Emodin inhibits the proliferation of PC3 prostate cancer cells *in vitro* via the Notch signaling pathway

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Abstract. The aim of the present study was to examine the anticarcinogenic effects of emodin on the Notch signaling pathway and vascular endothelial growth factor (VEGF) in the PC3 androgen-independent prostate cancer cell line *in vitro*. The cell viability was assessed using an 3-(4,5)-dime thylthiahiazo(-z-y1)-3,5-di-phenytetrazolium bromide assay. Cell apoptosis and cell cycle were detected using flow cytometry. Morphological alterations were observed using transmission electron microscopy. The mRNA and protein expression levels of Notch1, Jagged1, VEGF and bFGF were detected using reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Laser scanning confocal microscope (LSCM) immunofluorescence analysis was performed to detect the levels of expression and the sub-cellular localization of the Notch1 receptor protein. Growth of the PC3 cells was inhibited by emodin. Flow cytometry demonstrated that emodin induced apoptosis in the PC3 cells and arrested the cell cycle of the PC3 cells at the G2/M phase. The mRNA and protein expression of Notch1 in the PC3 cells was markedly increased, whereas the mRNA and protein expression levels of Jagged1, VEGF and bFGF were significantly decreased following exposure to emodin for 24 h. The LSCM assay revealed that the Notch1 was not only localized in the membrane and cytoplasm, but was also present in the nucleolus of the PC3 cells, and the expression of Notch1 in the nuclei gradually increased following treatment with emodin. These results demonstrated that emodin suppressed the growth of androgen-independent prostate cancer cell lines and induced apoptosis and cell cycle arrest. The Notch signaling pathway was activated in the PC3 cells following exposure to emodin, which suggested that the Notch signaling pathway is involved in the prostate tumor-suppressing mechanisms of emodin, the activation of which may depend on CBF1 protein in the nucleus by classic pathways. The antitumor function of emodin, attributed to the Notch signaling pathway, induced the downregulation of VEGF by suppressing tumorigenesis and angiogenesis, which indicated a novel mechanism underlying the emodin-mediated anti-prostate cancer effect.

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Introduction

Prostate carcinoma is the most common type of malignancy (28%) and the second leading cause of cancer-associate mortality (10%) in men in the United States. In the United States, ~238,590 new cases of prostate cancer were diagnosed and ~29,720 deaths were attributable to the disease in 2013 (1,2). This devastating disease has a significant impact on public health, however, current prostate cancer therapies, including surgery, chemotherapy and radiation therapy are of limited efficacy in progressive, recurrent and metastatic prostate cancer, particularly in hormone-refractory prostate cancer (HRPC) (3-6). In investigations of alternative and preventive therapies for prostate cancer, attention has focused on natural products. Plant-derived compounds have been an important source of several clinically useful anti-cancer agents (7). Emodin (1,2,8-trihydroxy-6-methylanthraquinone), a natural compound extracted from *Rheum palmatum* L. of the family Polygonaceae, has received significant attention for its potent anti-cancer activity. A number of studies have demonstrated that emodin can cause a marked decrease in cell proliferation and an increase in apoptosis in several types of cancer cells, including prostate cancer (LNCaP), lung cancer (A549, H460, CH27 and WI-38), acute myelogenous leukemia (HL-60), colorectal (WiDr) and pancreatic cancer (PANC-1) cells (6,8-12). The previous studies by Yu *et al* (6) and Cha *el al* (13) revealed that the emodin inhibits prostate cancer cell growth and downregulates the androgen receptor and p53-p21 pathways. Expression of the Notch receptor, which has been widely demonstrated to be responsible for cell fate determination during normal development, is implicated in human T-cell leukemia and mouse mammary carcinoma, and can significantly affect development of the prostate and cancer cell growth (14,15). The present study aimed to elucidate the potential molecular mechanisms of emodin-mediated inhibition of cell growth and apoptotic induction in PC3 prostate cancer cells, and the involvement of the Notch signaling pathway.

