

Expression and roles of NUPR1 in cholangiocarcinoma cells

Ki-Sun Kim¹, Du-Il Jin¹, Sik Yoon¹, Sun-Yong Baek¹, Bong-Seon Kim¹, Sae-Ock Oh^{1,2}

¹Department of Anatomy, Pusan National University School of Medicine, ²Medical Research Center for Ischemic Tissue Regeneration, Pusan National University, Yangsan, Korea

Abstract: Nuclear protein-1 (NUPR1) is a small nuclear protein that is responsive to various stress stimuli. Although NUPR1 has been associated with cancer development, its expression and roles in cholangiocarcinoma have not yet been described. In the present study, we found that NUPR1 was over-expressed in human cholangiocarcinoma tissues, using immunohistochemistry. The role of NUPR1 in cholangiocarcinoma was examined by its specific siRNA. *NUPR1* siRNA decreased proliferation, migration and invasion of human cholangiocarcinoma cell lines (HuCCT1 and SNU1196 cells). From these results, we conclude that NUPR1 is over-expressed in cholangiocarcinoma and regulates the proliferation and motility of cancer cells.

Key words: NUPR1, Cholangiocarcinoma, Migration, Invasion, Proliferation

Received December 30, 2011; Revised January 25, 2012; Accepted February 1, 2012

Introduction

Nuclear protein-1 (NUPR1/Com1/p8) is a small nuclear protein that is responsive to various stress stimuli [1-3]. During the acute phase of pancreatitis, it is reported to be induced in pancreatic acinar cells [4]. Later studies have shown that various kinds of injuries such as systemic lipopolysaccharides or CCl₄ treatments [5, 6], demyelinating agents [7], serum withdrawal [8], and cell cycle arrest [9] may cause NUPR1 induction. Moreover, many molecules such as transforming growth factor β [10], tumor necrosis factor α [11], glucose [12], cannabinoids [13], angiotensin [14] and endothelin [14], have been shown to induce NUPR1 expression. The lack of prominent phenotypes in *NUPR1* knockout mice, despite the fact that it is a single copy gene and has no apparent

homologs, suggests that it plays a critical role in acquired pathological conditions [2, 15].

Structurally, human NUPR1 encodes an 82 aminoacid polypeptide with a theoretical molecular mass of 8,872.7 Da [16]. Alternative splicing produces two isoform: a and b. Isoform a has 18 additional amino acids whose functional significance is not yet known [1]. At its N-terminal, it has a PEST domain, suggesting regulation by the ubiquitin/proteasome system [17]. Its C-terminal has a basic helix-loop-helix motif [17]. The absence of a stable secondary structure of NUPR1, as shown by nuclear magnetic resonance and circular dichroism analyses, suggests that it functions by interacting with other molecules [18]. For example, NUPR1 binds to and is acetylated by the general coactivator of transcription p300 [19].

NUPR1 has been shown to be involved in tumorigenesis. Several studies have suggested the role of NUPR1 in the proliferation of cancer cells. Forced over-expression of NUPR1 increased proliferation of pancreatic cancer-derived and HeLa cells [4, 16]. Moreover down-regulation of NUPR1 decreased colony-formation of pancreatic cancer-derived cells [20]. Furthermore, transformed *p8*^{+/+} cells formed

Corresponding author:

Sae-Ock Oh

Department of Anatomy, School of Medicine, Pusan National University, Beomeo-ri, Mulgeum-eup, Yangsan 626-870, Korea
Tel: +82-51-510-8045, Fax: +82-51-510-8049, E-mail: hedgehog@pusan.ac.kr

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colonies at high frequency in soft agar assay [21]. By contrast, transformed *p8^{-/-}* cells were not able to form colonies. An involvement of NUPR1 in metastasis of cancer cells has also been suggested. NUPR1 was significantly up-regulated in the metastasis formed in the central nervous system after injection of breast cancer cells, suggesting that it facilitates tumor establishment in a secondary organ [22, 23]. Moreover, the NUPR1 expression level was directly linked to lymph node metastasis in thyroid cancer [24]. Furthermore, a recent study showed that NUPR1 regulates motility of pancreatic cancer cells [20].

