Chemosensitivity Induced by Down-regulation of MicroRNA-21 in Gemcitabine-resistant Pancreatic Cancer Cells by Indole-3-Carbinol

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Abstract. *Background/Aim: Overexpression of microRNA-21 (miR-21) indicates chemoresistance in pancreatic cancer. We evaluated the change of chemosensitivity to gemcitabine through the down-regulation of miR-21 in human pancreatic cancer cells (Panc-1). Materials and Methods: The efficacy of indole-3-carbinol (I3C) in suppressing miR-21 expression and its anticancer effect in combination with gemcitabine were investigated. Results: Down-regulation of miR-21 by I3C was positively-correlated in a time- and dose-dependent manner. I3C and gemcitabine combination therapy increased cytotoxicity in Panc-1 cells. Transfection of miR-21 mimic abrogated I3C-induced sensitivity to gemcitabine. DNA fragmentation and terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) assays showed that pre-treatment with I3C enhanced apoptosis and this effect was attenuated by miR-21 transfection. The expression of programmed cell death-4 (PDCD4) was increased by I3C and reduced by miR-21 transfection. Conclusion: I3C would be effective for enhancing sensitivity of pancreatic cancer cells to gemcitabine via down-regulation of miR-21. Such enhanced chemosensitivity might be explained by the increased expression of PDCD4, which is a downstream target which miR-21 negatively regulates.*

Pancreatic cancer is a very aggressive human cancer and has a dismal prognosis, with only 6% of patients surviving five years after diagnosis (1). Surgical resection is the only potentially curative treatment in pancreatic cancer, however, only 15% of patients are candidates for resection (2, 3). Gemcitabine became the standard chemotherapeutic agent for pancreatic cancer after randomized trials in 1997, however, this type of cancer is highly resistant to chemotherapy (4, 5). Therefore, new therapeutic targets for pancreatic cancer are required and microRNA (miRNA) may be one of the most specific targets.

miRNAs are 18-24-nucleotide single-stranded RNA molecules which regulate the translation of specific mRNAs and play an important role in various physiological and pathological processes (6). Several miRNAs are associated with prognosis of pancreatic cancer (7-9). High expression of miR-21, which is known to be an 'oncomir', is associated with poor clinical outcome and indicates chemoresistance, including that against gemcitabine (10).

Indole-3-carbinol (I3C) is a natural compound present in some fruits and cruciferous vegetables, such as broccoli and cabbage. I3C has been shown to exert anticancer properties in various types of cancer (11), and it was recently demonstrated that I3C reduced miR-21 expressions in vinyl carbamate-induced mouse lung tumors (12). The aim of this study was to evaluate the effect of down-regulation of miR-21 by I3C on gemcitabine-induced cytotoxicity in human pancreatic cancer cells.

Materials and Methods

Human pancreatic cancer cells and treatment conditions for I3C and gemcitabine. The Panc-1 human pancreatic cancer cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Panc-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen) and 1% penicillin-streptomycin. In order to determine the specific I3C (Sigma-Aldrich, St Louis, MO, USA) concentration which induces minimal

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Key Words: Pancreatic neoplasm, indole-3-carbinol, gemcitabine, microRNA, chemotherapy, Panc-1 cells.