Materials and methods

Chemicals and reagents. Emodin was purchased from JRDUN biotechnology (Shanghai, China). The 3-(4,5)-dimet hylthiahiazo(-z-yl)-3,5-di-phenytetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), RNase and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA); M-MLV Reverse Transcriptase was obtained from Toboyo Co., Ltd. (Osaka, Japan); TaqplusDNA Polymerase, dNTP and TRIzol were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA); Goat anti-human polyclonal antibodies against Notch1 (sc-6014), Jagged1 (sc-6011), VEGF (sc-1836), bFGF (sc-1360), immunoglobulin (Ig)G-FITC and peroxidase-conjugated goat anti-human IgG (H+L) (sc-2012) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); All other chemicals were of reagent grade.

Cell culture. PC3 cells were obtained from the China Center for Type Culture Collection (Wuhan, China) and were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin in a humidified CO_2 incubator at 37˚C.

Cell viability assays. An MTT assay was used to assess the effect of emodin on PC3 cell viability. In brief, the PC3 cells were seeded in 96-well culture plates at a cell density of $5x10^4$ cells/ml and treated with emodin (10, 20, 40, 60 and 80 μg/ml) or remained untreated (control) for 12, 24, 36, 48, 72 and 96 h incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Subsequently, the cell viability was evaluated using an MTT assay (5 mg/ml MTT). The absorbance was measured at a test wavelength of 490 nm using a Tecan Sunrise Elisa-Reader (Tecan Group, Ltd., Mannedorf, Switzerland).

Assay to analyze cell cycle distribution. The PC3 cells were cultured in triplicate in 96-well plates at a density of $5x10^4$ cells/ml and treated with emodin (0, 20, 40, 60 and 80 μg/ml) or remained untreated (control) for 24 h. The cells were trypsinized, and washed three times with phosphate-buffered-saline (PBS), and then fixed in 75% ethanol overnight at 4˚C. The fixed cells were washed three times with PBS, incubated with 10 μl RNase for 30 min at 37˚C, and stained with 10 μl PI, followed by incubation for 30 min at 4˚C in the dark. Data acquisition and analysis were performed using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of apoptitic cells and cell cycle analysis were performed using ModFit LT software for Windows (Version V3.2; Verity Software House, Inc., Topsham, ME, USA).

Assessment of cell morphological changes. The PC3 cells were plated at a density of $4x10^5$ cells/well and treated with 60 μmol/l emodin for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2, washed with PBS, collected in an Eppendorf tube and fixed with 2.5% glutaraldehyde for 30 min. The PC3 cells were then washed with PBS and resuspended, and the ultrastructure of the PC3 cells was observed under a transmission electron microscope (JEM2000; JEOL, Ltd., Tokyo, Japan) at 100 kV.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The PC3 cells ($5x10^6$ cells/100-mm dish) were treated with emodin (10, 20, 40, 60 and 80 μg/ml) or remained untreated (control) for 24 h. The total RNA was isolated from the PC3 cells using TRIzol reagent, according to the manufacturer's instructions. Total RNA (2 μg) was converted into cDNA in a series of standard RT reactions using M-MLV Reverse Transcriptase. The resulting cDNA mixture (3 μl) was then used for enzymatic amplification. The primer sequences of Notch1, Jagged1, VEGF, bFGF and β-actin, and the thermal cycling conditions are shown in Table I. The reaction products were visualized by electrophoresis on a 1.2% agarose gel (Promega Corporation, Madison, WI, USA), containing ethidium bromide, followed by ultraviolet (UV) light illumination using a UVP Gel Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. The expression levels of Notch1, Jagged1, VEGF and bFGF apoptosis-associated proteins were detected using western blot analysis to clarify the mechanism underlying the induction of PC3 cell apoptosis by emodin, according to the manufacturer's instructions and the methods of a previous study (16). The PC3 cells ($5x10^6$ cells/100-mm dish) were treated with emodin (10, 20, 40, 60 and 80 μg/ml) for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2, and the protein extracts were prepared, as described by Schreiber *et al* (17). In brief, the cells were washed twice with cold PBS and lysed in radioimmunoprecipitation assay buffer (1X TBS, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1% MSF, 1% sodium orthovanadate and 1% protease inhibitor cocktail; Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions. The cell lysates were agitated for 1 h at 4˚C followed by a 15 min centrifugation at 10,000 x g. The protein concentrations were determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc.). For western blot analysis, the proteins were subjected to 12% SDS-PAGE at 60 V for 1 h and 120 V for 2 h. The proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.) at 30 mA for 1.5 h. The membranes were then blocked with 5% skimmed milk for 1 h and incubated with goat anti-human polyclonal anti-Notch1 (1:1,000), anti-Jagged1 (1:1,000), anti-VEGF (1:1,000) and anti-bFGF (1:1,000) antibodies. The membranes were washed three times in Tris-buffered saline with 0.2% Tween 20 (TBST) for 10 min and incubated with the appropriate peroxidase-conjugated secondary antibodies in TBS. Following another three washes with TBST for 10 min, the membranes were visualized using 3,3'-diaminobenzidine (Sigma-Aldrich), and were exposed to films (Kodak, Rochester, NY, USA). The protein levels were quantified by scanning densitometry using an Scanning Densitometry/Image Analysis System (Scion Corporation, Frederick, MD, USA).