Cholangiocarcinoma is a malignant tumor originating from bile duct epithelial cells [25]. Depending on location, it can be classified as intrahepatic or extrahepatic. Klatskin tumors, which originate from the bifurcation of the hepatic duct, are generally considered extrahepatic. Intrahepatic cholangiocarcinoma is the second most common primary hepatic malignancy worldwide [26]. The main cause of cholangiocarcinoma in Asian countries has been associated with infections, especially infections with the liver flukes *Clonorchis sinensis* [27, 28]. Despite advances in surgical and medical therapy, its survival rate is still poor. The main reasons for this poor prognosis are diagnostic difficulty, extensive local tumor invasion at diagnosis and multi-drug resistance. Molecular and pathophysiological understanding of the disease will improve the diagnosis and prognosis.

The expression status and role of NUPR1 in cholangiocarcinoma has not yet been examined. In the present study, we showed that NUPR1 was over-expressed in cholangiocarcinoma and could regulate motility of cancer cells.

Materials and Methods

Cell culture and transfection

HuCCT1 and SUN1196 cell line were purchased from the Health Science Research Resources Bank (Osaka, Japan) and the Korean Cell Line Bank (Seoul, Korea) respectively. HuCCT1 and SNU1196 cells were cultured with RPMI1640, 10% fetal bovine serum (FBS) and 1× penicillin/streptomycin at 37°C and 5% CO₂ incubator. NUPR1 siRNA (Bioneer, Daejeon, Korea) and scrambled (SCR) siRNA (Dharmacon, Lafayette, CO, USA) were purchased. Cells were transfected with 100 nM of NUPR1 siRNA, or SCR siRNA with Dharmafect reagent (Dharmacon) in accordance with manufacturer protocol. SCR siRNA was used as a negative

control. The sequences of NUPR1 siRNA duplex were as follows: 5'-CAG ACA AAG CGU UAG GAG A-3', 5'-CAG AGA CAG ACA AAG CGU U-3', and 5'-CUC CAA CCC UAG CAA AAG U-3'.

Immunohistochemistry

After deparaffinization and rehydration, slides were subjected to 0.3% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. Blocking was performed with 10% normal donkey serum, 1% bovine serum albumin (BSA) in 1× phosphate buffered saline (PBS). Anti-NUPR1 antibody (Abcam, Cambridge, UK) binding was performed at 1 : 200 dilution in blocking buffer overnight at 4°C, and secondary antibody (1 : 200, horseradish peroxidase [HRP]-conjugated anti-rabbit) binding for two hours at room temperature, detection with HRP (Vector Laboratories, Burlingame, CA, USA) using the DAB substrate kit (Vector Laboratories).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer protocol. cDNA was synthesized with MMLV reverse transcriptase (Promega, Madison, WI, USA), dNTP and oligo-dT primers. Then, PCR was carried out using the following primers: NUPR1 (F: 5'-CAC CTT CCC ACC AGC AAC CAG CG-3', R: 5'-GTG GGC ATA GGC ATG ATG AGA GGC CC-3'), and β -actin (F: 5'-CAA GAG ATG GCC ACG GCT GC-3', R: 5'-TCC TTC TGC ATC CTG TCG GC-3'). β -actin was used as a loading control and all signals were normalized to β -actin.

Western blotting

Cell lysates were run onto 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a PVDF membrane. Blocking was carried out with 3% BSA in PBS for 1 hour at room temperature. NUPR1 antibody (Abcam, Cambridge, UK) was diluted to 1 : 500 in 3% BSA in PBS, and incubated overnight at 4°C. β -actin antibody (Abcam, Cambridge, MA, USA) was diluted to 1 : 2,000 in 3% BSA in PBS. HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was diluted to 1 : 2,000 in PBST and incubated for 2 hours at room temperature. Blots were visualized by enhanced chemiluminescence (Amersham Bioscience, Freiburg, Germany).

Proliferation assay

Three or seven days after the transfection of *NUPR1* siRNA into cells in a 96-well plate, 10 μ l of Ez-Cytox (ITSBIO, Seoul, Korea) were added and incubated for 2 hours under normal cell culture conditions. Cell viability was measured by absorbance at 450 nm using an enzyme-linked immunosorbent assay reader (TECAN, Mannedorf, Switzerland).