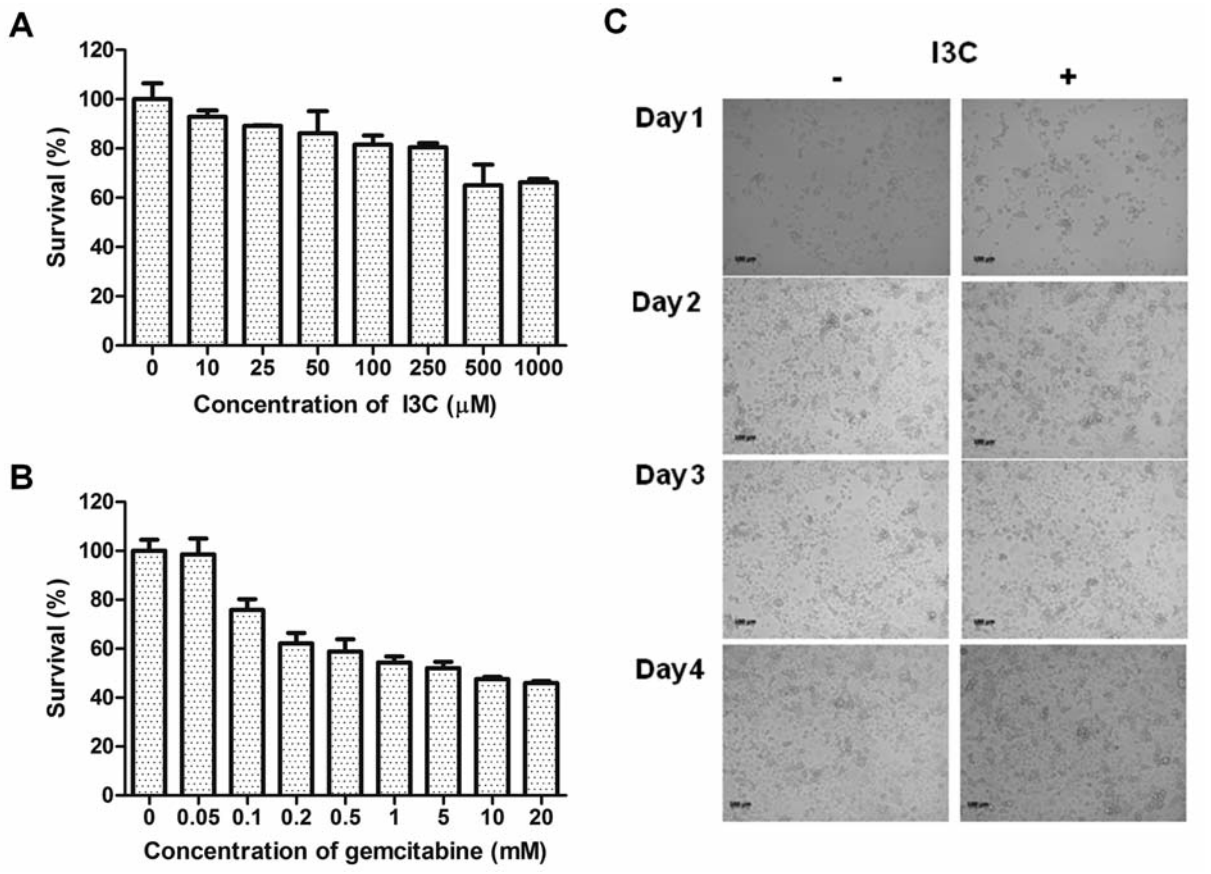

Figure 1. *Cytotoxicity of indole-3-carbinol (I3C) (A and C) and gemcitabine (B) towards Panc-1 cells. Panc-1 cells were forward to be gemcitabine-resistant because the half-maximal inhibitory concentration (IC_{50}) of gemcitabine was 9 mM (B). However, treatment of 100 μM I3C was minimally cytotoxic towards Panc-1 cells (A and C). Values were obtained from at least three separate experiments. I3C, Indole-3-carbinol.*

cytotoxicity, Panc-1 cells were cultured with serial concentrations of I3C (0 to 1000 μM). Subsequently, the cells were cultured for different exposure times (0, 24, 48 and 72 h) to choose an appropriate exposure time for adequate I3C suppression of miR-21 expression. miR-21 expression was measured quantitatively. The half-maximal inhibitory concentration (IC_{50}) of gemcitabine (Eli Lilly & Co., Indianapolis, IN, USA) was obtained by culture with serial concentrations of gemcitabine (0, 50 μM, 100 μM, 200 μM, 500 μM, 1 mM, 5 mM, 10 mM, and 20 mM). Cells were then pre-treated with 100 μM I3C for 24 h and transfected with miR-21 mimic or negative control. After another 24 h, cells were washed and treated with 9 mM gemcitabine for 72 h.

RNA isolation and real-time quantitative RT-PCR for miR-21. After exposure to I3C and/or gemcitabine, total RNA from cells was extracted by using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The quality and quantity of the RNA was assessed at A260/A280 nm using a NanoDrop1000 spectrophotometer (Thermo Scientific Inc., Bremen, Germany). Expression levels of miR-21 were analyzed by the NCode SBGR qRT-PCR kit (Invitrogen) using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers were designed and purchased from Invitrogen. The miR-21 primer sequence was TAGCTTATCAGACTGATGTTGA and U6 (as an internal control) primer sequence was GGCAGCACATATACTAAAATTGGAA.

Transfection of miR-21 mimic. miR-21 mimic and CY3-labeled negative control were designed by and purchased from Ambion (Austin, TX, USA). Before the experiments, the duration of miR-21 expression after transfection of miR-21 mimic in the cytoplasm was ascertained under fluorescence microscopy (Axio Observer, Carl Zeiss, Jena, Germany). Panc-1 cells were then pre-treated with 100 μM I3C for 24 h and transfected with 30 nM miR-21 mimic or CY3-labeled negative control using G-Fectin (Genolution Pharmaceuticals, Seoul, Korea), according to the manufacturer's instructions. Briefly, 1-2 μl of G-Fectin and miR-21 mimic or negative control were added to 100 μl of phosphate buffered saline (PBS) in a tube and then incubated for 10 min at room temperature. The mixture was then applied to cells in 24-well plates directly. The transfected cells were used after 24 h. The miR-21 mimic sequence was UAGCUUAUCAGACUGAUGUUGA.

Cell viability assay. Cells were dispersed at 8×10^3 per well in 96-well plates. The cells were then treated as follows: no treatment, 9 mM gemcitabine only, 100 μM I3C only, or combination of I3C and gemcitabine at the respective concentrations. After 72 h, cells were

