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Anti-Proliferative Activity of Nodosin, a Diterpenoid from *Isodon serra*, via Regulation of Wnt/β-Catenin Signaling Pathways in Human Colon Cancer Cells

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Abstract

Colorectal cancer (CRC) is one of the most malignant type of cancers and its incidence is steadily increasing, due to life style factors that include western diet. Abnormal activation of canonical Wnt/β-catenin signaling pathway plays an important role in colorectal carcinogenesis. Therefore, targeting Wnt/β-catenin signaling has been considered a crucial strategy in the discovery of small molecules for CRC. In the present study, we found that Nodosin, an *ent*-kaurene diterpenoid isolated from *Isodon serra*, effectively inhibits the proliferation of human colon cancer HCT116 cells. Mechanistically, Nodosin effectively inhibited the over-activated transcriptional activity of β-catenin/T-cell factor (TCF) determined by Wnt/β-catenin reporter gene assay in HEK293 and HCT116 cells. The expression of Wnt/β-catenin target genes such as Axin2, cyclin D1, and survivin were also suppressed by Nodosin in HCT116 cells. Further study revealed that a longer exposure of Nodosin induced the G_2/M phase cell cycle arrest and subsequently apoptosis in HCT116 cells. These findings suggest that the anti-proliferative activity of Nodosin in colorectal cancer cells might in part be associated with the regulation of Wnt/β-catenin signaling pathway.

Key Words: Nodosin, *Isodon serra*, Wnt/β-catenin signaling pathway, Anti-proliferative activity, Apoptosis, Colon cancer cells

INTRODUCTION

During the last decades, the incidence and mortality of colorectal cancer (CRC) have gradually increased and among all cancers worldwide, CRC is now ranked third (Maharjan *et al*., 2019; Siegel *et al*., 2019). However, current chemotherapies for CRCs, such as 5-fluorouracil and irinotecan have shown the limitation of a relative lack of selectivity against cancer cells compared to normal cells, and the occurrence of acquired drug resistance after repeated exposures of the drug to patients (Yao *et al*., 2017). Therefore, there is still the urgently necessity to discover novel small molecules for CRCs.

The aberrant activation of canonical Wnt/β-catenin signaling pathways is frequently found in CRC patients with a relatively poorer clinical outcome (Fodde *et al*., 2001; Yoshida *et al*., 2015). The Wnt/β-catenin signaling is an evolutionarily conserved pathway that regulates critical biological processes such as cell fate determination, migration, polarity, and organogenesis (Reya and Clevers, 2005; Komiya and Habas, 2008). In the absence of the Wnt ligand, Axin (Lee *et al*., 2003), glycogen synthase 3β (GSK-3β), and adenomatous polyposis coli (APC) form destruction complex to induce phosphorylation of β-catenin (Behrens *et al*., 1998) and β-catenin undergoes proteasomal degradation by the β-TRCP and E3 ubiquitin ligase subunit (He *et al*., 2004). This event subsequently hinders the translocation of β-catenin into nucleus and further its binding with T cell factor/lymphoid enhancer factor (TCF/LEF) family of proteins which blocks the capacity of the transcriptional activity (Tolwinski and Wieschaus, 2004; MacDonald *et al*., 2009). However, when Wnt proteins bind to a receptor complex consisting of a member of the Frizzled family, and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) (Hua *et al*., 2018), this results in the subsequent recruitment and phosphorylation of the Dishevelled protein (DVL). This in turn induces the translocation of β-catenin into nucleus and initiates the transcription of its downstream tar-

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get genes (Kobayashi *et al*., 2000; Komiya and Habas, 2008). Defects of the Wnt signaling components such as mutations of APC, Axin, or β-catenin itself result in β-catenin stabilization (Yang *et al*., 2016), and thus these events are known to contribute to carcinogenesis of many types of cancers, especially colorectal cancer (Pai *et al*., 2017; Schatoff *et al*., 2017). In view of this, the discovery of compounds that specifically target the Wnt signaling pathway is considered to be a strategy to treat colorectal cancers.

Isodon serra (Labiatae) has been known as a traditional Chinese medicine for the treatment of toxification and inflammation (Sun *et al*., 2006). *Isodon serra* has also been used for diseases such as cholecystis and the protection of liver disorder (Wang *et al*., 2012; Chen *et al*., 2014). Phytochemical study revealed that Nodosin is one of the major components in the aerial parts of *I. serra* (Yan *et al*., 2008). Previous studies for the biological activities of Nodosin exhibited anti-inflammatory (Li *et al*., 2010), anti-melanogenesis (Satooka *et al*., 2012), and immunosuppressive effects (Zhang *et al*., 2005). Nodosin was also reported with the perfusion protection of liver by induction of the cytoprotective enzyme heme oxygenase-1 *in vivo* (Wang *et al*., 2012).