Boyden chamber assay

A modified Boyden chamber (Neuro Probe, Gaithersburg, MD, USA) migration assay was used. The 8.0 μ m pore polycarbonate filter was coated with type-I collagen (10 μ l/ml). Two days after transfection with SCR or *NUPR1* siRNA, the cells were trypsinized. Cells suspended in 30 μ l of serum-free medium were added to the upper chamber with a density of

5×10^4 cells per well. The lower chamber was filled with 30 μ l of medium containing 10% FBS or 100 ng/ml of epidermal growth factor (EGF) as chemoattractant. After treatment with mitomycin C at 5 μ g/ml (Sigma-Aldrich, St. Louis, MO, USA), cells were incubated at 37°C for four hours. After incubation, the cells in the upper surface of the membrane were removed with cotton swab and the cells that migrated to the lower surface of the membrane were fixed and stained with Diff Quik Solution (Sysmex, Kobe, Japan). The migrated cells on the lower surface of the membrane filter were scored from nine random fields under microscopy (200 \times magnification). Experiments were performed in triplicate and repeated thrice.

Wound-healing assay

One day after the transfection of *NUPR1* siRNA into cells in a 6-well plate, cells were transferred and cultured to a 48-

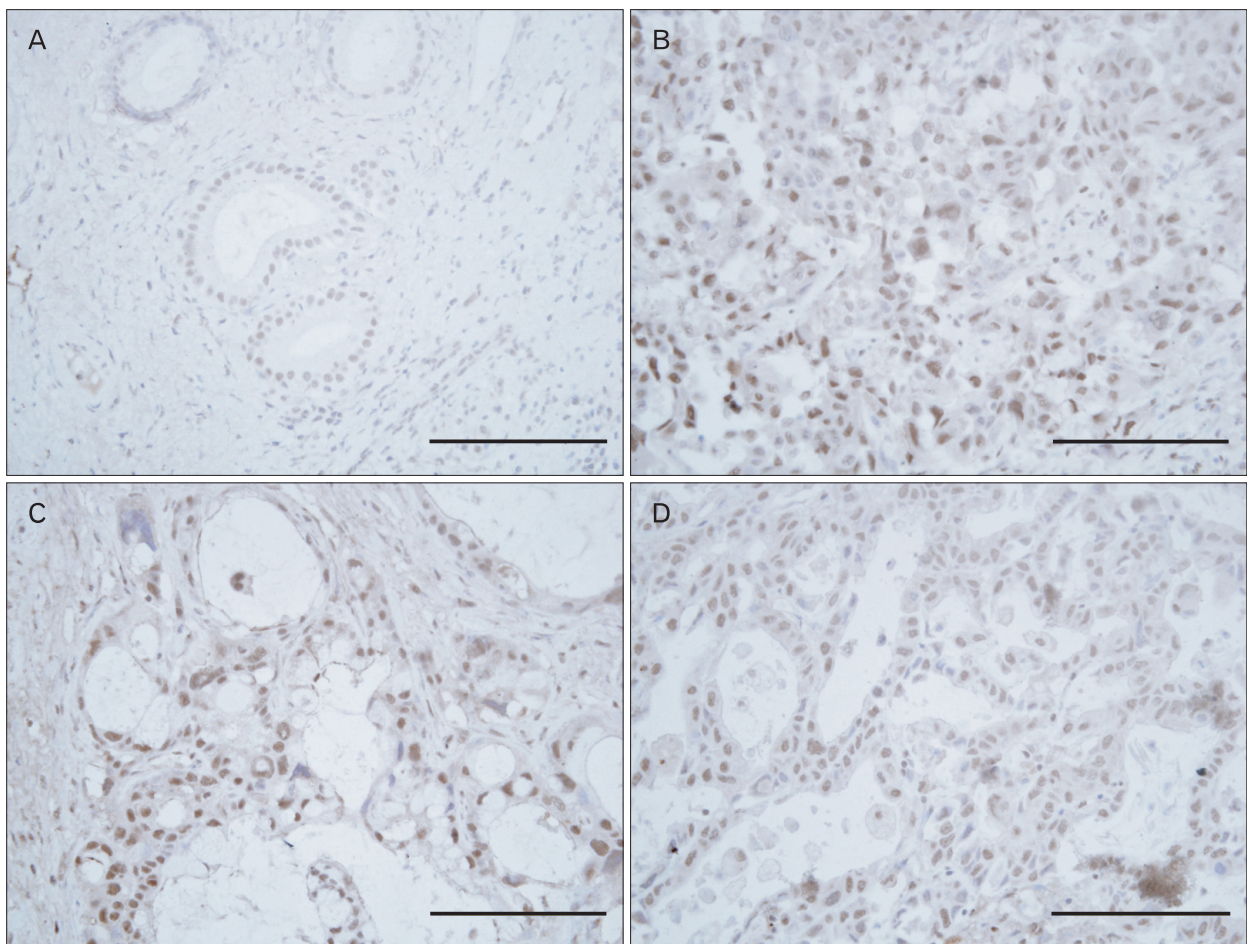


Fig. 1. Nuclear protein-1 (NUPR1) was over-expressed in cholangiocarcinoma. Immunohistochemistry was carried out using cholangiocarcinoma patient tissues (n=10) to examine the expression of NUPR1. Representative stainings from normal (A) or cancerous areas (B-D) are presented. Hematoxylin and eosin staining was used for counterstaining. Scale bars=50 μ m (A-D).

well plate until confluent. Three hours following treatment with mitomycin C at 5 $\mu\text{g}/\text{ml}$ (Sigma-Aldrich), cells were scratched using 200 μl yellow tips and moved to fresh media to incubate for 24 hours.

Matrigel invasion assay

As described by Baek et al. [29], following two days of transfection with SCR or *NUPR1* siRNA into cells, transfected cells were seeded to a 24-well BioCoat Matrigel chamber inserts (BD Biosciences, San Jose, CA, USA). Eighteen hours after treatment with mitomycin C at 5 $\mu\text{g}/\text{ml}$ (Sigma-Aldrich), the cells on the insert insides were removed with cotton tips, and the invaded cells on the insert outers were visualized using hematoxylin/eosin staining.