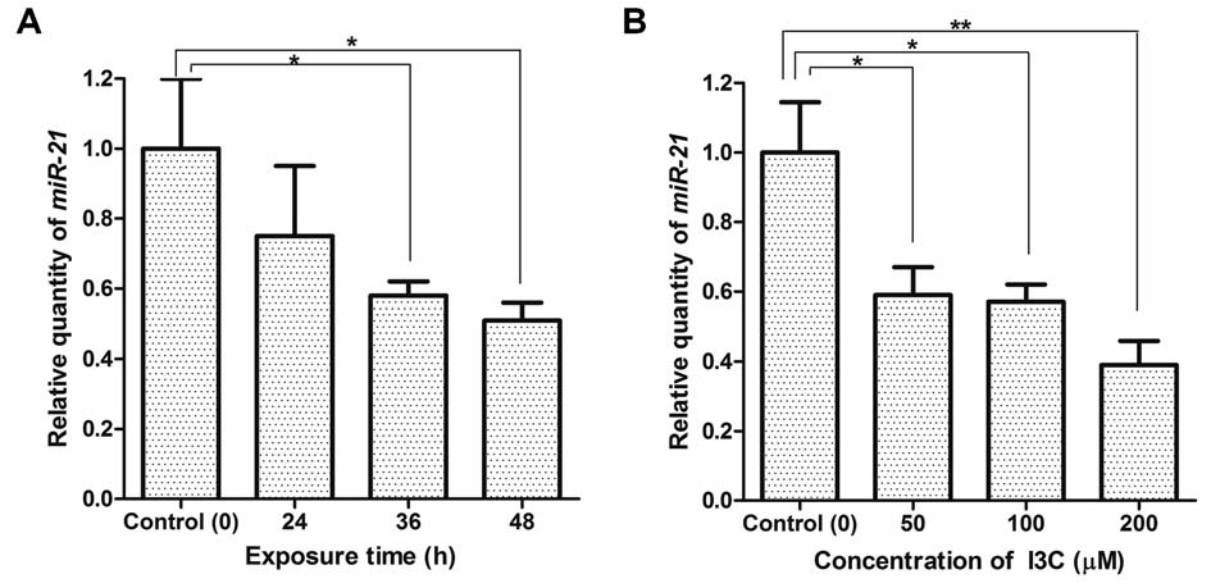

Figure 2. *microRNA-21 (miR-21) expression after indole-3-carbinol (I3C) treatment of Panc-1 cells according to time of exposure to (A) and concentration of (B) I3C. I3C concentration was 100 μM in (A) and the time of exposure was 24 h in (B). miR-21 expression was reduced by I3C in a time- and dose-dependent manner. *p<0.05; **p<0.01.*

monitored by the xCELLigence-RTCA DP system (Roche Applied Science, Mannheim, Germany) for real-time cell analysis.

Cell viability was also determined by the cell counting kit-8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan). Cells were dispersed at 5×10^4 per well in 96-well plates. The cells were then cultured in the presence or absence of 100 μM of I3C for 24 h and 9 mM of gemcitabine for 72 h. After treatment, 10 μl of the CCK-8 solution were added to each well of the plates and they were incubated for 1 h at 37˚C. The density of cells was measured at 450 nm using a SpectraMax plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Cell-cycle analysis, Western blotting and apoptosis assay. We used NucleoCounter NC-3000 from Chemometec (Allerød, Denmark) to analyze the cell-cycle distribution according to the manufacturer's specifications. Cells were harvested and rinsed by PBS then resuspended with 10 mg/ml 4`,6-diamidino-2-phenylindole (DAPI). Cells were subsequently incubated at 37˚C for 5 min then 30 ml of suspended cells was loaded onto an 8-chamber slide (NC-Slide A8, LI-COR Biosciences, Lincoln, NE, USA). The cell cycle distribution was analyzed by using the provided software.

Cells were treated as specified and then lysed in mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA). A BCA assay (Sigma-Aldrich) was used for the quantitation of protein. Whole-cell lysate was heated at 100˚C for 3 min and denatured before being run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblotting was performed with each of the following primary antibodies against: phosphatase and tensin homolog (PTEN) (Cell Signaling Tech., Danvers, MA, USA), phospho-PTEN (Ser380/Thr382/383) (Cell Signaling Tech.), serine/threonine-specific protein kinase (AKT) (Abcam Inc., Cambridge, UK), phospho-Akt (Abcam Inc.), programmed cell death 4 (PDCD4) (Cell Signaling Tech.) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated with secondary antibody (anti-rabbit IgG, Abcam Inc; anti-mouse IgG, Cell Signaling Tech.). The immune complex in polyvinylidene difluoride (PVDF) membrane was detected with a Novex chemiluminescence reagent (Invitrogen) and Chemidoc XRS system (Bio-Rad, Richmond, CA, USA).

Apoptosis was detected by an enhanced apoptotic DNA ladder detection kit (Biovision, Mountain View, CA, USA). Cells were dispersed at 5×10^4 cells per well in 96-well plates. The cells were treated as specified above and were then harvested. Total DNA was extracted according to the manufacturer's instructions. The DNA was separated on a 1.8% agarose gel and visualized by Chemidoc XRS system (Bio-Rad) by dye staining. Apoptosis was also detected by an *in situ* cell death detection kit, with fluorescein (Roche Applied Science). Cells treated as specified on coverslips were fixed with 4% paraformaldehyde and were permeabilized by 0.1% Triton X-100. Cells were then incubated with terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) reaction mixture. Coverslips were observed under fluorescence microscopy.