Immunofluorescence and confocal microscopy analysis. The PC3 cells were maintained on glass coverslips in 24-well plates at a density of $1x10^5$ cells/well for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. Following treatment with emodin (0, 10, 20, 40, 60 and 80 μg/ml), the cells were fixed with ice-cold dehydrated ethanol/acetone. Subsequently, the cells were washed with cold PBS for 10 min and blocked with 5% normal goat serum for 30 min at room

Table I. Primers and thermal cycling conditions used for reverse transcription-quantitative polymerase chain reaction analysis.

Target gene	Primer sequence	Length (bp)	Thermal cycling conditions
Notch1	5'-GACATCACGGATCATATGGA-3' 5'-CTCGCA TTGACCA TTCAAAC-3'	666	95˚C 1 min, 60˚C 2 min, 72˚C 1.5 min, 34 cycles
Jaggedl	5'-AACTGGTACCGGTGCGAA-3' 5'-TGATGCAAGATCTCCCTGAAAC-3'	216	95˚C 1 min, 54˚C 1 min, 72˚C 1 min, 34 cycles
VEGF	5'-TCGGGCCTCCGAAACCATGA-3' 5'-CCTGGTGAGAGATCTGGTTC-3'	516	94˚C 30 sec, 55˚C 40 sec, 72˚C 1 min, 34 cycles
bFGF	5'-GGAGAAGAGCGACCCACA-3' 5'-CCAGTTCGTTTCAGTGCC-3'	234	94˚C 30 sec, 43˚C 30 sec, 72˚C 30 min, 30 cycles
β-actin	5'-TCTACAATGAGCTGCGTGTG-3' 5'-CAACTAAGTCATAGTCCGCC-3'	878	95˚C 1 min, 60˚C 2 min, 72˚C 1.5 min, 34 cycles

VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

temperature, followed by incubation with primary anti-Notch1 (1:100) overnight at 4˚C. The cells were washed again and then incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:100) for 1 h in the dark. Following several additional washing steps, the coverslips were mounted using Fluoromount-G™ mounting media with DAPI, and fluorescence was visualized using Leica confocal software (Leica Microsytems, Inc., Buffalo Grove, IL, USA).

Statistical analysis. The data are presented as the mean ± standard deviation (n=6). Differences between experimental groups were assessed using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of emodin on PC3 cell proliferation. The proliferation of the cells was determined using an MTT assay. As shown in Table II, emodin treatment inhibited the proliferation of the PC3 cells at 40, 60, 80 μmol/l significantly between 12 and 96 h ($P<0.05$). Treatment with emodin inhibited PC3 cell proliferation in a time- and dose-dependent manner.