In our continuous study to explore the anticancer agents from natural products, the extract of *Isodon serra* was found to exhibit a potential growth-inhibitory activity against human cancer cell lines. Although the extract and its isolated compounds have previously exerted several pharmacological effects including anti-inflammatory activity, the anti-proliferation and its underlying mechanisms of actions in cancer cells have not been reported yet. In the present study, we found that Nodosin, a major component of the plant, exhibited a potent anti-proliferative activity in a panel of cancer cell lines. In particular, the human colorectal cancer cell HCT116 was the most sensitive to Nodosin among the tested cell lines. Since the aberrant activation of canonical Wnt/β-catenin signaling pathway was highly associated with colorectal cancer cells, therefore, we employed the HCT116 colon cancer cells (β-catenin mutated cell line) for elucidation of the anti-proliferative activity and its underlying molecular mechanisms of actions with Nodosin in the anti-proliferative activity of the cancer cells.

MATERIALS AND METHODS

Reagents and chemicals

Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotic/antimycotic solution, TRI reagent, Lipofectamine 2000 and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), sulforhodamine B (SRB), trichloroacetic acid (TCA), bovine serum albumin (BSA), isoprophanol and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The luciferase reporter plasmids (TOPflash and FOPflash), Renilla luciferase reporter vectors, and pcCDNA β-catenin- and TCF4-expression vectors were obtained from Upstate Biotechnology (Lake Placid, NY, USA). The Reverse Transcription Kit was purchased from Toyobo (Osaka, Japan). The Dual Luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA). The iQ SYBR Green Supermix was obtained from Bio-Rad Laboratories (Hercules, CA, USA). For real-time PCR, the gene-specific primers were designed by Roche (Basel, Switzerland) and synthesized by Bioneer (Dajeon, Korea). The goat anti-rabbit IgG-HRP, anti-mouse IgG-HRP, anti-β actin, anti-TCF4, anti-cyclin B1, anti-cyclin A, anti-CDK2, anti-CDC2, anti-PARP, anti-p21 and anti-p27 were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). Anti-β-catenin, anti-survivin, anti-Axin1, anti-Axin2, anti-c-Myc, anti-cyclin D, anti-GSK-3β, anti-cleaved caspase3 and anti-cleaved caspase9 were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-cleaved poly (ADP-ribose) polymerase (PARP) and Annexin V-Fluorescein isothiocyanate (Annexin V-FITC) Apoptosis Detection Kit I were purchased from BD Bioscience (Franklin Lakes, NJ, USA).

Isolation of Nodosin from *Isodon serra*

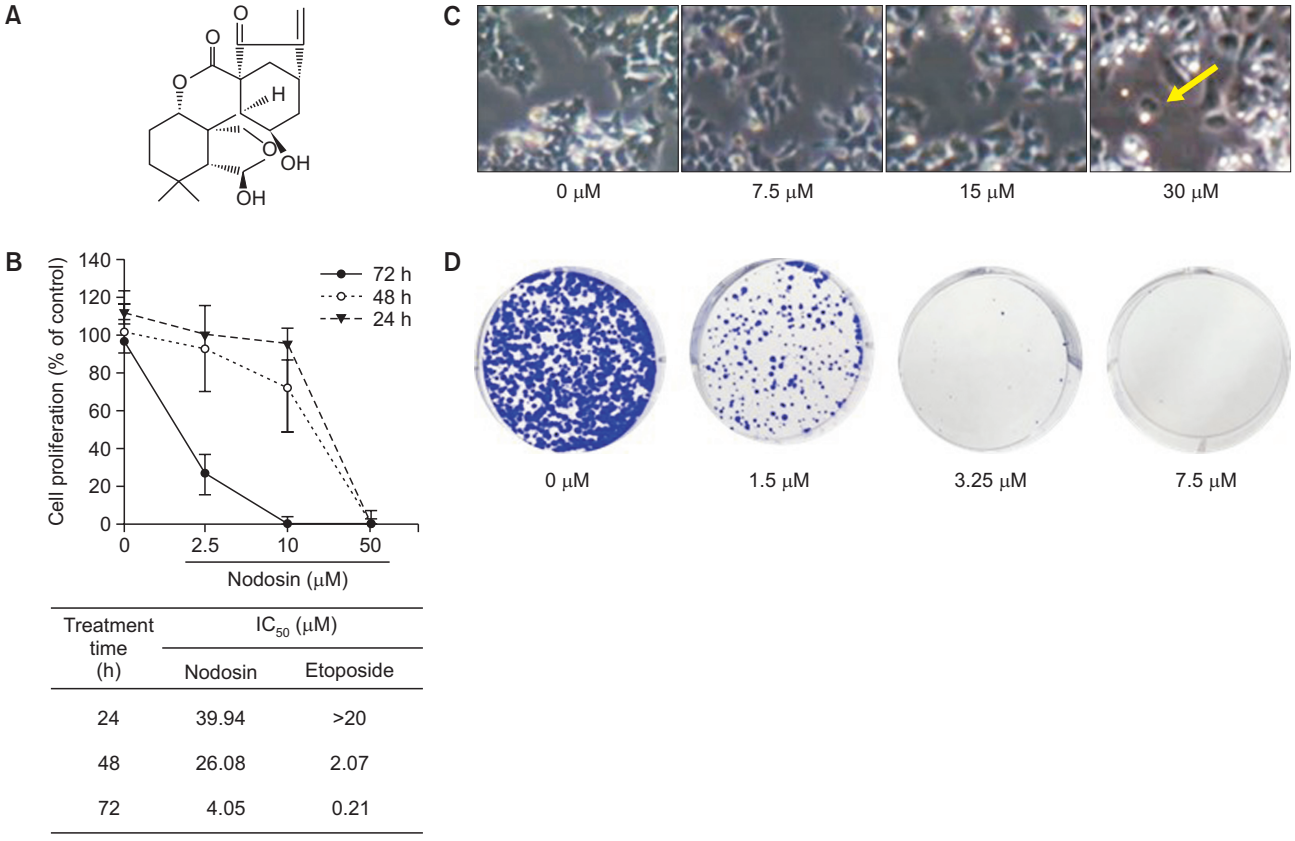
Dried whole plant materials (1,150 g) were pulverized and then extracted with MeOH (twice 10 L each) in room temperature (RT), yielding a crude extract of 123.2 g. The crude extract (119.8 g) was dissolved in deionized water (500 mL) and then partitioned with EtOAc (500 mL×2), furnishing 35 g of EtOAc soluble extract. A portion of the EtOAc-soluble extract (32 g) was subjected to silica gel column chromatography (620 g) eluted with a gradient mixture of chloroform and MeOH (100:0 to 0:100) and gave 11 subfractions (JaE1-JaE11). JaE3 (10.4 g) was subjected to silica gel column chromatography (200 g), eluting with a gradient mixture of chloroform and MeOH (40:1 to 0:1), and furnished 16 subfractions (JaE3-1 to JaE3-16). From JaE3-14, Nodosin (23.2 mg, Fig. 1A) was precipitated. The structure of Nodosin was confirmed by comparing the measured values with the published values (Yan *et al*., 2008).