Statistical analysis. All the experiments were performed four times. Statistical analysis was performed using SPSS v.18.0 (IBM Corp., Armonk, NY, USA) by Mann Whitney *U*-test. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Different concentrations of I3C (10 to 1000 μM) and gemcitabine (50 μm to 20 mM) were used to assess their cytotoxicities upon a human pancreatic cancer cell line (Panc-1 cells). I3C and gemcitabine exhibited concentration-dependent cytotoxicities, and the IC_{50} of gemcitabine was

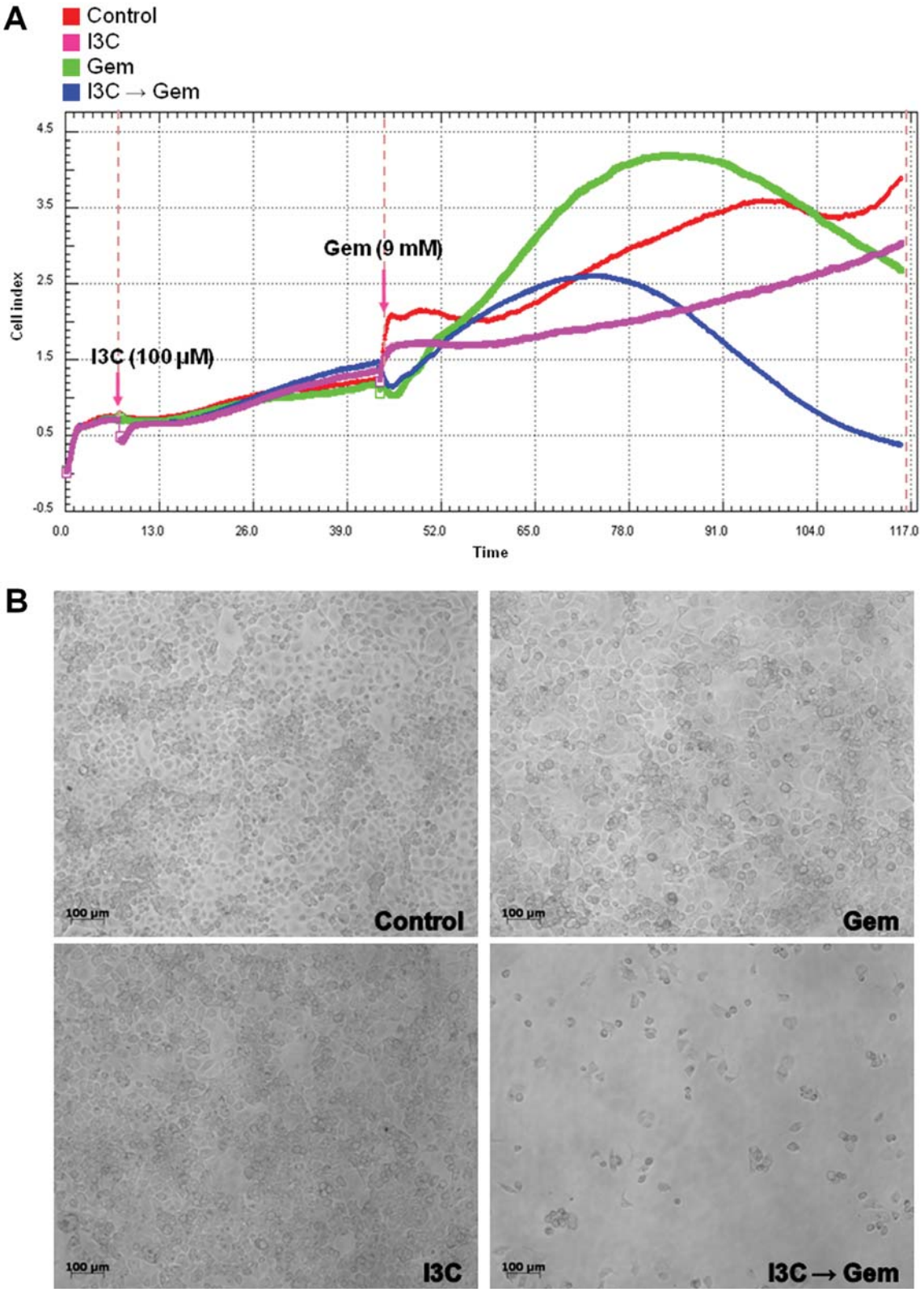

Figure 3. *Real-time cell analyzer assay according to gemcitabine and/or indole-3-carbinol (I3C) treatment. The assay demonstrated the enhancement of cytotoxicity after treating cells with I3C and gemcitabine (blue line), compared to gemcitabine alone (green line) (A). In microscopic findings, gemcitabine with pre-treatment of I3C led to fewer viable cells than with gemcitabine or I3C alone (B). Gem, Gemcitabine.*