Effects of emodin on PC3 cell cycle and apoptosis. To examine the mechanism responsible for cell proliferation inhibition, apoptosis and cell cycle distribution were evaluated using flow cytometry. As shown in Table III, a significant increase of apoptosis was observed at 24 h following treatment with different doses of emodin (0-60 μmol/l) in the PC3 cells. No significant effect on the proportion of cells in the G0/G1 phase was observed following treatment with different concentration of emodin (0-80 μmol/l) for 24 h, however, the proportion of the cells in the G2/M phase increased significantly in a dose-dependent manner, and the proportion of cells in the S phase decreased as the concentration of emodin increased. The cell cycle analysis results revealed G2/M phase arrest in the emodin-treated PC3 cells. Typical morphological features of apoptotic cell death, including cell shrinkage, membrane blebbing and nuclear fragmentation were also observed in the majority of the emodin-treated cells on analysis using transmission electron microscopy. A representative image containing these features is shown in Fig. 1.

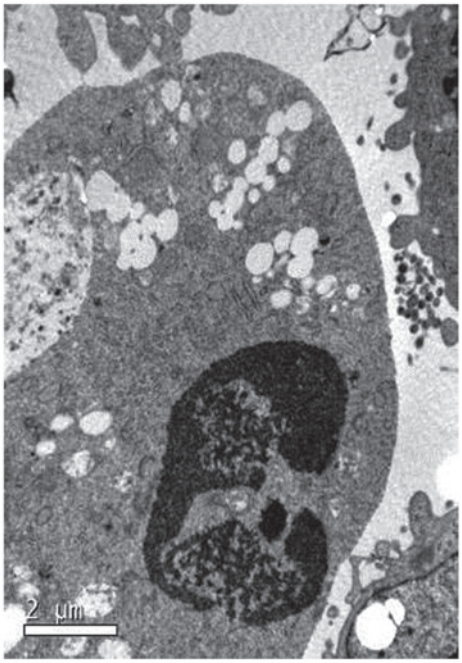

Figure 1. Ultrastructure of PC3 cells treated with emodin (60 μmol/l) for 24 h, observed using a transmission electron microscope (magnification, x10,000). Typical morphological features of apoptotic cell death were observed, with cell shrinkage, membrane blebbing and nuclear fragmentation.

Emodin increases the mRNA expression of Notch1 and decreases the mRNA expression levels of Jagged1, VEGF and bFGF in the PC3 cells. The mRNA expression levels of Notch1, Jagged1, VEGF and bFGF were examined using RT-qPCR to access the effect of emodin on the Notch pathway in PC3 cell apoptosis. As shown in Fig. 2, following treatment with different concentrations of emodin (10-80 μmol/l) for 24 h, the mRNA expression of Notch1 increased considerably, in a dose-dependent manner, in the PC3 cells. However,

Table II. Effects of emodin on PC3 cell proliferation.

	Time (h)					
Dose (μmol/l)	12	24	36	48	72	96
0	0.19±0.02	1.09±0.06	3.02±0.12	5.07±1.02	6.10±1.21	6.65±2.12
20	0.32±0.12	2.27±0.93	4.48±1.93	8.33±0.97	9.92±2.09	12.98±2.19
40	3.31±0.73[a]	6.13±1.42[a]	12.23±2.08[a]	16.88±3.12[a]	25.64±2.51[a]	38.42±2.94[a]
60	9.29±1.95[a]	17.64±1.23[a]	26.59±3.62[a]	39.25±3.87[a]	52.32±3.41[a]	63.02±5.12[a]
80	16.01±2.03[a]	25.21±1.59[a]	38.93±3.08[a]	56.77±2.80[a]	68.34±4.14[a]	79.13±3.34[a]

Data are expressed as the mean ± standard deviation (n=6). [a]P<0.05 and [b]P<0.01, compared with the control group.

Table III. Effects of emodin on PC3 cell cycle and apoptosis.