Cell culture

Human embryonic kidney cells (HEK293), human normal lung fibroblast cell (MRC-5), human colorectal cancer cells (HCT116), human breast cancer cells (MDA-MB-231), human lung cancer cells (A549), human liver cancer cells (SK-HEP-1), and human gastric cancer cells (SNU-638) were purchased from the American Type Culture Collection (Manassas, VA, USA). HEK293, MRC5, MDA-MB-231 and SK-HEP-1 were maintained in DMEM, while HCT116, A549 and SNU-638 were cultured in RPMI-1640 media supplemented with 10% FBS and antibiotics-antimycotics (PSF: 100 units/mL sodium penicillin G, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B) (Thermo-Scientific, MA, USA) in a humidified incubator containing 5% CO_2 at 37°C.

Cell proliferation assay

Cells were seeded in 96-well plates and then treated with samples for 24, 48, or 72 h. After incubation, the cells were fixed with 10% TCA for 30 min, dried overnight, and stained with 0.4% SRB in 1% acetic acid for 2 h. The unbound dye was washed out using 1% acetic acid, after which the stained cells were dried and then were resuspended in 10 mM Tris (PH 10.0) (Kang *et al*., 2016). The absorbance value was measured at 515 nm, and the cell proliferation rates were determined using the following equation: cell proliferation (%)=(average absorbance$_{sample}$–average absorbance$_{day\ zero}$)/(average absorbance$_{control}$–average absorbance$_{day\ zero}$)×100 (Kim *et al*., 2018). The IC_{50} values were calculated using non-linear regression analysis using TableCurve 2D v5.01 software (Systat Software Inc., San Jose, CA, USA).



Treatment time (h)	IC_{50} (μM)	
	Nodosin	Etoposide
24	39.94	>20
48	26.08	2.07
72	4.05	0.21

Fig. 1. Anti-proliferative activity of Nodosin in HCT116 human colon cancer cells. (A) Chemical structure of Nodosin. (B) HCT116 cells were treated with the indicated concentrations of Nodosin for 24-72 h. The cell proliferation activity was determined by SRB assay as described in the Materials and Methods. Data are presented as the means ± SD (n=3). (C) The cells were treated with Nodosin for 24 h. Morphological changes were detected by phase-contrast microscopy (arrow indicates rounded shape of cells, 200×magnification). (D) The effect of Nodosin on colony formation was evaluated with the treatment of the indicated concentrations of Nodosin for 72 h, and then incubated for an additional 2 weeks. The colony formation was stained with crystal violet solution and photographed under inverted microscopy (40×magnification).