Data analysis

All data are presented as means \pm SD. All experiments were repeated at least four times. The difference between the mean values of the two groups was evaluated using Student's t-test (unpaired comparison). For comparison of more than three groups, we used one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparison. A *P*-value of <0.05 was considered statistically significant.

Results

First of all, we examined the expression status of *NUPR1* in cholangiocarcinoma patients' tissues ($n=10$). Normal areas in patient tissues were used as a control. Low level of *NUPR1* expression was observed in the nuclei of normal bile duct

cells (Fig. 1A). However, in cancerous areas, significant over-expression of *NUPR1* was observed (Fig. 1B-D).

Next, we studied the functional significance of *NUPR1* in cholangiocarcinoma cells. To examine the role of *NUPR1* in HuCCT1 cholangiocarcinoma cells, we knock-downed it using the specific siRNA. RT-PCR or western blotting (Fig. 2) showed the RNA and protein level of *NUPR1* was significantly reduced by *NUPR1* siRNA. To reveal the role of *NUPR1* in the proliferation of cholangiocarcinoma cells, we performed WST-1 survival assay, three and seven days after knock-down. In the presence of 1% or 10% FBS, 100 nM *NUPR1* siRNA reduced the proliferation of HuCCT1 by 23.2% and 17.8% respectively compared to SCR siRNA seven days after knock-down (Fig. 3).

To examine the role of *NUPR1* in the migration of HuCCT1 cholangiocarcinoma cells, we performed Boyden chamber assays (Fig. 4) and wound healing assays (Fig. 5). To rule out the effect of proliferation on migration assays, we added mitomycin C into the media. In the Boyden chamber assay, *NUPR1* siRNA reduced EGF- or FBS-induced migration by 21.1% and 34.6% respectively, compared to SCR siRNA (Fig. 4). Moreover, we also observed similar effects of *NUPR1* siRNA on FBS-induced migration of SNU1196 cholangiocarcinoma cells (data not shown). This reduction of migration by *NUPR1* siRNA was further confirmed by wound healing assays (Fig. 5).