		PC3 cell cycle phase		
Dose (μmol/l)	Apoptosis (%)	G0/G1	S	G2/M
0	1.17±0.47	45.12±1.24	53.91±0.93	0.37±0.02
10	2.03±0.77	43.96±1.91	49.53±1.21	6.51±0.31[a]
20	5.32±1.97[a]	44.18±1.25	41.72±2.12[a]	14.1±1.01[a]
40	10.21±1.31[a]	41.01±1.93	36.42±1.83[a]	22.57±1.54[a]
60	21.15±1.54[a]	43.37±1.42	18.24±1.39[a]	38.39±1.91[a]
80	15.61±1.17[a]	42.71±1.76	11.63±1.69[a]	45.66±2.07[a]

Data are expressed as the mean ± standard deviation (n=6). [a]P<0.05 and [b]P<0.01, compared with the control group.

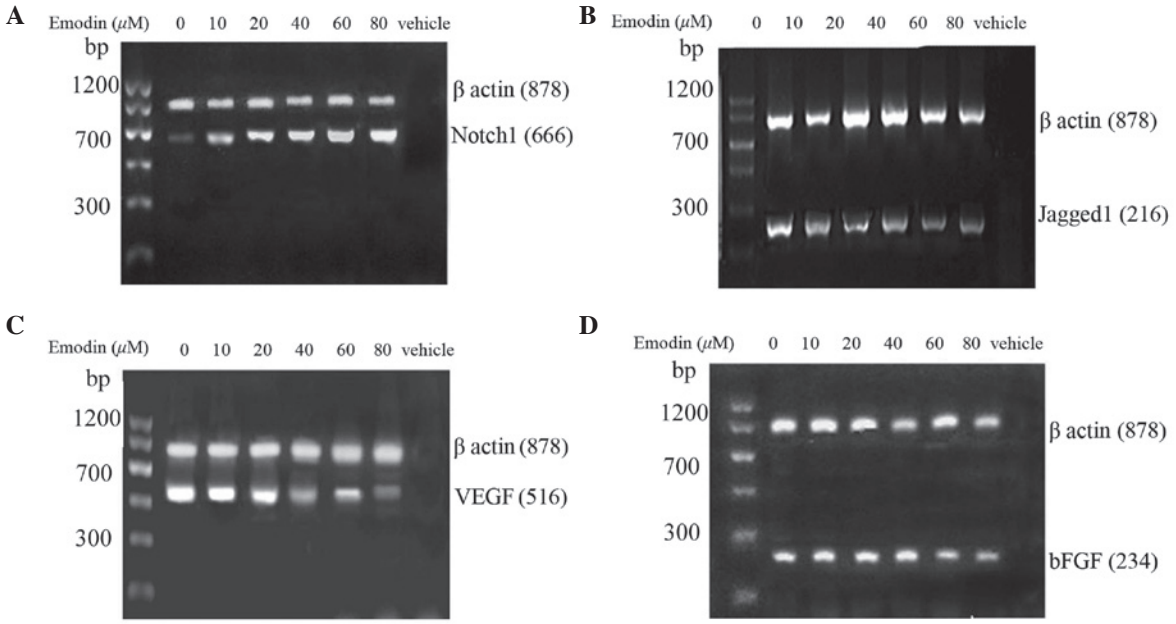

Figure 2. Effects of emodin on the mRNA expression levels of (A) Notch1, (B) Jagged1, (C) VEGF and (D) bFGF in the PC3 cells. Following treatment with or without emodin (0, 10, 20, 40, 60 and 80 μmol/l), the mRNA expression levels of Notch1, Jagged1, VEGF and bFGF in the PC3 cells were assessed using western blot analysis. β-actin was used to normalize the quantity of cDNA. VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

following treatment with emodin (10-80 μmol/l), the mRNA expression levels of Jagged1, VEGF and bFGF decreased significantly, compared with the control (0 μmol/l) group, which also occured in a dose-dependent manner. These results suggested that emodin may increase the mRNA expression of Notch1 and inhibit the mRNA expression levels

