followed by incubation with the peroxidase-linked avidin-biotin complex. Diaminobenzidine was used as the chromogen, and the slides were then lightly counterstained with Mayer's hematoxylin. As a negative control, the slides were treated and the primary antibody was replaced by non-immune serum.

EVALUATION OF IMMUNOHISTOCHEMICAL STAINING

Scoring of the TMA was performed independently by two pathologists. In the event of disagreement, the two reached a consensus by jointly re-evaluating the TMA using a multi-head microscope. Immunostaining intensity was graded in three categories: 1+ (weak), 2+ (moderate), or 3+ (strong). However, if the number of immunostained cells was less than 10%, we considered the case negative for staining. Each two replicate core tumor tissues were combined and calculated as one case.

SILENCING OF NLK EXPRESSION

The NLK siRNA and scrambled siRNA were purchased from Ambion Inc (Ambion, Austin, TX). The targeted NLK sequences were 5′-GGGUCUUCCGGGAAUU-GAAtt-3′ (sense) and 5′-UUCAAUUCCCG-GAAGA-CCCtt-3′ (anti-sense). The cells were harvested by Trypsin/EDTA, replated at 1.5×10^5 cells in a 60 mm dish, and allowed to grow overnight at 37°C in a humidified incubator at 5% CO_2. After 16–18 h of plating, the cells were transfected with none, reagent only, 50 nmol/L scrambled siRNA, 50 nmol/L or 100 nmol/L of NLK-specific siRNA in Opti-MEM (Invitrogen). Transfection was carried out using 10 μl of lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's specifications. After 4–6 h of

transfection, the medium was replaced by new fresh RPMI 1640 medium supplemented with 10% FBS.

CELL PROLIFERATION ASSAY

For the cell proliferation assay, the cells were plated in a 24-well culture plate at a density of 2×10^4 cells per well with RPMI 1640 medium with 10% FBS and maintained for 16–18 h. Four hours after the NLK-specific siRNA transfection, RPMI 1640 medium with 10% FBS was replaced in each of the 24-wells in the culture plate and the cells were maintained at 37°C in a 5% CO_2 humidified incubator. To determine cell proliferation, the cells were incubated with 200 μl of the CellTiter 96® AQueous One Solution Cell Proliferation Assay solution (Promega, Madison, WI) at each indicated time (0, 1, 2, and 3 days). Three hours after incubation, absorbance of the cells was determined with a VICTOR3™ Multilabel plate reader (PerkinElmer Inc, Boston, MA).

CELL CYCLE ANALYSIS

Transfected Hep3B cells were harvested by trypsinization at 48 h after transfection, washed with cold PBS and fixed in 70% alcohol for 1 day at −20°C. After fixation, the cells were washed again with cold PBS twice and incubated for 30 min in PBS containing 10 mg/ml of RNase A at 37°C. After RNase A treatment, the nuclei were stained with 5 mg/ml of propidium iodide (PI), and stained cells were measured by fluorescence-activated cell sorting (FACS) on a FACScan apparatus. The data obtained was analyzed by Cell-Quest FACS analysis software (BD Biosciences, Franklin Lakes, NJ). To measure the change of cell cycle transition by NLK siRNA transfection, gated single cell population from all cell population in the FL2-A/FL2-W plot was analyzed according to Cell-Quest FACS analysis software protocol.

APOPTOSIS ASSAY

The Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) was used to quantify the level of apoptosis in the samples. Briefly, the cells were trypsinized, washed twice with cold PBS and resuspended in 1× binding buffer at a concentration of 1×10^6 cells/ml. The cells were transferred, 100 μl of the cell suspension (1×10^5 cells), to a 5 ml culture tube and 5 μl of Annexin V-FITC and 10 μl of PI solution was added. After 15 min of incubation at room temperature in the dark, 400 μl of 1× binding buffer was added to each of the culture tubes; determination of the apoptotic fraction of cells was then performed by Cell-Quest FACS analysis software on a FACScan flowcytometer (BD Biosciences).

REVERSE TRANSCRIPTION-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and quality control was performed using RNA 6000 Nanochips on an Agilent 2001 Bioanalyzer (Agilent Technologies, Germany). Then, 1 μg RNA was used for cDNA synthesis reaction using RNA PCR Core Kit (Roche, Branchburg, NJ, USA). cDNA was used per reverse transcription-PCR (RT-PCR) reaction. RT-PCR program was 95°C 30 s, 53°C 30 s, and 72°C 30 s for 35 cycles. RT-PCR primer sequences are as follows: NLK forward 5′-GCT GGA TAT TGA GCC GGA TA, reverse 5′-CAT CTT CAA TTC CCG GAA GA; GAPDH forward 5′-ACC AGG TGG TCT CCT CTG AC, reverse 5′-TGC TGT AGC CAA ATT CGT TG.