Transfection and luciferase reporter gene assays

Transient transfections were conducted using Lipofectamine 2000 (Invitrogen). HEK293 and HCT116 cells were seeded in 48-well plates, and then the cells were transfected with 0.1 μg of a luciferase reporter plasmid (TOPflash or FOPflash) and 0.005 μg of the Renilla luciferase vector for normalization. HEK293 cells were also co-transfected with 0.02 μg of the pcDNA β-catenin expression vector and 0.004 μg of the TCF4 expression vector to activate the Wnt pathway (Kang *et al*., 2012; Kim *et al*., 2017). After 24 h transfection, the cells were treated with a test compound. After incubation for an additional 24 h, the cells were lysed and were subjected to a dual luciferase activity assay using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. pTOP/FOPflash and Renilla reporter plasmids were obtained from Upstate Biotechnology. TCF4 and pc-β-catenin expression plasmids were gifted by Dr. Meeldijk of the University Medical Center (Kim *et al*., 2017). The levels of luciferase activity were measured and calculated relative to that of the vehicle control.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells using TRI reagent (Invitrogen). RNA extracts were reverse-transcribed using the Toyobo Reverse Transcription System (Toyobo). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The primer sequences were as follows: *CCND1* (sense: 5′-GAA GAT CGT CGC CAC CTG-3′/antisense: 5′-GAC CTC CTC GCA CTT CT-3′), *Axin2* (sense: 5′-CAA CAC CAG GCG GAA CGA A-3′/antisense: 5′-GCC CAA TAA GGA GTG TAA GGA CT-3′), *BIRC5* (sense: 5′-CCG ACG TTG CCC CCT GC-3′/ antisense: 5′-TCG ATG GCA CGG CGC AC-3′), *CTNNB1* (sense: 5′-GTC TTA CCT GGA CTC TGG AA-3′/antisense: 5′-GGT ATC CAC ATC CTC TTC CTC AG-3′), *ATCB* (sense: 5′-CCA ACC GCG AGA TGA-3′/ antisense: 5′-CCA GAG GCG TAC AGG GAT AG-3′).

Western blotting analysis

Total cell lysates were mixed with lysis buffer, and boiled for 10-20 min at 100°C. The protein concentrations of the cell lysates were determined by the BCA protein assay (Byun *et al*., 2019). Samples containing equal amounts of protein

Table 1. Anti-proliferative activity of Nodosin in a panel of human cancer cell lines

IC_{50} (μM)	SNU638	SK-HEP-1	A549	HCT116	MDA-MB-231	MRC-5
Nodosin	7.53	5.33	13.73	4.05	7.12	>20
Etoposide	0.39	0.77	0.25	0.21	7.1	>20

[a]Cancer cell lines: SNU638 (stomach), SK-HEP-1 (liver), A549 (lung), HCT116 (colon), MDA-MB-231 (breast), MRC-5 (normal lung epithelial cell); [b]Etoposide was used as a positive control.

were subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were electrically transferred to PVDF membranes (Millipore, Darmstadt, Germany) activated with 100% methanol. The membranes were blocked using 5% bovine serum albumin (BSA, Sigma-Aldrich) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 30 min at least at room temperature. The desired primary antibodies were diluted in 2.5% BSA (1:500-1:10,000) overnight at 4°C. The membranes were washed three times with PBST and were then incubated with the appropriate secondary antibodies diluted in TBST for 2 h at room temperature. The membranes were washed three times with PBST, and then were visualized using an enhanced chemiluminescence detection kit (Intron Biotechnology, Sungnam, Korea). The membranes were analyzed using an ImageQuant LAS 4000 imager (Fujifilm Corp., Tokyo, Japan).

Flow cytometry analysis of cell cycle distribution

The cells were seeded at a density of 2×10^6 cells in 100 mm cell culture dish. After 24 h incubation, the cells were treated with Nodosin and washed with PBS, and then the cell pellets were fixed with 70% ethanol overnight at –20°C. Fixed cells were pelleted and washed with PBS. The cells were resuspended in 100 μg/mL RNase A and 50 μg/mL of propidium iodide (PI) in the dark for 30 min at room temperature. The DNA content of the fluorescence binding cells was analyzed using a flow cytometry FACScalibur flowcytometer (BD Bioscience, Franklin Lakes, NJ, USA). The distribution of cell contents was measured with 5,000 cells in each group and the results were represented as histograms of the DNA content (Jung *et al*., 2015; Byun *et al*., 2018).

Annexin V-FITC/propidium iodide (PI) double staining

The cells were treated with Nodosin for 48 h and then stained with Annexin V-FITC and PI using an annexin V-FITC apoptosis detection kit (BD Bioscience) according to the manufacturer's instruction. Briefly, the incubated cells were harvested and resuspended with 1×binding buffer. Annexin V-FITC and PI (5 μL) were added to cell suspensions and further incubated in the dark for 15 min at room temperature. Stained cells were immediately analyzed with 1× binding buffer using a flow cytometer (Kim *et al*., 2016).