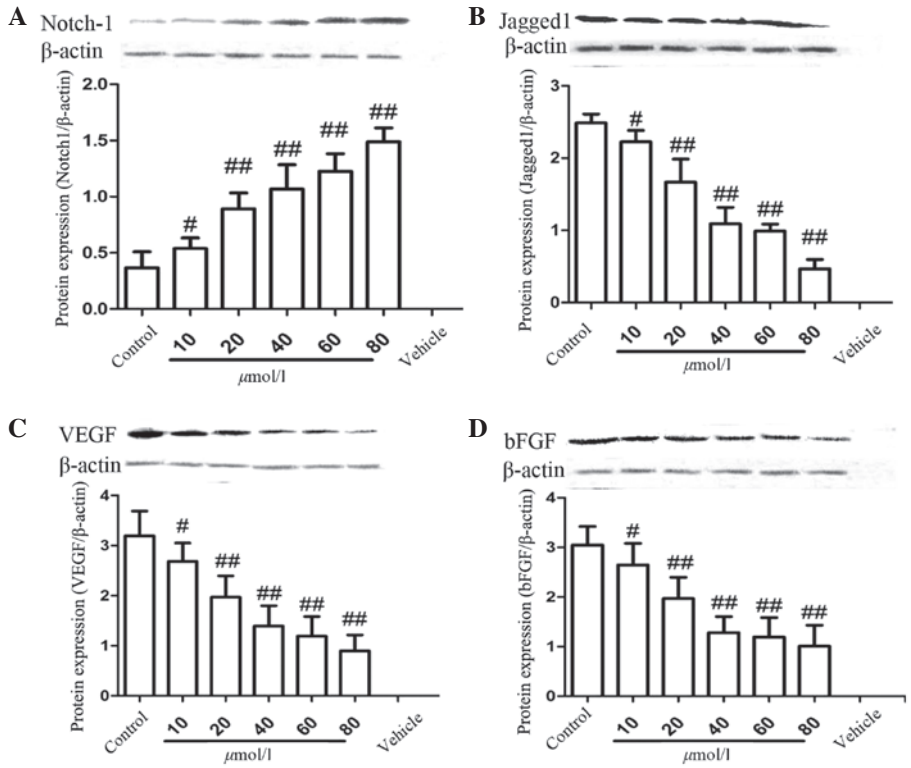

Figure 3. Effects of emodin on the protein expression levels of (A) Notch1, (B) Jagged1, (C) VEGF and (D) bFGF in the PC3 cells. Following treatment with or without (control) emodin (0, 10, 20, 40, 60 and 80 μmol/l), the protein expression levels of Notch1, Jagged1, VEGF and bFGF in PC3 cells were assessed using western blot analysis. The data are expressed as the mean ± standard deviation. $^{\#\#}$P<0.01 and $^{\#}$P<0.05, vs. control group. VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.

of Jagged1, VEGF and bFGF at the transcriptional level in emodin-induced PC3 cell apoptosis.

Emodin increases the protein expression of Notch1 and decreases protein expression levels of Jagged1, VEGF and bFGF in PC3 cells. The effect of emodin on the protein expression levels of Notch1, Jagged1, VEGF and bFGF in the PC3 cells were examined using western blot analysis. As shown in Fig. 3, treatment with emodin (10-80 μmol/l) for 24, led to a significant increase in the protein expression of Notch1 and a significant decrease in the protein expression levels of Jagged1, VEGF and bFGF. The increasing mRNA and protein expression levels of Notch1 and the decreasing mRNA and protein expression levels of Jagged1, VEGF and bFGF indicated that the Notch signaling pathway is involved in emodin-suppressing prostate tumor mechanisms.

Immunofluorescence and confocal microscopic analysis of Notch1 subcellular localization. The effects of emodin on the expression and subcellular localization of Notch1 receptor were examined using immunofluorescence and confocal microscopy. The confocal microscopy revealed pseudopodal shrinkage, cells turned round with poor adherence, clustering and apoptosis in the PC3 cells following treatment with emodin (0-80 μmol/l) for 24 h (Fig. 4). The protein expression of Notch1 was detected in all the groups of PC3 cells, however, the fluorescent intensity increased gradually following treatment with emodin for 24 h, which occurred in a dose-dependent manner. The fluorescence intensities of the PC3 cells treated with emodin at 0, 10, 20, 40, 60 and 80 μmol/l, were 8.73±2.54, 15.31±3.47, 22.64±4.34, 66.32±12.92, 112.64±16.26 and 163.21±19.18, respectively. Treatment with emodin (10-80 μmol/l) increased the fluorescence intensity significantly, compared with the control group (P<0.05). The intracellular location assay revealed mottled expression of Notch1 in the nucleus in addition to the cytoplasm and cell membrane. Notch1 localization was gradually increased in the nuclear membrane and nucleus at emodin concentrations >40 μmol/l (Fig. 4). It has been suggested that nuclear translocation or activation of Notch1 is occurring when nuclear fluorescence intensity is increased (18).