Finally we examined the role of *NUPR1* in the invasion of HuCCT1 cholangiocarcinoma cells using Matrigel invasion assays. In this, *NUPR1* siRNA reduced EGF- or FBS-induced invasion by 75.4% and 41.4% respectively, compared to SCR siRNA (Fig. 6). Moreover, we also observed a similar effect

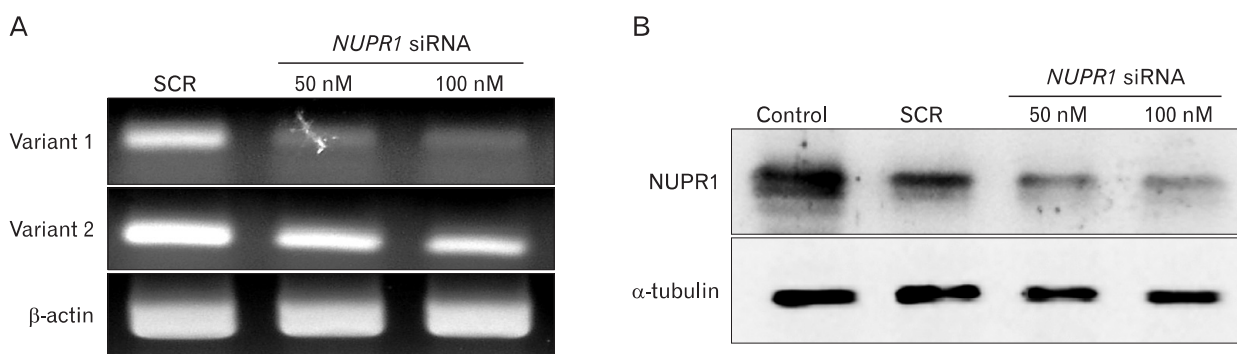


Fig. 2. Nuclear protein-1 (*NUPR1*) siRNA specifically down-regulated *NUPR1* expression in HuCCT1 cholangiocarcinoma cells. The mRNA level of *NUPR1* was reduced by siRNA (A). The protein level of *NUPR1* was also reduced by siRNA (B). Protein and RNA were extracted two days after transfection with indicated concentrations of the siRNA. Three independent experiments were repeated. β -actin and α -tubulin were used for normalization of RNA and protein loading respectively. SCR, scrambled.

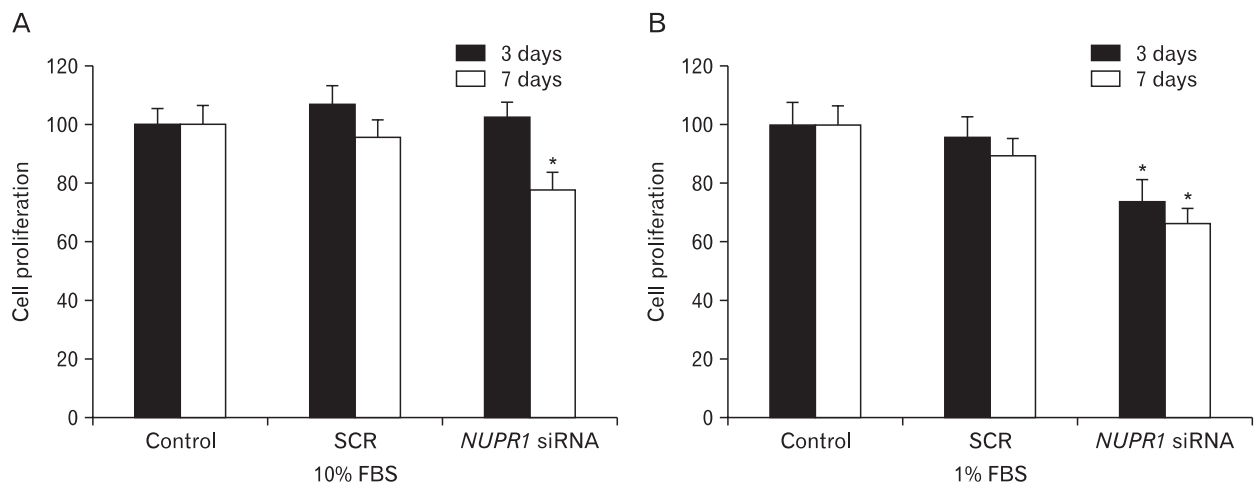


Fig. 3. Effects of knock-down of nuclear protein-1 (NUPR1) on proliferation of HuCCT1 cholangiocarcinoma cells. Cell proliferation was measured three and seven days after the siRNA transfection using WST-1 assay. In the presence of 10% (A) or 1% fetal bovine serum (FBS) (B), proliferation was examined. Data show the means±SDs of three independent experiments performed in quintuplicate (**P*<0.05). SCR, scrambled.