Statistical analysis

Data are presented as the mean values ± standard deviation for the indicated number of independently performed experiments. All data are representative of the results of at least three independent experiments. Statistical significance (*p<0.05, **p<0.01, ***p<0.001) was assessed using Student's *t*-test, or one-way analysis of variance coupled with Dunnett's *t*-test.

RESULTS

Effect of Nodosin on cancer cell proliferation

To evaluate whether Nodosin inhibits the growth of human cancer cells, a cell proliferation assay was conducted in a panel of cancer cell lines. Nodosin effectively inhibited the growth of various solid cancer cell lines. Among the cancer cell lines tested, the HCT116 colon cancer cells were shown to be the most sensitive in the growth-inhibitory activity of Nodosin with the IC_{50} values of 4.05 μM after 72 h incubation (Table 1). In addition, Nodosin did not significantly inhibit the growth of MRC5, a normal lung fibroblast cell line (IC_{50} value >20 μM), suggesting that Nodosin exhibits a relatively selective growth inhibition against cancer cells compared to a normal cell. We further extended to determine the growth inhibitory activity of Nodosin in the HCT116 cells after incubation of 24 h and 48 h treatment. Nodosin showed growth inhibition of the cells in a time- and concentration-dependent manner (Fig. 1B). The morphological change of the cancer cells treated with Nodosin was also observed under phase-contrast microscopy after 24 h treatment. The treatment of Nodosin (30 μM) evoked morphological changes with rounded shapes and the cells were floated in culture media (Fig. 1C). Moreover, the effects of Nodosin on colony formation were evaluated in the HCT116 cells. The cells were treated with Nodosin for 72 h, and then media were replaced two or three times per week for approximately 2 weeks. As shown in Fig. 1D, Nodosin significantly suppressed colony formation in the HCT116 cells. These data suggest that Nodosin effectively inhibits cancer cell proliferation and colony formation in human colorectal cancer cells.

Suppressive effect of Nodosin on the Wnt signaling pathways in HCT116 cells

To further confirm whether the growth-inhibitory activity of Nodosin against HCT116 cells is associated with the suppression of the Wnt signaling pathways, the effect of Nodosin on Wnt/β-catenin-mediated transcriptional activity was primarily evaluated. Using the T-cell factor (TCF) reporter gene (TOPflash), luciferase reporter gene assay was conducted in HCT116 cells and HEK293 cells. Nodosin significantly inhibited TOPflash activity in the TCF/β-catenin-stimulated luciferase activity in HEK293 cells (Fig. 2A). In addition, Nodosin also effectively suppressed overactivated TOPflash activity in the intrinsic β-catenin-mutated HCT116 colon cancer cells (Fig. 2B). We further detected the effect of Nodosin on the nuclear localization of β-catenin in HCT116 cells. As shown in Fig. 2C, immunocytochemical analysis revealed that overexpression of β-catenin in the nuclear fractions was significantly suppressed by the treatment of Nodosin (30 μM) in HCT116 cells. Real-time PCR analysis also suggested that the suppression of Wnt/β-catenin signaling by Nodosin is involved in the down-regulation of mRNA expression of Wnt target genes,