WESTERN BLOT ANALYSIS

Whole-cell extracts were prepared with radio-immunoprecipitation assay (RIPA) lysis buffer (50 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mmol/L phenylmethane-sulfonylfluoride containing protease inhibitors, Roche, Mannheim, Germany). Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL) and absorbance of the protein samples were read at 570 nm with the VICTOR3™ Multilabel Plate Reader (PerkinElmer). RIPA lysates containing 10 μg or 15 μg of protein were separated by SDS–PAGE and transferred onto a polyvinylidene difluoride membrane (Amersham Hybond™-P, Little Chalfont, Buckinghamshire, UK) and kept in 5% skim milk (BD Biosciences) in TBS solution containing 0.05% Tween-20 (Usb Corporation, Cleveland, OH) overnight to protect against non-specific binding. The membranes were incubated with each of the primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce). The ECL plus Western blotting detection system (Amersham) was used to detect immobilized specific antigens conjugated to HRP labeled antibodies. Antibodies for the related cell-cycle (p21, p15, p16, p27, cyclin D1, and CDK2) and NLK antibodies were purchased from Cell Signaling (Cell Signaling Technology Inc, Beverly, MA) and Abcam (Abcam Inc. Cambrige, MA). The membrane was exposed to LAS 3000 (Fuji Photo Film Co. Ltd, Japan).

SOFT-AGAR COLONY FORMATION ASSAY

Forty-eight hours after transfection with NLK siRNA, ~5,000 cells in 1 ml of 0.4% agarose with RPMI-1640 were plated in each well on the top of existing 0.8% bottom agarose in 35 mm dishes in triplicate for each treatment condition. The plates were covered with 500 μl of medium with 10% FBS and incubated at 37°C in a 5% CO_2 incubator for 3 weeks. The covering medium was replaced every week. At the end of 3 weeks, cell colonies were stained with 0.05% crystal violet and colonies >0.1 mm in diameter were counted under a microscopic field at 40× magnifications. Means were based on numbers from triplicate wells for each treatment condition and were analyzed using one-sided Student's *t*-test.

RESULTS

ABERRANT REGULATION OF NLK IN HEPATOCELLULAR CARCINOMA

Accumulating evidence has shown that NLK negatively regulates the β-catenin pathway. In addition, it has also been shown that over-expression of NLK, by wild-type vector, was associated with decreased growth activity and induced apoptosis in the human colon cancer cell line, DLD-1 [Yasuda et al., 2003]. Thus, NLK appears to act as tumor suppressor in colon cancer. However, no further studies have been performed in other types of cancers. Since we have previously reported large-scale molecular changes as part of a multi-step process in hepatocarcinogenesis, from the dysplastic nodule to HCC, by expression profilings, we predicted that down-regulation of NLK would be present in HCC. However, in contrast to our expectation, we noted that NLK expression was gradually

increased from dysplasia, a precancerous lesions, to HCC based on our previous findings [Nam et al., 2005]. These findings are inconsistent with previous reports; therefore, to confirm our results, we investigated the expression of NLK in HCC by RT-PCR and Western blot analysis.

We first examined the mRNA expression of NLK by RT-PCR in five selected HCCs and matched normal liver tissue samples obtained from patients that had surgical resection of HCC. As shown in Figure 1A, the mRNA expression of NLK was up-regulated by at least twofold in the HCC samples compared to the corresponding normal liver tissues. Similarly, when NLK protein expression was assessed by Western blot analysis, expression of NLK was markedly up-regulated in all tested HCCs (Fig. 1B). To confirm the finding that NLK was over-expressed in the HCCs, we next examined NLK expression by immunohistochemical staining using HCC TMA. The immunostaining results for the replicated tumor punches, in the TMA, are presented in Table I, and representative images of the immunostaining are shown in Figure 2. As summarized in Table I, among the 20 samples tested of normal hepatocytes, 16 (80%) had weak positive or no detectable to NLK antibody, while 17 (54%) out of 30 tested HCCs showed moderate or strong positive to NLK antibody staining. As shown in Figure 2, NLK localized to the cytoplasm and nucleus in all of the HCC cases (Fig. 2C and D).

TARGETED-DISRUPTION OF NLK SUPPRESSED GROWTH RATE OF HEP3B CELLS

Because we observed highly over-expression of NLK in HCC tissues, we next examined and compared NLK expression levels of human hepatoma cell lines. We took 10 different human hepatoma cell lines which were originally established from HCCs or hepatoblastomas, and determined expression of NLK by RT-PCR and Western blot analysis. Among the hepatoma cell lines, Hep3B cells were appeared to be highest expression level of NLK in both RT-PCR and Western blot analysis (Fig. 3A).