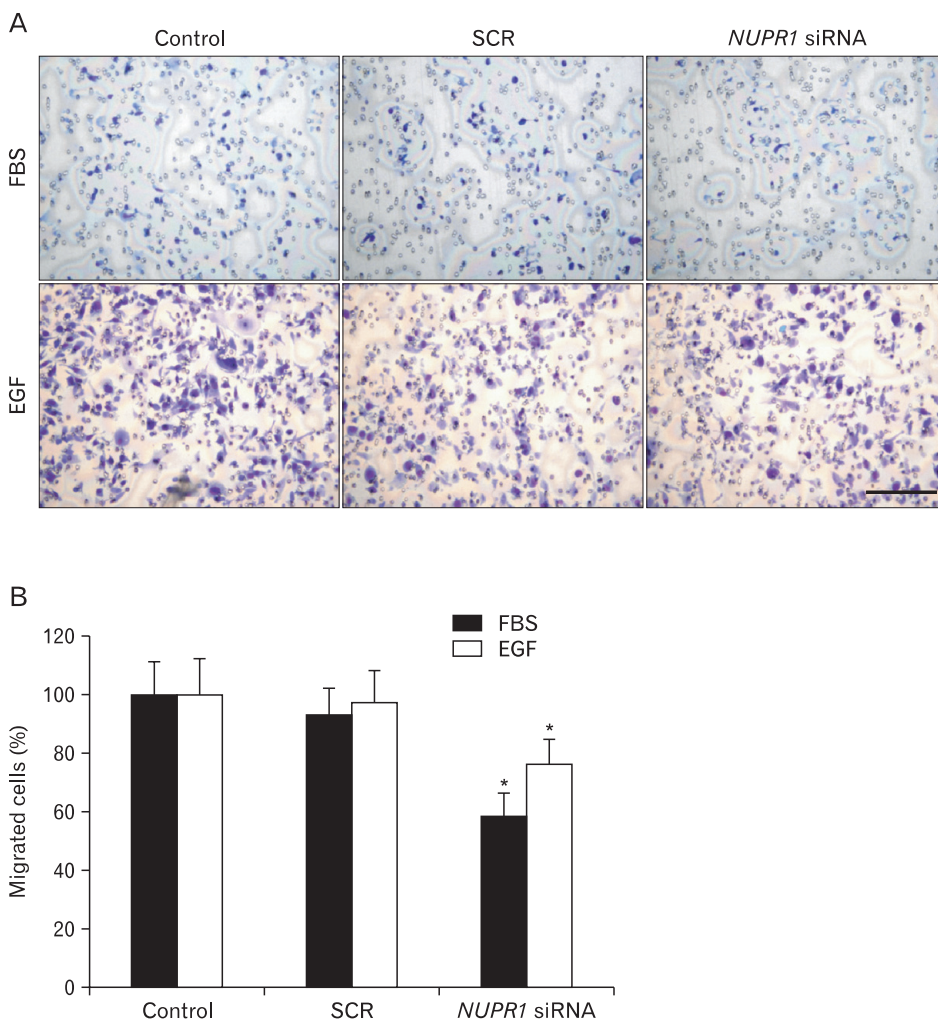


Fig. 4. Nuclear protein-1 (NUPR1) affected epidermal growth factor (EGF)- or serum-induced migration of HuCCT1 cholangiocarcinoma cells. To examine effects on migration, Boyden chamber assays were performed. Two days after siRNA transfection, 5×10^4 cells were seeded into Boyden chambers. Addition of 100 nM EGF or 10% fetal bovine serum (FBS) into lower but not upper Boyden chambers induced migration of the cells. To rule out effects of proliferation, mitomycin C (5 μ g/ml) was added. Four hours later, cells were fixed, stained with Diff-quick solution and pictured. The representative staining of migrated cells is presented (A). Scale bar=50 μ m. (B) Migrated cells were counted and the data are presented as a graph. Data show the means±SDs of three independent experiments performed in triplicate (**P*<0.05). SCR, scrambled.