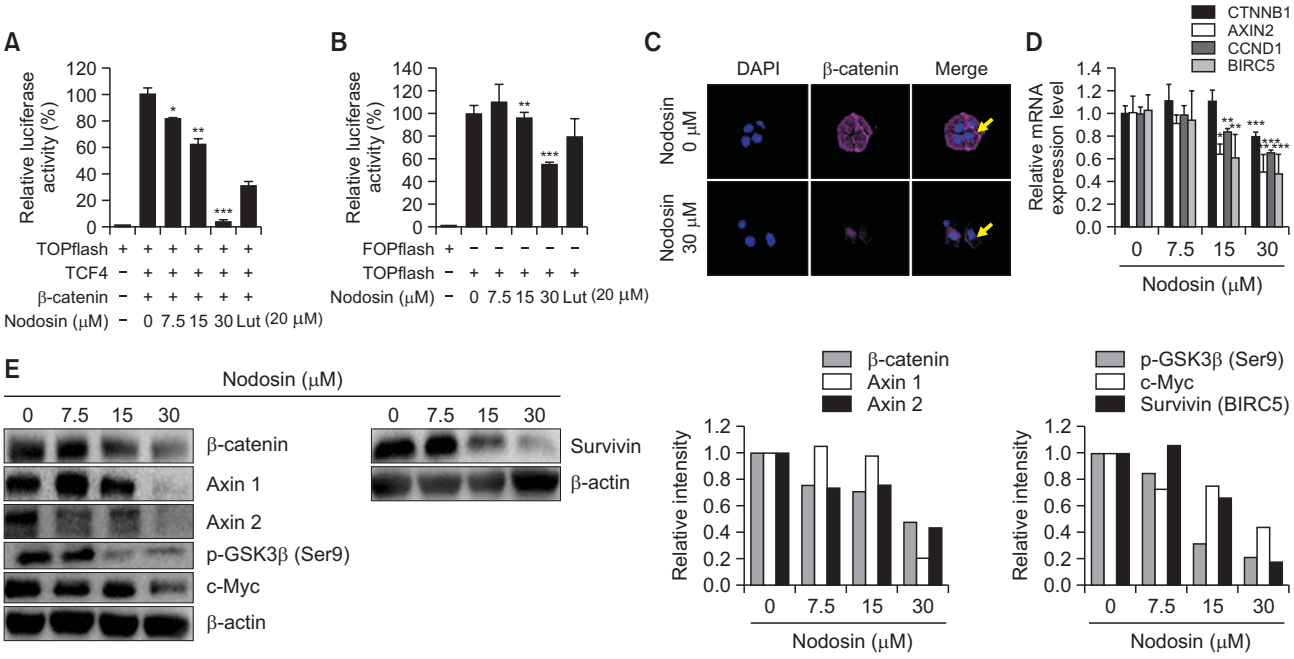


Fig. 2. Effects of Nodosin on the Wnt/β-catenin signaling pathways. (A) Luciferase activities were determined in HEK293 cells that were transiently transfected with TOPflash and TCF4-, β-catenin- expression vectors and a *Renilla* reporter gene vector for 24 h and then were treated with Nodosin for an additional 24 h. Luteolin (Lut, 20 μM) was used as a positive control. (B) Luciferase activities were determined in HCT116 cells that were transiently co-transfected with the TOPflash (or FOPflash) and a *Renilla* reporter gene vector for 24 h and then were treated with Nodosin for an additional 24 h. Luciferase activities were normalized with the *Renilla* values, and expressed as relative luciferase activity (%). Luteolin (Lut, 20 μM) was used as a positive control. (C) The effect of Nodosin on the nuclear level of β-catenin. HCT116 cells were treated with Nodosin for 24 h and then the cells were incubated with β-catenin antibody and detected by Alexa fluor 594 secondary antibody under confocal microscopy. (D) The mRNA expressions of the Wnt signaling target genes such as *CTNNB1*, *CCND1*, *BIRC5*, and *AXIN2* in HCT116 cells were determined by real-time PCR analysis after the treatment of Nodosin for 12 h. β-actin gene was used for normalization. (E) The protein expressions of the Wnt signaling pathways were determined by western blot analysis after the treatment of Nodosin for 18 h. β-actin was used as an internal standard. Data are expressed as the mean values ± SD (*n*=3) and are representative of three separate determinations (**p*<0.05, ***p*<0.01 or ****p*<0.001).

such as CCND1 (cyclin D1), AXIN2, BIRC5 (survivin), and CTNNB1 (β-catenin), which are key regulators of cancer cell proliferation, survival, and progression (Fig. 2D). Furthermore, Nodosin also significantly suppressed Wnt/β-catenin target protein expressions including β-catenin, p-GSK-3β, c-Myc, and Survivin in HCT116 cells (Fig. 2E). Collectively, these data indicated that the growth-inhibitory activity of Nodosin against colorectal cancer cells may be in part associated with the effective suppression of endogenously activated Wnt signaling pathways in HCT116 colorectal cancer cells.

Induction of G_2/M cell cycle arrest by Nodosin in HCT116 cells

The Wnt signaling pathway plays a pivotal role in cell proliferation and survival. To further clarify the involvement of anti-proliferative activity of Nodosin with the modulation of the Wnt signaling pathway, we primarily determined cell cycle distribution with the treatment of Nodosin in HCT116 cells. Flow cytometry analysis revealed that the treatment of Nodosin for 24 h in cancer cells significantly induced G_2/M phase cell cycle arrest in a concentration-dependent manner. In particular, the cell populations of G_2/M phase were manifestly increased from 13.87% in the vehicle-treated control cells, to 45.88% in the Nodosin (30 μM)-treated cells (Fig. 3A). The cell cycle is finely controlled by the regulation of cell cycle checkpoint proteins. In the G_2/M phase, the SCF E3 ubiquitin ligase consisting of S phase kinase-associated protein 1 (Skp1) and cullin-1 (Cul-1), and a member of the F-box protein, are actively involved in the cell cycle progression (Willems *et al*., 2004). Therefore, we determined whether Nodosin affected Skp2 in cancer cells. Nodosin effectively suppressed the expression of Skp2 protein levels in HCT116 cells. Further study revealed that additional checkpoint protein expressions in the G_2/M phase were also modulated by Nodosin: the cyclin-dependent kinase inhibitors p21 and p27 protein expressions were up-regulated, while the expressions of checkpoint proteins cyclin B1 and cdc25C were down-regulated (Fig. 3B). These data suggest that the anti-proliferative activity of Nodosin in cancer cells may in part be associated with cell cycle arrest followed by inhibition of the Wnt signaling pathway in colon cancer cells.