Next, to explain the biologic consequences of aberrant expression of NLK in HCC, NLK was endogenously disrupted by the RNA interference-mediated protein knock-down method in Hep3B cells. NLK silencing was carried out by using NLK specific siRNAs, and the efficiency of the NLK-targeted siRNA-mediated down-regulation was assessed by RT-PCR and Western blot analysis. The results showed that both 50 and 100 nM of the NLK siRNA could effectively knock-down the expression of NLK in the Hep3B cells compared to non-silencing (scrambled sequence) in the controls (Fig. 3B). To determine the biologic effects of the disruption of NLK expression, the growth rate of the Hep3B cells was determined by MTS assay. As shown in Figure 3B, silencing of NLK significantly decreased the cell growth rate at both the 50 and 100 nM of NLK siRNA concentrations compared to cells treated with the scrambled siRNA. To verify the fact that NLK promotes hepatic tumor growth, we took another cell lines and disrupted NLK expression. As shown in Figure 3C and D, when the expression of NLK was suppressed in SNU-423 cells which showed relatively high expression level of NLK, SNU-423 cells growth rate was reduced like Hep3B cells did. However, SNU-368 cells, which showed very low expression level of NLK, did not display the reduced growth rated. This result indicates that aberrant regulation of NLK stimulates tumor cell growth in human HCCs.

The anti-mitogenic effect on Hep3 cells and SNU-423 cells could be partially explained by disturbance of cell-cycle regulation or augmentation of cellular senescence and apoptosis. Thus, we next examined the effect of NLK, in the Hep3B cells, on the cell cycle as well as apoptosis. As shown in Figure 4A, when the Hep3B cells were analyzed by flow cytometry, 48 h after transfection with or without NLK siRNA, the PI stained cells showed that the cell population during the G1-S phase was increased by NLK siRNA transfection. It

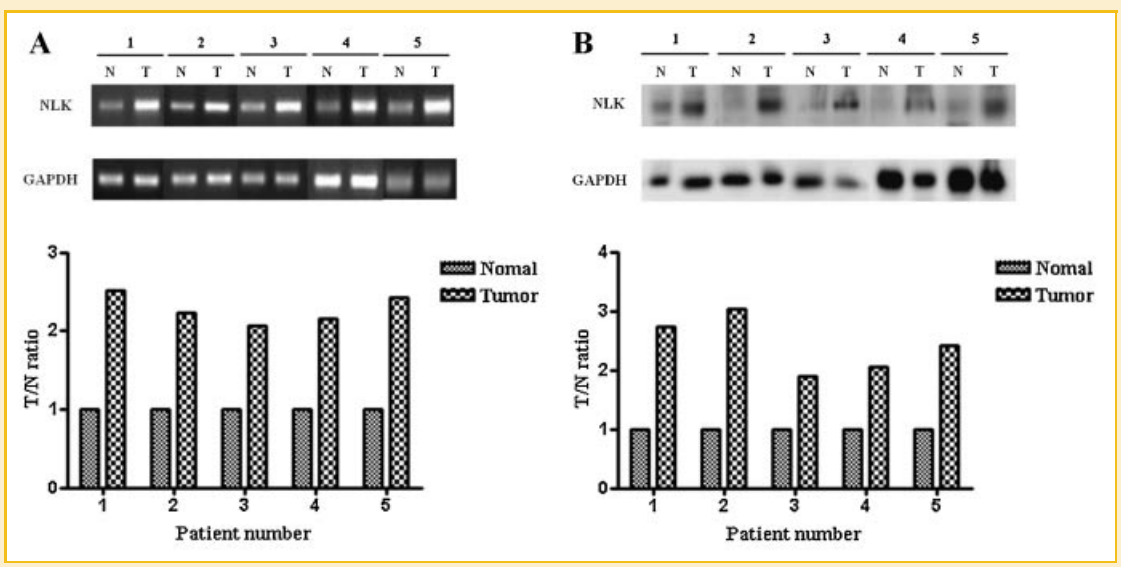

Fig. 1. Expression of NLK in hepatocellular carcinomas. A: The mRNA expression of NLK in hepatocellular carcinoma was assessed by RT-PCR analysis. NLK mRNA levels were normalized against GAPDH and the tumor/normal (T/N) ratios calculated (bar graph). B: Under the same condition as A, the protein expression of NLK was determined by Western blot analysis. NLK protein levels were normalized against GAPDH and the tumor/normal (T/N) ratios calculated (bar graph).

TABLE I. Summary of Immunohistochemical Staining Analysis of NLK in HCC TMA.

Parameters	Normal	Hepatocellular carcinoma (HCC) $P = 0.034^{*}$
[a]Weak or absent	16 (80%)	13 (46%)
Moderate	4 (20%)	16 (50%)
Strong		1 (4%)
Total	20 (100%)	30 (100%)

[a]Number and percentage of normal and tumor samples scored in each category for NLK.
*Chi-square test.

appeared that the NLK knock-down augmented G1 cycle arrest (51.79%) at the 100 nmol/L concentration of NLK siRNA compared to the non-silencing control (Scr, 40.84%). Next, for assessment of cellular apoptosis, the Annexin V staining of cells, the siRNA transfectants were measured by flow cytometry. As shown in Figure 4B, the flow cytometry analysis with PI and Annexin V staining, for the dead cells or apoptotic cells, indicated that cellular apoptosis (upper right in dot plot graphs) was not affected by NLK silencing. These results suggest that targeted-disruption of NLK in Hep3B cells suppressed cell growth by causing cell cycle arrest, not by apoptosis. This raised the question of how NLK regulated the G1/S cell cycle transition.