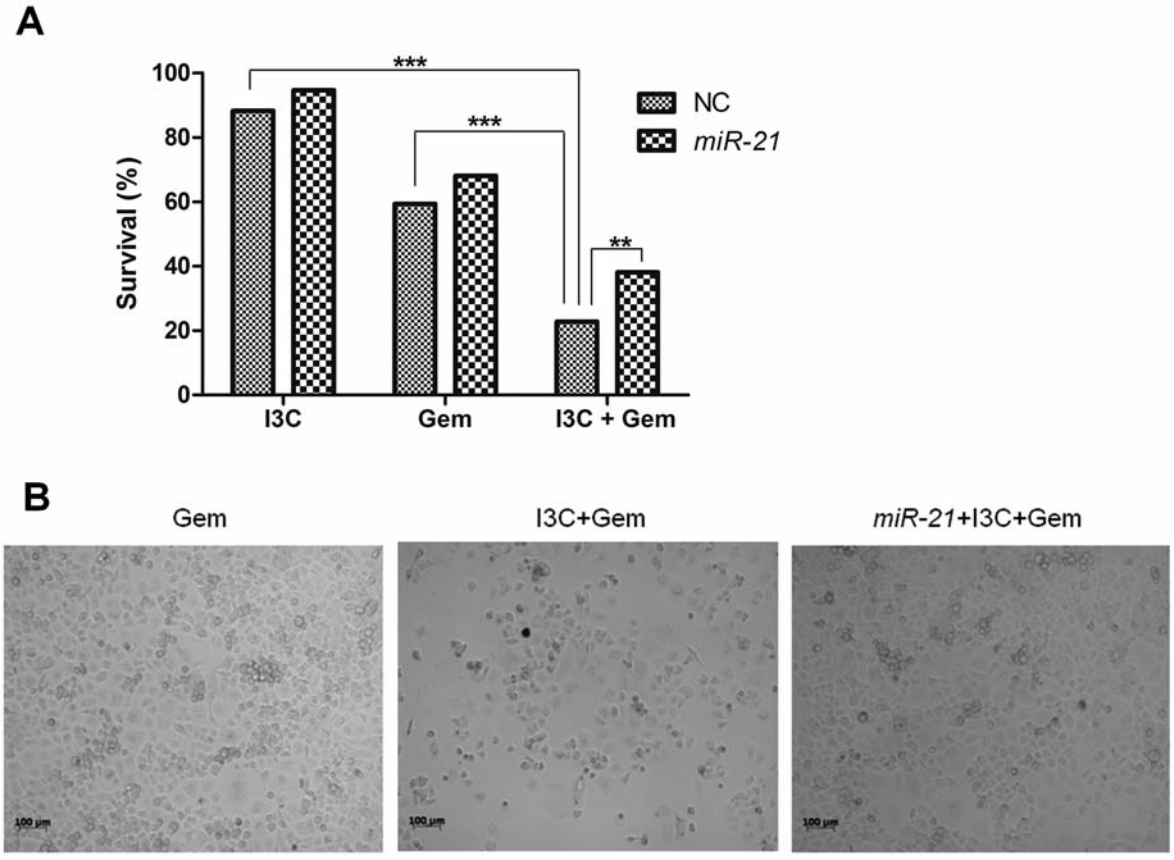

Figure 4. *Gemcitabine resistance of Panc-1 increased after miR-21 transfection. A: CCK-8 assay. B: Microscopic findings. When miR-21 was transfected into Panc-1, indole-3-carbinol (I3C)-induced gemcitabine cytotoxicity was significantly weakened. Gem, Gemcitabine; NC, negative control. **p<0.01; ***p<0.001.*

9 mM for Panc-1 cells (Figure 1A and B). I3C was relatively less cytotoxic compared to gemcitabine, and at a concentration of 100 μM I3C, the number of viable cells did not differ from that of control cells microscopically (Figure 1C). The cytotoxic effect of I3C reached a plateau above a concentration of 500 μM.

I3C down-regulated miR-21 expression in a time- and dose-dependent manner (Figure 2). miR-21 expression was decreased by half after 48 h treatment (Figure 2A), whereas miR-21 expression was augmented to 0.6-fold of its basal level after treatment with 100 μM I3C in Panc-1 cells (Figure 2B).

The cytotoxic effect of gemcitabine was increased by pre-treatment with I3C compared to both I3C and gemcitabine treatments alone by real-time cell analysis (Figure 3A). The system measures the electronic impedance on cell-containing tissue culture plates, which indicates the number of viable attached cells. Pancreatic cancer cells began to float after 24-48 h of gemcitabine treatment with pre-treatment of I3C (Figure 3B). After transfection of miR-21 mimic, expression of miR-21 was time-limited; miR-21 expression was increased by about 2.5-fold up to 24 h after transfection, but after 72 h of transfection, the miR-21 expression was reduced to 1.5-times the initial level (data not shown). Combination of I3C and gemcitabine inhibited proliferation of Panc-1 cells and only 15% of pancreatic cancer cells were viable, as shown by the CCK-8 assay, with the combination showing more significant cytotoxicity than both I3C and gemcitabine treatments alone. However, when miR-21 was transfected into Panc-1 cells, I3C-induced gemcitabine cytotoxicity was significantly reduced (Figure 4).

In cell-cycle analysis, there was no significant difference in cells arrested after administration of I3C and/or gemcitabine (Figure 5). There was little change after I3C administration in the sub-G_1 phase, thus I3C-alone had minimal cytotoxicity towards Panc-1 cells.