Discussion

Emodin is a natural anthraquinone derivative, isolated from *Rheum palmatum* L (19). Pharmacological studies have demonstrated that emodin has antitumor, antibacterial, diuretic and vasorelaxant effects, however, its antitumor activity has received more attention (20-23). The curative effects and mechanisms of emodin in prostate cancer cells have been previously investigated to a certain extent, and include the inhibition of proliferation and induction of apoptosis (24), regulation of the cell cycle (25), enhanced cytotoxicity of chemotherapeutic drugs in cells (26), suppression of angiogenesis and metastasis (27) and downregulation of androgen receptors (13). However, the molecular mechanisms underlying the induction of apoptosis by emodin in prostate cancer cells remain to be fully elucidated.

Notch-mediated cell-cell interaction and signaling are important for stem cell maintenance, cell fate determination, cell proliferation, differentiation and apoptosis in a variety of

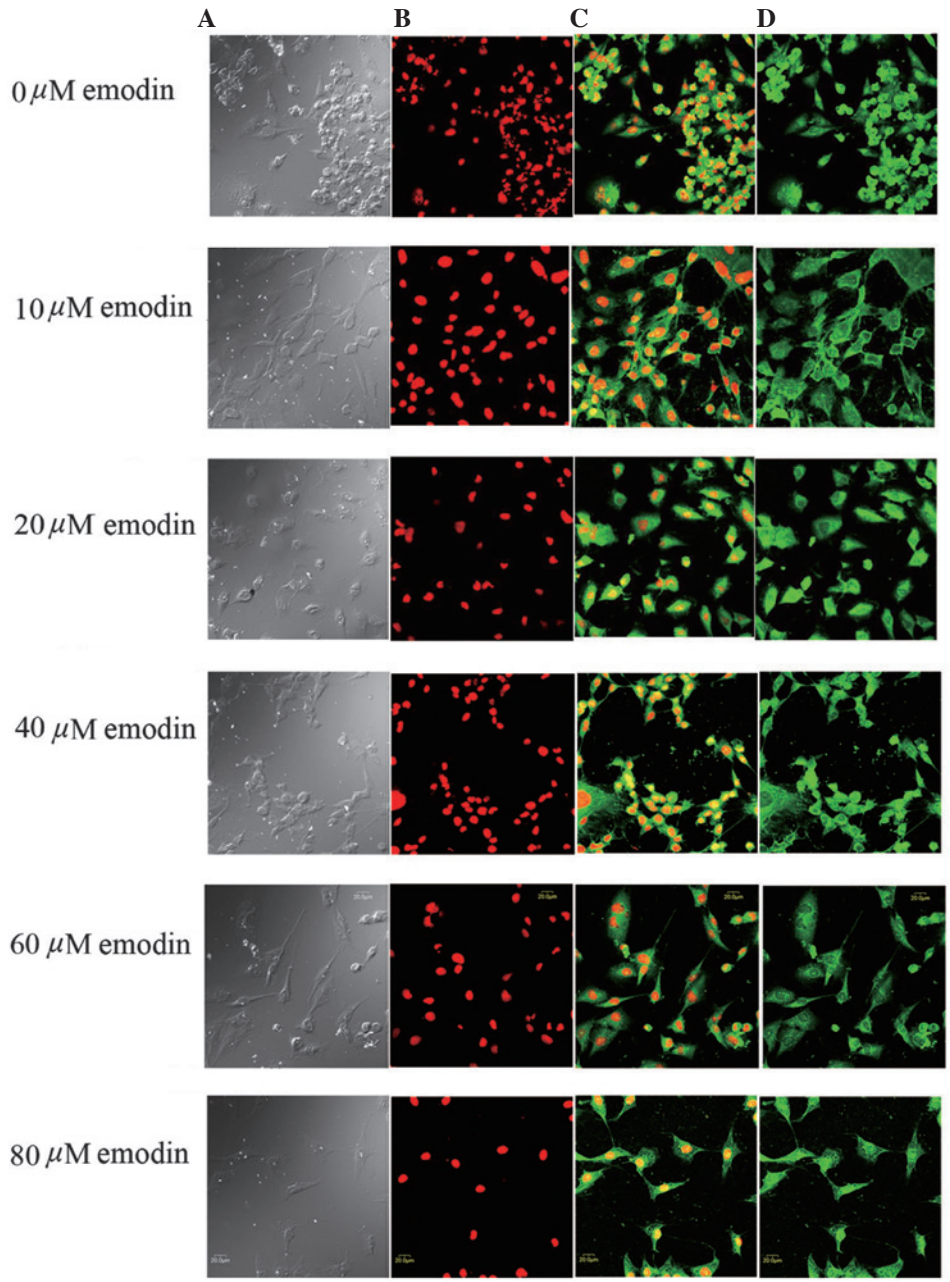

Figure 4. Expression and subcellular localization of the Notch1 receptor in PC3 cells treated with emodin (magnification, x200). Following treatment with or without emodin (0, 10, 20, 40, 60 and 80 μmol/l), the expression and subcellular localization of Notch1 receptor in the PC3 cells were assessed using immunofluorescence and confocal microscopy. (A) Bright fields; (B) red fluorescent staining (DAPI) (C) merged image (of B and D); (D) green fluorescent staining (anti-Notch1 receptor).

tissues (28). Cross talk between Notch and nuclear factor-κB (NF-κB) has been extensively investigated, and has been found to have important roles in tissue development and disease progression (29,30). It has been revealed that the gene expression of Notch1 and its effector, Hey-1, is significantly downregulated in human prostate adenocarcinoma tissue, compared with normal prostate tissue, and constitutively over-expressed active Notch1 can inhibit the proliferation of various prostate cancer cell lines (31,32). The results of the present study suggested that emodin upregulated the expression of Notch1, and downregulated the expression levels of Jagged1, VEGF and bFGF, which may have contributed to the induction of proliferation inhibition and apoptosis in the PC3 cells.