REGULATORY MECHANISMS OF NLK IN THE G1/S CELL CYCLE TRANSITION

The fact that the suppression of NLK caused regression of Hep3B cell growth implies that NLK is involved in the regulation of the cell-cycle circuit. In addition, FACS analysis with PI staining of cells showed an increase in the proportion of cells in the G1-S phase in the NLK siRNA transfectants (Fig. 4A). Because cyclin dependent kinases (CDKs), CDK inhibitors (CDKIs), and cyclins are the core components of the cell cycle clock, we next examined the effects of NLK suppression on these regulatory components in the G1/S phase of the cell-cycle. In the G1/S phase transition of the cell-cycle, it has been well established that negative cell-cycle regulators such as $p21^{WAF1/CIP1}$, $p15^{INK4B}$, $p16^{INK4A}$, and $p27^{Kip1}$ are the key modulators that suppress cyclin D1/CDK4, 6, or cyclin E/CDK2 complexes [Xiong et al., 1993; Grana and Reddy, 1995; Soto Martinez et al., 2005]. When these negative cell cycle modulators were examined in the NLK siRNA transfectants, p16 and p27 were non-detectable compared to MKN-1 cells as positive control and no significant changes of expression were observed in all tested negative regulators (Fig. 5A). However, knock-down of NLK elicited down-regulation of cyclin D1 and CDK2 (Fig. 5A). These result suggests that over-expression of NLK, in HCC cells, might concomitantly activate expression of both cyclin D1 and CDK2. Thus, to verify direct relationship between NLK and CDK2 and cyclin D1 expression, we next evaluated phosphorylation status of p130, a pRb protein, when NLK was knock-downed in Hep3B cells. As shown

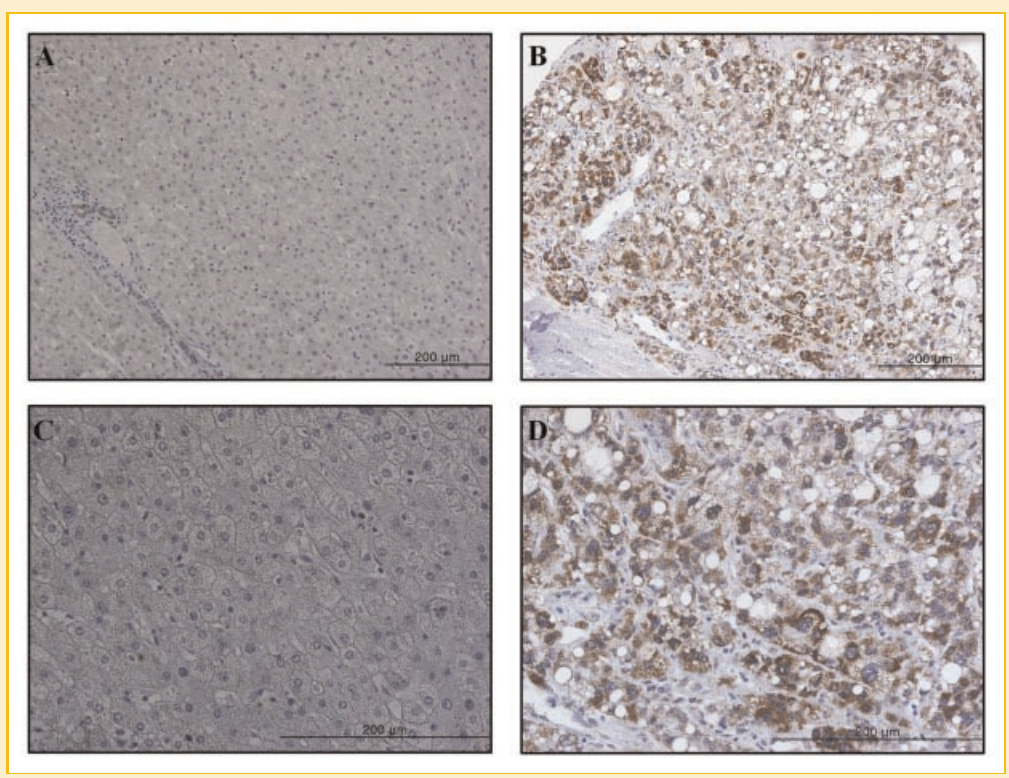

Fig. 2. Representative images of immunohistochemical analysis of NLK on hepatocellular carcinomas. A and C: Representative normal liver hepatocytes were negative for immunostaining against NLK antibody at different magnification. B and D: Example of hepatocellular carcinoma, showing strong positive staining against NLK antibody. The upper panel shows 200× magnification and the lower panel shows 400× magnification.