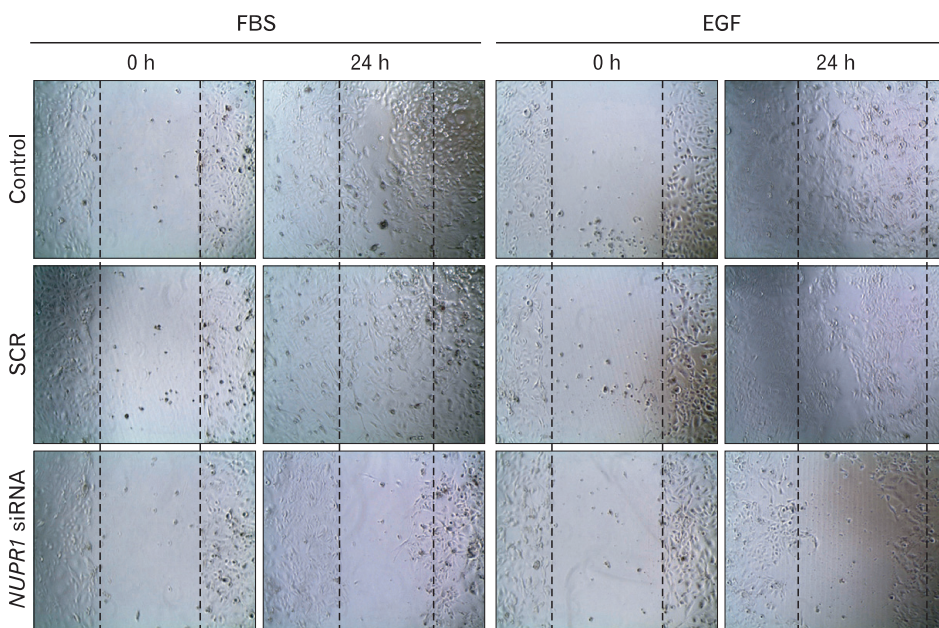


Fig. 5. Nuclear protein-1 (NUPR1) affected epidermal growth factor (EGF)- or serum-induced migration of HuCCT1 cholangiocarcinoma cells. To examine the effects on migration, wound healing assays were performed. To rule out effects of proliferation, mitomycin C (5 µg/ml) was added. A day after scratch with a yellow tip, migration was documented. Three independent experiments were carried out. FBS, fetal bovine serum; SCR, scrambled.

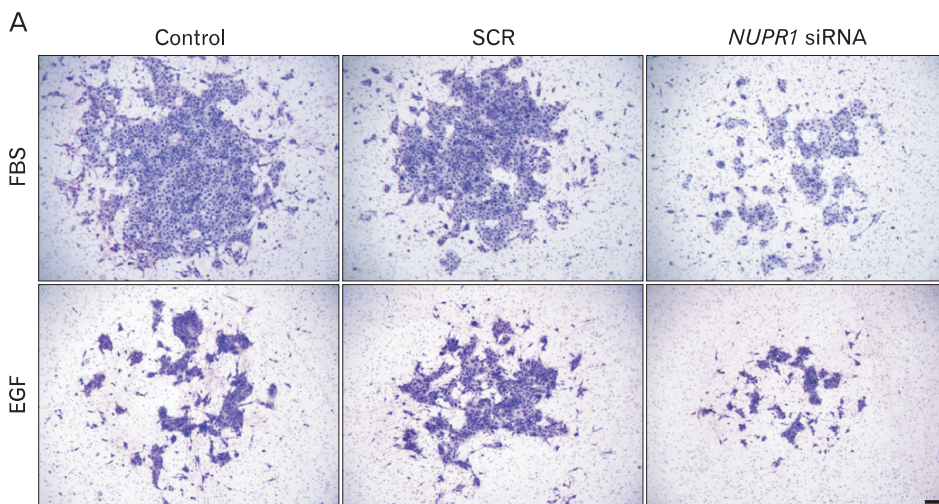
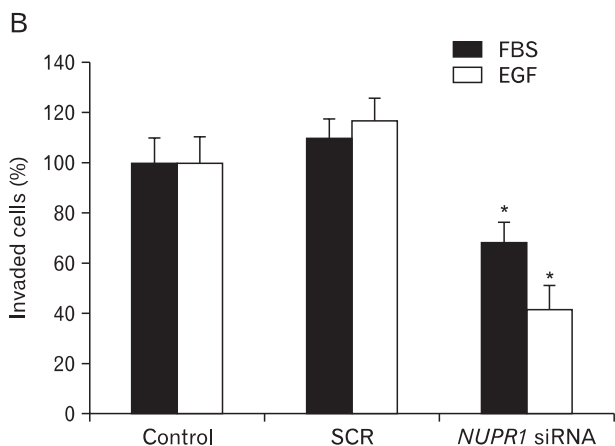