The expressions of apoptosis-related proteins were examined after treating the cells with I3C and/or gemcitabine. The expression of PDCD4 which induces apoptosis was increased after 48 h of treatment with I3C or

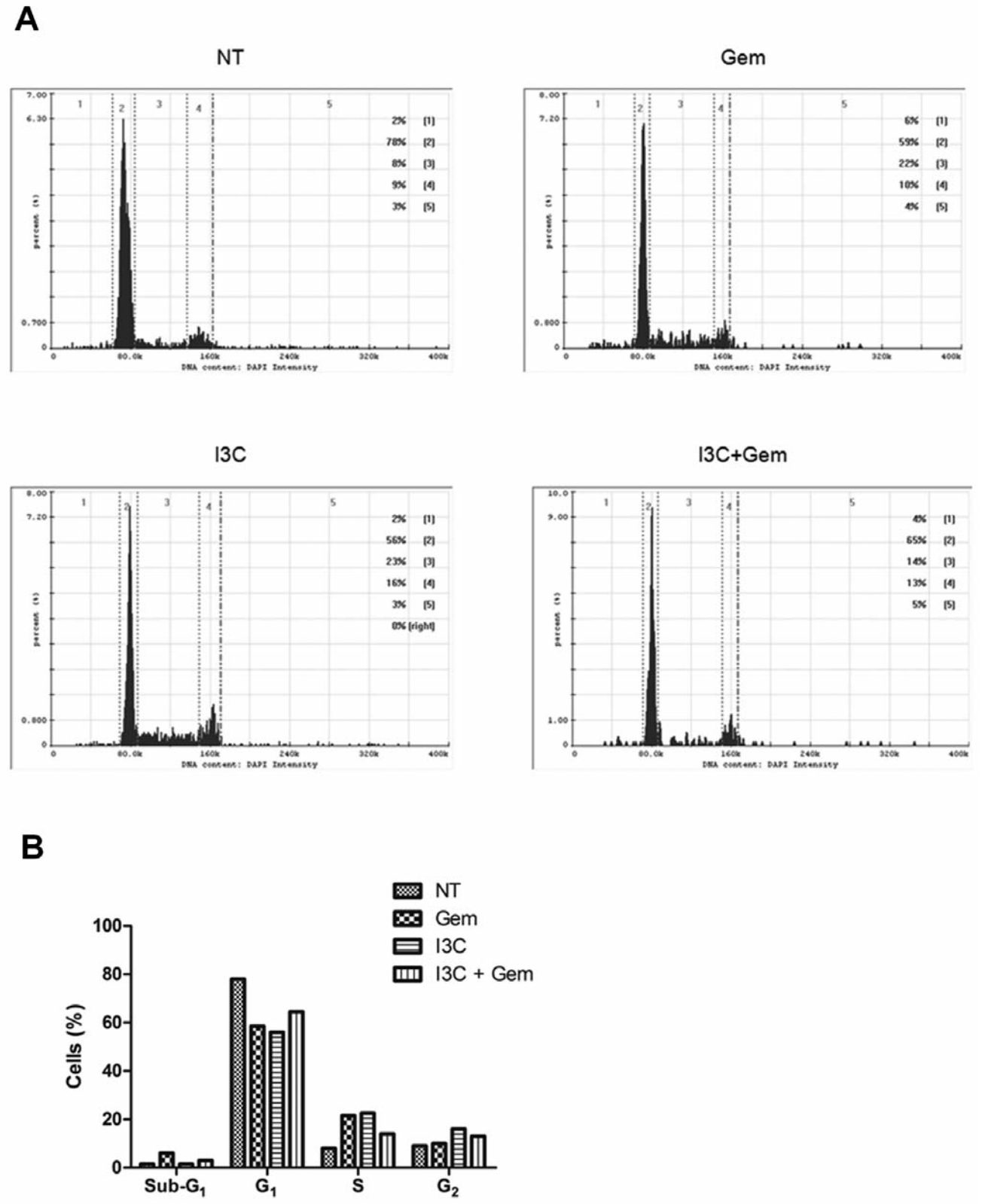

Figure 5. *Cell-cycle analysis. There was no significant difference in cell arrest after administration of gemcitabine and/or indole-3-carbinol (I3C). Moreover, there was little change in sub-G_1 phase after I3C administration, thus I3C-alone had minimal cytotoxicity towards Panc-1 cells. NT, No treatment; Gem, gemcitabine.*