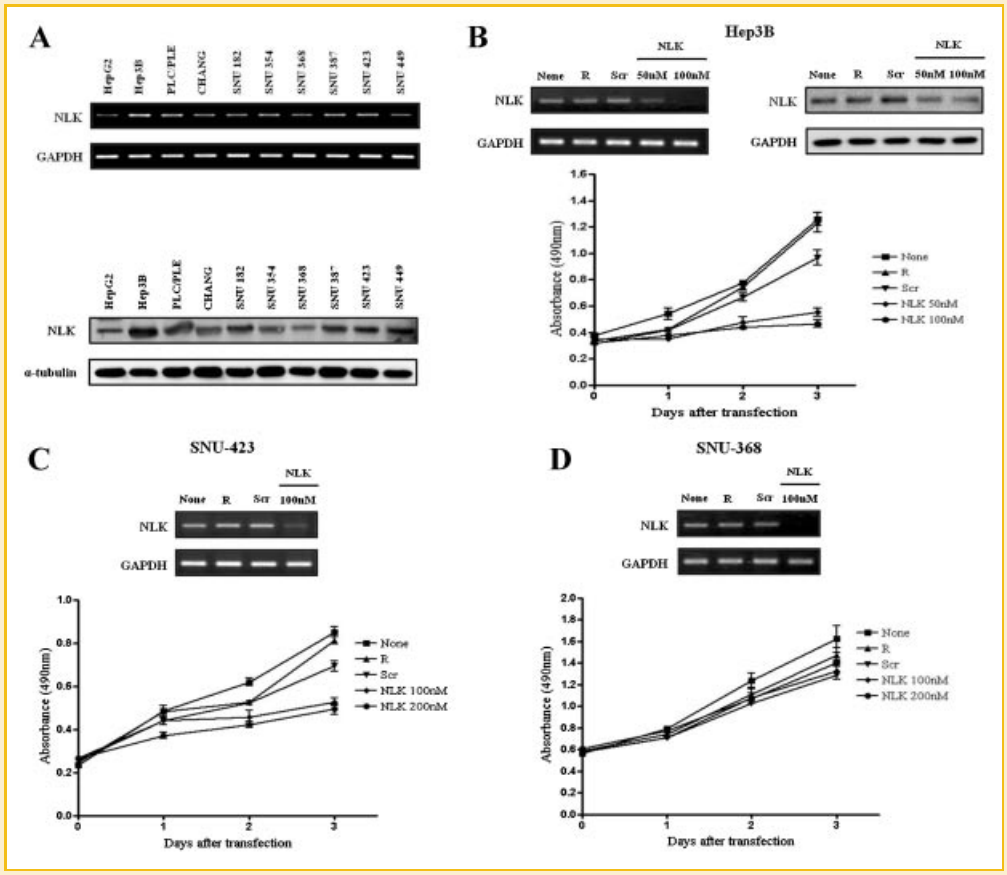

Fig. 3. Effect of NLK suppression on cell growth of human HCC cell lines. A: Endogenous expression of NLK in human hepatoma cell lines were determined by RT-PCR and Western blot analysis. B: Hep3B cells were transfected with none (None), reagent only (R), 50 nmol/L scramble siRNA (Scr), 50 and 100 nmol/L NLK-specific siRNA (NLK 50 nM and NLK 100 nM), respectively. The NLK expression was determined by both RT-PCR and Western blot analysis on 48 h after transfection. C and D: SNU-423 and SNU-368 cells were transfected with none (None), reagent only (R), 50 nmol/L scramble siRNA (Scr), 100 nmol/L NLK-specific siRNA (NLK 100 nM), respectively. The cell growth rate was assessed by MTS cell viability testing on NLK specific siRNA-transfected Hep3B, SNU-423, and SNU-68 cells. Four hours after transfection, the cells were maintained with complete media and the relative growth rate was determined by MTS assay at each indicated time (0, 1, 2, and 3 days). The data are presented as mean ± standard error for three experiments.

in Figure 5A, disruption of NLK expression elicited hypo-phosphorylation of p130 implying aberrant regulation of NLK affects phosphorylation of pRb protein family via transcriptional activation of CDK2 and cyclin D1 in HCCs. This suggests that simultaneous regulation of CDK2 and cyclin D1 by NLK exerts very potent mitogenic stimulation causing uncontrolled cell growth during liver cancer progression.

Previous studies reported that NLK negatively regulates Wnt signaling via the phosphorylation of TCF/LEF. However, we were also noted that disruption of NLK suppressed the expression of β-catenin in Hep3B cells (Fig. 5A). This suggests another possible mechanism of NLK that regulates β-catenin stability in Hep3B cells. Lastly, to investigate neoplastic potential of NLK, soft-agar colony formation assay was carried out by using NLK knock-down cells. As shown in Figure 5B, knock-down of NLK resulted in the reduction of colony formation number compared to corresponding controls.

DISCUSSION

The results of this study suggest that aberrant regulation of NLK occurs in HCC. Our findings demonstrated that targeted-disruption of NLK suppressed simultaneous expression of CDK2 and cyclin D1, suggesting that NLK might have mitogenic potential during hepatocarcinogenesis.