Fig. 6. Nuclear protein-1 (NUPR1) affected epidermal growth factor (EGF)- or serum-induced invasion of HuCCT1 cholangiocarcinoma cells. To examine the effects on invasion, matrigel invasion assays were performed. Two days after siRNA transfection, 5×10^4 cells were seeded into matrigel-coated transwells. To rule out effects of proliferation, mitomycin C (5 µg/ml) was added. Eighteen hours later, the cells were fixed, stained with Diff-quick solution, and pictured. The representative staining of invaded cells is presented (A). Scale bar=50 µm. (B) Invaded cells were counted and the data are presented as a graph. Data show means±SDs of three independent experiments performed in triplicate (* $P < 0.01$). FBS, fetal bovine serum; SCR, scrambled.



of *NUPR1* siRNA on FBS-induced invasion of SNU1196 cholangiocarcinoma cells (data not shown).

Discussion

The extensive invasion of cholangiocarcinoma cells greatly affects the prognosis of patients. Molecular understanding about the motility of cholangiocarcinoma cells will improve diagnosis and therapy. In the present study, we documented over-expression of *NUPR1* in cholangiocarcinoma and its role in cell proliferation and motility.

The over-expression of *NUPR1* in cholangiocarcinoma patient tissues was presented in the current study for the first time. *NUPR1* has been shown to be over-expressed in various cancers including those of the pancreas, breast, pituitary and thyroid. Interestingly, the expression of *NUPR1* in thyroid cancers was directly linked to lymph node metastasis [24]. In addition, cellular localizations of *NUPR1* were related to biological characteristics of papillary carcinoma. In aggressive tumors, *NUPR1* is localized in the cytoplasm, whereas in less aggressive tumors, it is in the nucleus [1, 30]. In future study, the relationship between *NUPR1* over-expression and patient prognosis needs to be examined.

There have been contradictory reports about the role of *NUPR1* in cell proliferation. Its over-expression in Cos-7 or AR4-2J cells promoted cell proliferation [4]. Knock-down of *NUPR1* in L β T2 cells decreased *in vivo* tumorigenicity and increased expressions of cycle regulators such as p21 and p57 [31]. Moreover, *NUPR1* siRNA decreased colony formation and tumorigenicity of pancreatic cancer-derived cells [20]. However *NUPR1* over-expression decreased the growth of prostate tumors *in vivo* [32, 33]. Moreover, *NUPR1* negatively regulated the cell cycle and promoted myogenic differentiation in C2C12 myoblasts [34, 35]. One suggested mechanism by which *NUPR1* may regulate cell proliferation is through interaction with Jab1, a component of the COP9 signalosome complex. The *NUPR1* and Jab1 complex induces translocation of p27 from the nucleus to the cytoplasm where it is degraded [36]. In the present study, *NUPR1* siRNA slightly reduced proliferation of cholangiocarcinoma cell lines.

The role of *NUPR1* in the motility of cancer cells was first described in pancreatic cancer-derived cells [20]. Knock-down of *NUPR1* in pancreatic cancer cells *in vitro* decreased migration and invasion while increasing cell adhesion.

Moreover, its over-expression showed opposite effects. This suggested that CDC42 might mediate the regulation of motility by *NUPR1*. In the current study, we showed for the first time that *NUPR1* siRNA decreased migration and invasion of cholangiocarcinoma cell lines. In future study, the underlying mechanisms through which *NUPR1* regulates cell motility need to be examined.

In the present study, FBS-induced migration was more sensitively inhibited by *NUPR1* siRNA than was EGF-induced migration. However, EGF-induced invasion was more sensitively inhibited by *NUPR1* siRNA than was FBS-induced invasion. Molecules that can degrade the extracellular matrix also contribute to the invasion process of cancer cells in addition to those molecules which regulate the migration of cancer cells [37]. Therefore the different sensitivity to FBS or EGF in migration and invasion may involve different regulation of the molecules involved. Further studies need to be conducted to examine this different sensitivity.

Acknowledgements

This work was supported by a Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-331- E00047).

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