Induction of apoptosis by Nodosin in HCT116 cells

To further evaluate whether Nodosin is able to induce apoptosis for prolonged exposure in HCT116 cells, the cells were treated with Nodosin for 48 h. Flow cytometric analysis was performed after double staining with Annexin V-FITC/PI. As shown in Fig. 4A, the populations of apoptotic cells including early and late apoptosis were induced in a concentration-dependent manner. In particular, Nodosin significantly exhibited the population of apoptotic cell death with up to 61.3%

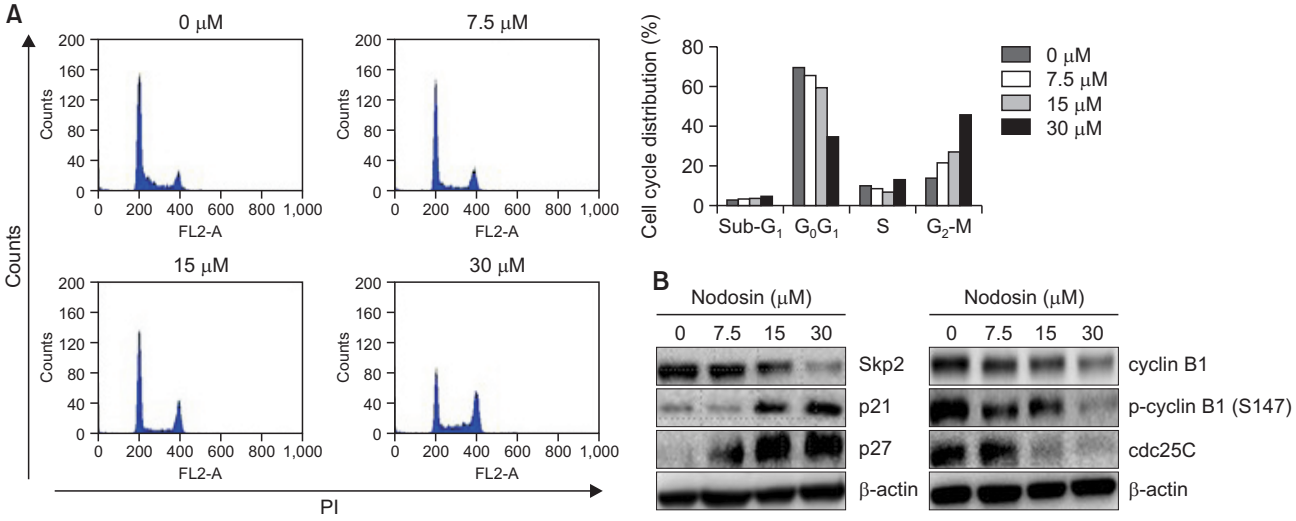


Fig. 3. The effects of Nodosin on the cell cycle distribution in HCT116 cells. (A) The cells were treated with the indicated concentrations of Nodosin for 24 h and then fixed with 70% ethanol for overnight. The cells were stained with PI, and then cell cycle distribution was determined by a flow cytometry. (B) The effect of Nodosin on the protein expressions associated with cell cycle checkpoint proteins. The cells were treated with Nodosin for 24 h, and then the levels of protein expressions were detected by western blot analysis. β-actin was used as an internal standard.