Wnt signals are transduced to the planar cell polarity (PCP) pathway or β-catenin pathway [Kanei-Ishii et al., 2004]. Wnt/PCP signaling pathway controls tissue polarity and cell movement through the activation of RHOA, c-Jun N-terminal kinase (JNK), and NLK signaling cascades [Katoh, 2005]. Disheveled-independent Wnt/PCP signals are transduced to the NLK signaling cascade through TAK1. Canonical (β-catenin dependent) Wnt signaling, functions by stabilization of pools of β-catenin in the cytoplasm that are constitutively degraded in the absence of Wnt signaling. Stabilized β-catenin then translocates to the nucleus and forms a

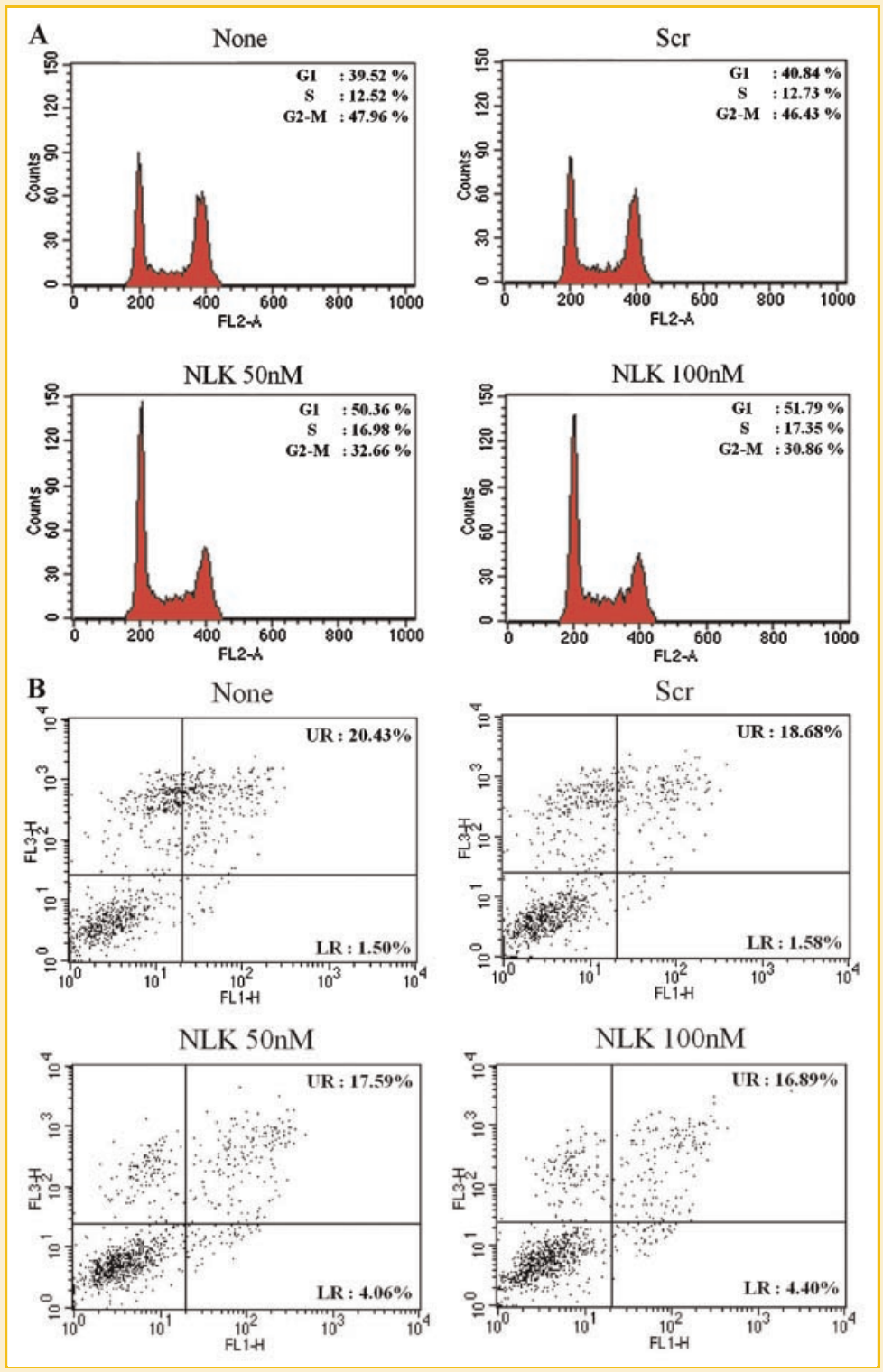

Fig. 4. Effect of NLK silencing on the cell cycle and apoptosis. A: The cell cycle analysis was measured by propidium iodide (PI) staining of NLK silencing Hep3B cells. Hep3B cells were transfected with none (None), 50 nmol/L scramble siRNA (Scr), 50 and 100 nmol/L NLK-specific siRNA (NLK 50 nM and NLK 100 nM), respectively. Forty-eight hours after transfection, cells were trypsinized, fixed in 70% alcohol, and incubated for 30 min in PBS containing 10 mg/ml of RNase A at 37°C, after incubation, the cells were stained with 5 mg/ml PI. B: Apoptosis analysis was by Annexin V-FITC labeling after suppression of NLK. FITC-labeled Annexin V-positive cells (upper right and lower right) were considered to be apoptotic cells. Single staining with Annexin V-FITC indicates cells in the early stage of apoptosis (lower right), and double staining with Annexin V and PI indicates cells in the late stage of apoptosis (upper right). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