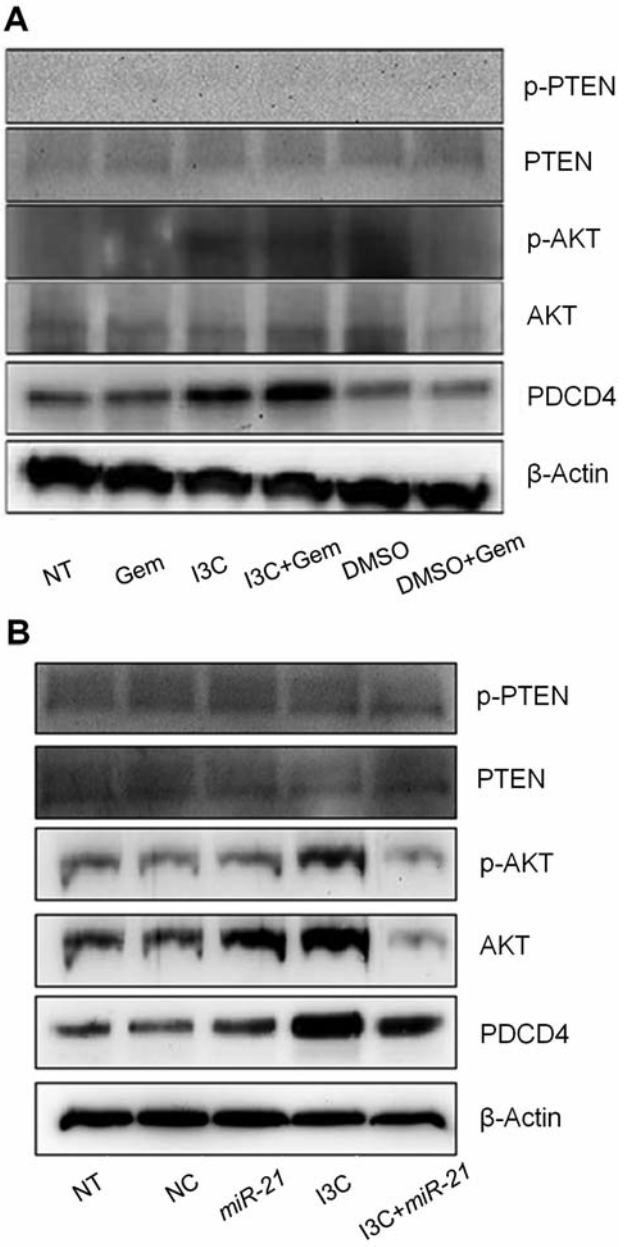

Figure 6. *Western blots demonstrating the increase of programmed cell death-4 (PDCD4) expression in Panc-1 cells after 48 h treatment with indole-3-carbinol (I3C) alone and after 24 h treatment of gemcitabine after I3C (A). On the contrary, PDCD4 expression decreased after transfection of miR-21 (B). β-Actin was used as a loading control. NT, No treatment; Gem, gemcitabine; NC, negative control.*

24 h treatment with gemcitabine after exposure to I3C (Figure 6A). However, PDCD4 expression decreased after miR-21 transfection (Figure 6B). The DNA fragmentation and the TUNEL assays also showed enhancement of apoptosis by I3C and gemcitabine treatment, and apoptosis inhibition after miR-21 transfection (Figure 7).

Discussion

This study suggests that I3C enhances cytotoxicity of gemcitabine towards human pancreatic cancer cells by down-regulation of miR-21. Down-regulation of miR-21 increased the expression of PDCD4, which is known to be a tumor suppressor and a marker of apoptosis. Therefore, those results imply that the down-regulation of miR-21 by I3C increased apoptosis in gemcitabine-resistant pancreatic cancer cells through the up-regulation of PDCD4. Panc-1 was selected for this study because it is one of the most chemoresistant human pancreatic cancer cell lines and had a relatively high gemcitabine IC_{50} compared to other human pancreatic cancer cell lines (13). In this study, I3C exhibited a minor cytotoxicity up to 250 μM and had no additional cytotoxicity at more than 500 μM. Thus, we determined the concentration of I3C for use to be 100 μM, which had minimal cytotoxicity but appropriate suppression of miR-21 expression. By doing so, we were able to establish that the enhanced chemosensitivity to gemcitabine induced by I3C is not due to cytotoxicity of I3C itself, but through regulation of miR-21 expression. Furthermore, miR-21 transfection significantly reduced the cytotoxicity of the I3C and gemcitabine combination therapy. These results support the notion that chemosensitivity of I3C occurs *via* miR-21 modulation in Panc-1 cells.

I3C has pleiotropic antitumor effects on multiple targets governing apoptotic signals, cell-cycle progression, hormonal homeostasis, DNA repair, angiogenesis, and multiple drug resistance (11, 14-16). I3C has been known to suppress the proliferation of various cancer cell lines at a concentration range of 50-100 μM (11). Previous studies reported the efficacy of I3C in human pancreatic cancer cells by inhibiting signal transducer and activator of transcription-3 (STAT3), reactivating $p16^{INK4A}$ and by up-regulation of human equilibrative nucleoside transporter 1 (17-19). To our knowledge, this is the first study about treatment of I3C targeting miR-21 in pancreatic cancer. We could suggest that I3C has a potential as a therapeutic agent for pancreatic cancer through interferance with various pathways of carcinogenesis.

PDCD4 is a tumor suppressor which regulates multiple proteins that are involved in cell survival, proliferation and metastasis (20). A recent study identified PDCD4 as a downstream target of miR-21 in pancreatic cancer (21). Our study also indicated that PDCD4 is directly regulated by miR-21, by which inhibition of miR-21 increases and transfection of miR-21 reduces PDCD4 expression in Panc-1 cells.

miR-21 is the most frequently increased oncomir in solid tumors (22). Up-regulation of miR-21 causes cell proliferation, apoptosis inhibition and tumor invasion by down-regulation of tumor suppressor genes such as PTEN, PDCD4, reversion-inducing-cysteine-rich protein with kazal motifs (RECK), tissue inhibitor of metalloproteinase-3 (TIMP3), p53 and transforming growth factor-β (TGF-β) (23-26). Low miR-21 expression implies a good response to adjuvant treatment for pancreatic