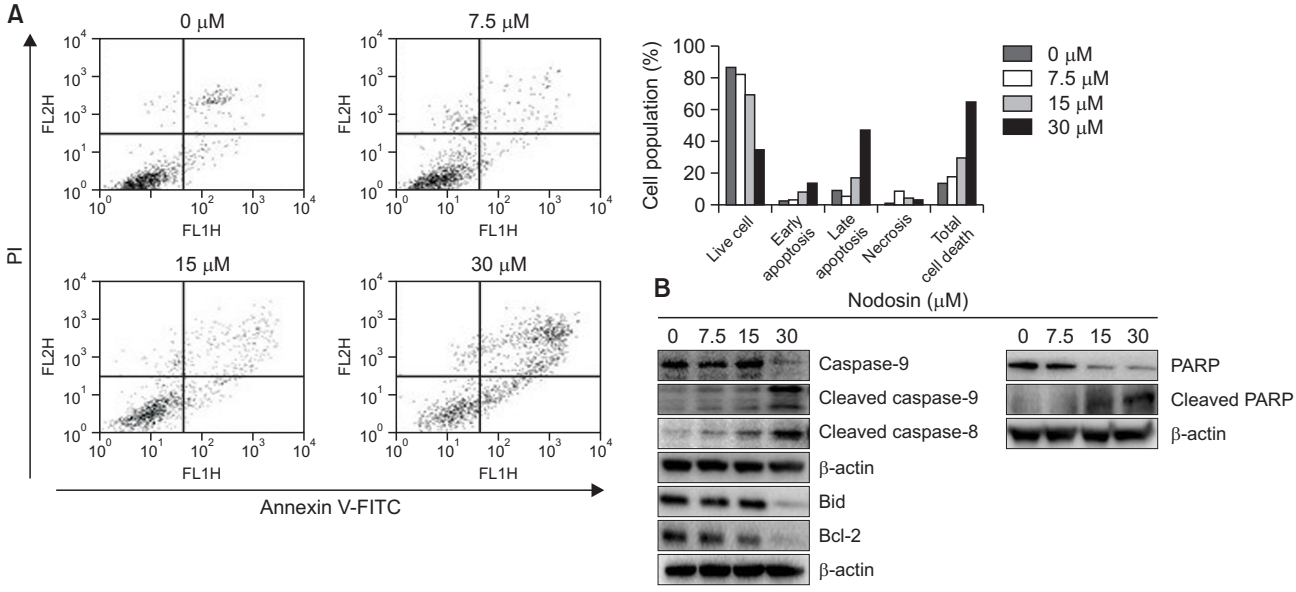


Fig. 4. The effect of Nodosin on the induction of apoptosis in HCT116 cells. (A) The cells were treated with the indicated concentrations of Nodosin for 48 h and then stained with Annexin V-FITC and PI solutions. The stained cells were then analyzed by flow cytometry, and cell populations are represented as histograms (%). (B) The effects of Nodosin on the expression of apoptosis-associated proteins in HCT116 cells. The cells were treated with the indicated concentrations of Nodosin for 48 h, and then the protein expressions were analyzed by western blotting. β-actin was used as an internal standard.

at the treatment of 30 μM Nodosin in HCT116 cells. In addition, the induction of apoptosis was in part correlated with the down-regulation of the anti-apoptotic proteins Bcl-2 and Bid expression, and up-regulation of pro-apoptotic proteins such as cleaved caspase-8, cleaved caspase-9 and cleaved PARP through the suppression of caspase-9 and PARP expression, respectively (Fig. 4B). These data suggest that Nodosin is able to induce apoptotic cell death via mitochondria-dependent pathway in HCT116 cells.

DISCUSSION

A body of evidence suggests that the constitutive activation of Wnt signaling is essential for the growth and survival of colorectal cancer cells (Zhan *et al*., 2017). Therefore, the

Wnt signaling pathway is considered an attractive target in the development of novel anticancer agents for treating colon cancers. In the present study, we explored whether the growth-inhibitory activity of Nodosin against colon cancer HCT116 cells is associated with the suppression of canonical Wnt/β-catenin signaling pathways in the cancer cells. Nodosin is an *ent*-kaurene diterpenoid isolated from the herb of *Isodon serra*, and a few of its biological activities such as anti-inflammatory (Li *et al*., 2010), anti-melanogenesis (Satooka *et al*., 2012), and immune-suppressive activity were reported (Zhang *et al*., 2005). The present study showed that Nodosin effectively inhibits the proliferation and colony formation of HCT116 colon cancer cells without affecting the growth-inhibitory activity against normal human lung fibroblast MRC-5 cells, indicating the selective anti-proliferative activity of Nodosin for cancer cells, compared to normal cells. Based on these findings, further study was designed to elucidate the involved mechanisms of actions for the growth-inhibitory activity of Nodosin in HCT116 colon cancer cells, a colon cancer cell line expressing β-catenin-mutation. Primarily, a luciferase reporter gene assay revealed that Nodosin significantly suppressed TCF/β-catenin-responsive transcriptional activity in HEK293 cells. In addition, the treatment of Nodosin also effectively inhibited the intrinsic overactivated transcriptional activity with a β-catenin mutation in HCT116 cells. The down-regulation of transcriptional activity by Nodosin subsequently suppressed the expressions of Wnt signaling components, such as β-catenin, axin, and GSK-3β. In particular, Nodosin significantly suppressed the expression of Axin2 mRNA and protein levels in a concentration-dependent manner. Although the function of Axin2 on regulation of Wnt/β-catenin signaling pathways is multifactorial, it is also involved in the regulation of cell cycle. Since Nodosin modulates Axin2, we extended investigation to evaluate the effect of Nodosin on cell cycle distribution in the cancer cells. Nodisin exhibited G_2/M phase cell cycle arrest and this event was in part correlated with the regulation of checkpoint proteins including Skp2, p21, and p27, in HCT116 cells. Further study revealed that the longer exposure of Nodosin in the cancer cells is able to induce apoptotic cell deaths, which is determined by the up-regulation of cleaved caspase-9 and cleaved PARP levels, which is the mitochondrial-mediated apoptosis pathway.

In summary, the present study for the first time demonstrates that Nodosin, a natural diterpenoid from *Isodon serra*, has the potential to inhibit the proliferation of colon cancer cells through effective suppression of the Wnt/β-catenin signaling pathways. Therefore, Nodosin might serve as a Wnt inhibitor for the development of anticancer agents targeting colon cancers with modulation of the Wnt/β-catenin signaling pathways.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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