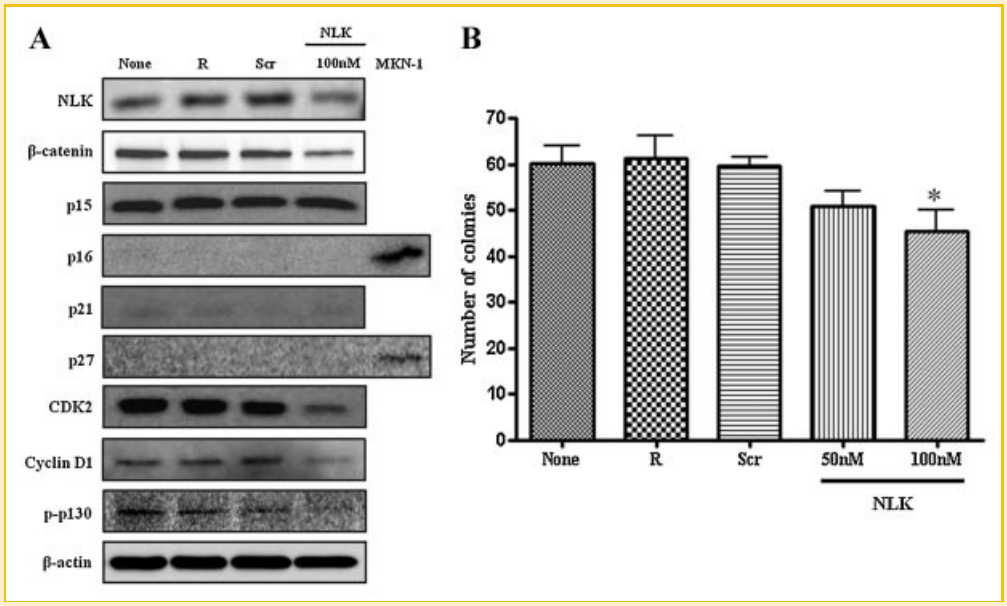

Fig. 5. Effect of NLK suppression on cell cycle regulatory components and soft-agar colony formation. A: Western blot analysis of cell cycle components in G1/S transition on NLK silencing in the Hep3B cells. Hep3B cells were transfected with none (None), reagent only (R), 100 nmol/L scrambled siRNA (Scr), 100 nmol/L NLK-specific siRNA (NLK 100 nM), respectively; β-actin was used as a protein loading control. B: Soft-agar colony assay. Hep3B cells were transfected with none (None), reagent only (R), 50 nmol/L scramble siRNA (Scr), 50 and 100 nmol/L NLK-specific siRNA (NLK 50 nM and NLK 100 nM), respectively. Cells (5,000/35 mm dishes) were then cultured in soft agar medium. Three weeks later, colonies were stained and counted. The graph indicates the average numbers of colonies in three wells with SEM. The data are presented as mean ± standard error for three experiments (unpaired Student's *t*-test, $^{*}P < 0.05$ vs. control).

nuclear complex with members of the TCF/LEF family of DNA-binding molecules, to activate transcription of target genes [Thorpe and Moon, 2004]. In colorectal cancer, inappropriate activation of the Wnt signaling pathway leads to stabilization of β-catenin, and several target genes, of TCF/β-catenin complexes that might function during the process of tumorigenesis have been identified [Polakis, 1999; Peifer and Polakis, 2000]. Mutations that increase β-catenin protein levels lead to changes in the fate of the cells during development and tumor formation in adult animals. Cells use various strategies to combat this condition, including the expression of negative regulators that modulate β-catenin levels such as *Naked* and *Axins*, [Zeng et al., 2000] expression of inhibitory Wnts such as Wnt5A [Topol et al., 2003] the expression of Siah and subsequent degradation of β-catenin by the proteosome [Liu et al., 2001] and the activation of the TAK1/NLK kinase pathway [Smit et al., 2004]. Thus, NLK positively regulates the Wnt/PCP pathway, and negatively regulates the Wnt/β-catenin pathway. In colorectal cancer, it has been shown that over-expression of NLK, by wild-type vector, decreased the growth activity and induced apoptosis in the human colon cancer cell line, DLD-1 [Yasuda et al., 2003]. Although, NLK has been reported to be a tumor suppressor in colon cancer, there has been limited study of Wnt signaling and NLK regulation in other human cancers.