The basic reason for the malignant proliferation of tumor cells is cell cycle imbalance. Notch1 inhibits the expression of antiapoptotic proteins and cell proliferation-associated proteins, including the downregulation of cyclin D1 and Bcl-2, and the upregulation of p21Cip1 and p27Kip1, which induce cell cycle arrest and apoptosis (33). In the present study, the results demonstrated that upregulated expression of Notch1 suppressed proliferation of the PC3 cells by inducing G2/M arrest and inducing apoptosis in a time- and dose-dependent manner. VEGF and bFGF are the most important factors in the promotion of endothelial angiogenesis and differentiation, and can be found in several types of solid tumor, including mammary cancer, hepatocarcinoma, gastric carcinoma, rectal cancer and prostate cancer (34). Previous evidence indicates that Notch signaling is important in determining the way in which an endothelial cell responds to VEGF (35). The results of the present study suggested that the Notch pathway

is involved in downregulating the expression levels of VEGF and bFGF, which may contribute to the inhibition of prostate cancer angiogenesis and metastasis by emodin.

The present study demonstrated that the Notch pathway was involved in the inhibition proliferation by emodin on PC3 cells. Regarding the localization of Notch1, as the emodin concentration increased, the distribution in the nuclear membrane and nucleus was gradually strengthened, which indicated the nuclear translocation or activation of Notch1.

In conclusion, emodin significantly inhibited PC3 human prostate cancer cell growth and induced apoptosis, in which the Notch1 and Jagged1-mediated classical pathway was important. Emodin may be a prospective candidate drug for HRPC and merits further investigation.

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