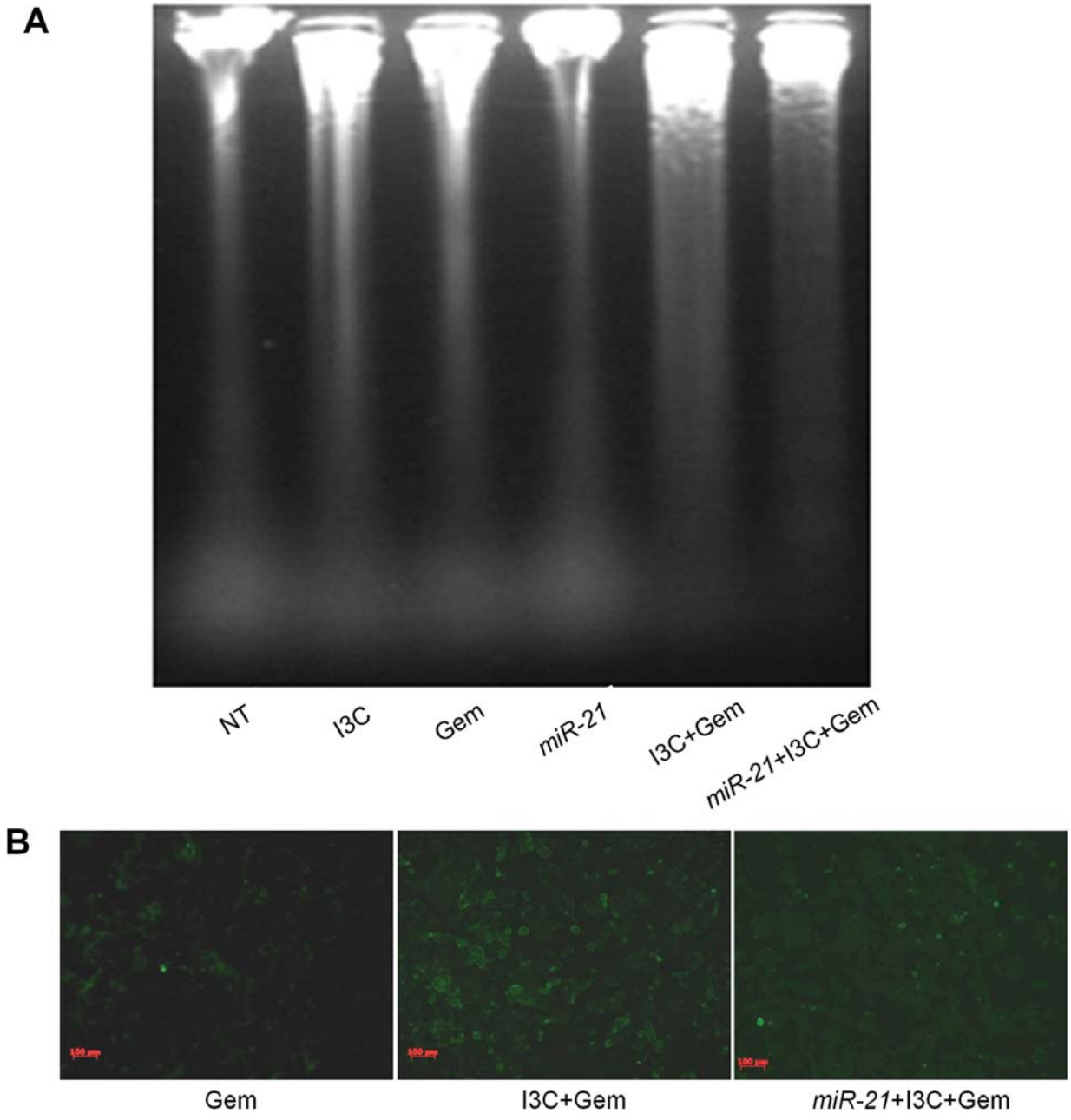

Figure 7. *DNA fragmentation assay (A) and TUNEL assay (B) showing apoptosis enhancement by gemcitabine (gem) and indole-3-carbinol (I3C) combination treatment. I3C down-regulates miR-21 and eventually increases the expression of programmed cell death-4 (PDCD4), which induces apoptosis. Inhibition of apoptosis after miR-21 transfection is clear. The green color in the TUNEL assay indicates apoptotic cells. NT, No treatment.*

cancer, and anti-miR-21 therapy enhances the chemosensitivity of pancreatic cancer cells (10). We found that miR-21 is a substantial target for the treatment of pancreatic cancer.

However, evaluation of various downstream targets of miR-21 still needs to be carried out, and *in vivo* studies demonstrating the efficacy of I3C and gemcitabine combination therapy in pancreatic cancer are still required.

In conclusion, this study showed that targeting miR-21 by I3C might have a role as a new approach to treatment of pancreatic cancer. The down-regulation of miR-21 by I3C leads to apoptosis of Panc-1 human pancreatic cancer cells through an increase of the levels of PDCD4 expression. We suggest that I3C may be a promising molecule for the treatment of pancreatic cancer.

Acknowledgements

This study was supported by grants from Korea Healthcare Technology R&D Project, Ministry of Health and Welfare, Korea (A110597) and from No. 03-2009-006 from the SNUBH Research Fund.

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Received January 21, 2013
Revised March 3, 2013
Accepted March 4, 2013