During the progression of liver cancer, recent studies have reported genetic alterations of tumor suppressor genes and oncogenes such as p53, pRb, β-catenin, and AXIN1 associated with hepatocarcinogenesis [de La Coste et al., 1998; Satoh et al., 2000; Thorgeirsson and Grisham, 2002; Ishitani et al., 2003a]. Although the frequency of mutations found in these genes appears to be very low, an average 20–30% of tumor tissues were found to over-express β-catenin in the HCCs. These findings suggest that the NLKs might lose their function during liver cancer progression in some HCCs. In addition, as we have previously reported that expression profiling showed large-scale molecular changes during the multistep process of hepatocarcinogenesis, from the dysplastic nodule to HCC [Nam et al., 2005]. These findings led us to predict that down-regulation of NLK likely occurs in HCC. However, in contrast to our expectation, we found that NLK expression was up-regulated in HCC based on the DNA microarray data.

To validate our previous observations based on expression profiling data with regard to multistep hepatocarcinogenesis, we determined the expression level of NLK in five selected HCCs and in the corresponding normal tissues (Fig. 1); the findings confirmed aberrant expression of NLK by immunohistochemical staining of HCC TMA (Table I and Fig. 2). NLK in the HCCs was over-expressed compared to normal tissues at both the mRNA and protein levels, based on the Western blot analysis and immunohistochemical analysis of TMA containing 30 HCCs and 20 normal tissues. We next used RNA interference-mediated gene silencing to determine the biologic consequences of aberrant regulation of NLK in the Hep3B cells. The silencing of endogenous NLK resulted in the regression of cell growth (Fig. 3B) and arrested cell cycle transition (Fig. 4). However, cells (SNU-368) that express relatively low level of NLK did not affected by silencing of NLK (Fig. 3D). These findings suggest that over-expression of NLK might act as an aggressiveness factor in the HCC cells by facilitating cell growth through resistance to cell cycle regulation.

Previous studies have suggested that over-expression of NLK induced apoptosis in human colon cancer cells [Yasuda et al., 2003]. By contrast, our results showed disruption of NLK induced cell cycle arrest in Hep3B cells. In the G1/S phase transition of the cell-cycle, it is well known that negative cell-cycle regulators such as $p21^{WAF1/CIP1}$, $p15^{INK4B}$, $p16^{INK4A}$, and $p27^{Kip1}$ are the key modulators responsible for suppressing cyclin D1/CDK4, 6, or cyclin E/CDK2 complexes [Xiong et al., 1993; Grana and Reddy, 1995; Soto Martinez et al., 2005]. Therefore, to identify the underlying mechanism associated with the neoplastic potential of NLK aberrant regulation, we investigated the regulatory role of NLK with regard to the key components of the G1/S cell cycle transition. During the regulation of the cell cycle, $p21^{WAF1/CIP1}$ inhibits the CDK/cyclin2 complex that induces hypo-phosphorylation of the Rb protein. In addition, cyclin D1 is also involved in regulating the Rb phosphorylation status in association with CDK4/6. As shown in Figure 5A, disruption of NLK simultaneously suppressed the expression of cyclin D1 and CDK2 in the Hep3B cells. Therefore, aberrant regulation of NLK may cause the induction of these two key modulators in the cell cycle, and this might exert synergistic effects on cell cycle transition and may result in uncontrolled cell cycle activity by inducing a hyperphosphorylated status of p130 during the development of HCC. With CDKs and the cyclins, the CDKIs, such as $p21^{WAF1/CIP1}$, $p15^{INK4B}$, $p16^{INK4A}$, and $p27^{Kip1}$, are very potent negative cell cycle regulators in cancer development and progression. However, our results indicated that NLK did not affect these molecules, based on the evaluation of their expression levels. Although these findings do not fully explain the mechanisms of NLK that are associated with the cell cycle, it is possible that NLK has a mitogenic role as a cell cycle regulator in Hep3B human HCC cells. This suggestion was partly verified by the fact that silencing of NLK suppressed colony formation potential (Fig. 5B). Furthermore, we were also noted that silencing of NLK suppressed β-catenin expression (Fig. 5A). Although it is remained to be elucidated for underlying mechanism that NLK regulate β-catenin fate, NLK may play a role in Wnt signaling pathway human of HCC development and progression.

In summary, the results of this study showed aberrant regulation of human HCCs. In addition, disruption of NLK suppressed tumor cell proliferation and transforming potential by the transcriptional inhibition of CDK2 and cyclin D1. This is the first study to report that NLK is dysregulated in human HCC, and to suggest that NLK might contribute to tumor cell growth through activation of cell cycle transition. The current data suggest that NLK might be associated with the development of aggressiveness in HCCs. Further studies will focus on the molecular mechanisms of NLK-induced tumor development as well as on strategies to down-regulate the protein level or inhibit its function for potential therapeutic applications.

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