# Inhibition of human colorectal cancer metastasis by notoginsenoside R1, an important compound from *Panax notoginseng*

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**Abstract.** *Panax notoginseng* (*P. notoginseng*) and its components are used as traditional Chinese medicine for cardiovascular disease, although studies concerning the anti-metastatic properties of these compounds are limited. The goal of this study was to investigate the effects of notoginsenoside R1 (NGR1), an important compound derived from *P. notoginseng*, on the metastasis of human colorectal cancer (CRC). The migratory, invasive, and adhesive abilities of cultured human CRC cells (HCT-116) treated with NGR1 and expression of metastasis-associated regulatory molecules were assessed. The migratory and invasive abilities of the HCT-116 cells were reduced after treatment with 75, 150 or 300 $\mu M$ NGR1 for 24 h. When HCT-116 cells were incubated with 150 or 300 $\mu$M NGR1 for 24 h, matrix metalloproteinase (MMP)-9 expression was reduced compared with that of the control group. In the adhesion reaction assays, treatment with 150 or 300 $\mu$M NGR1 led to significantly decreased adhesion of the HCT-116 cells to endothelial cells (EA.hy926 cells). Levels of integrin-1 protein were significantly decreased in the HCT-116 cells following treatment with 75, 150 or 300 $\mu$M NGR1, and levels of E-selectin and intercellular adhesion molecule 1 (ICAM-1) proteins were significantly decreased in the EA.hy926 cells treated with 75, 150 or 300 $\mu$M NGR1. Scanning electron microscopy examination indicated that HCT-116 cells treated with lipopolysaccharide (LPS) combined with 300 $\mu$M NGR1 exhibited a less flattened and retracted shape compared with cells treated with LPS alone, and this change in shape is characteristic of extravasation. Additionally, the transepithelial electrical resistance of the EA.hy926 endothelial cell monolayer increased after incubation with 150 or 300 $\mu$M NGR1 for 24 h. Overall, these results demonstrated the anti-metastatic properties of 150 or 300 $\mu$M NGR1, a compound that affects CRC metastasis by inhibiting cell migration, invasion, and adhesion and by regulating expression of metastasis-associated signalling molecules.

## Introduction

Human colorectal cancer (CRC) is one of the most common cancers and lethal malignancies in both Taiwan and worldwide. Approximately 1.4 million new cases of CRC are diagnosed annually, and approximately half of all CRC patients will develop metastatic cancer (1). Indeed, the major cause of death among CRC patients is metastasis (2). Thus, research concerning anti-metastatic agents is important for cancer prevention and therapy.

During metastasis, multistage progression and a series of regulatory events occur, with involvement of specific regulatory molecules. Major metastatic processes include migration and invasion, intravasation, circulation and adhesion, extravasation, and colonization (3). In the early stage of metastasis, migration and intravasation occur when the structure responsible for cell-cell contact, i.e., the extracellular matrix (ECM), is degraded by matrix metalloproteinases (MMPs) (4). After migration and intravasation, cancer cells leave the original tumor organ and enter the circulation. For extravasation to a metastatic organ, circulating cancer cells first need to adhere to endothelial cells, and adhesion molecules [e.g., integrin-1 in tumor cells and E-selectin and intracellular adhesion

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molecule 1 (ICAM-1) in endothelium cells] play a key role in regulating adhesion of tumor cells to endothelial cells (5,6). Thus, inhibition of cancer cell migration, invasion and adhesion and/or regulators of expression may be an effective strategy for suppressing CRC metastasis.

The root of *Panax notoginseng* (*P. notoginseng*) is used as a traditional Chinese medicine and functional food. Previous studies have shown that *P. notoginseng* suppressed liver metastasis when B16 melanoma cells were transplanted into the spleen of C57BL/6 mice (7). In addition, 20(S)-25-methoxyldammarane-3b, 12b, 20-triol (25-$OCH_3$-PPD), a ginsenoside from *P. notoginseng*, was active against the proliferation and migration of breast cancer cells via downregulation of mouse double minute 2 (MDM2) (8). Recently, we demonstrated that a *P. notoginseng* ethanol extract exerted a suppressive effect on metastasis by inhibiting migration, invasion and adhesion through alterations in the expression of associated regulatory molecules (9). *P. notoginseng* contains additional active ingredients, mainly consisting of dammarane-type saponins (10). The total saponin content of *P. notoginseng* is ~12%, with notoginsenoside, ginseng saponin and gynostemma glycosides being the three major types (10). Notoginsenoside R1 (NGR1), a unique major saponin of *P. notoginseng*, has been shown to effectively prevent cardiovascular disease, cerebrovascular disease, neurotoxicity and osteoporosis (11-14). Regarding cancer, some studies have demonstrated that NGR1 is beneficial for the prevention and treatment of colon cancer and leukaemia (15-17). However, the effects of NGR1 on CRC metastasis and the related regulatory mechanisms remain unclear.

The aims of this study were to investigate the possible anti-metastatic effects of NGR1 and to identify whether these effects are involved in regulating human CRC cell migration, invasion or adhesion. Wound healing and invasion assays and MMP-2 and MMP-9 expression analyses were performed using HCT-116 cells treated with NGR1. In addition, the effects of NGR1 on the adhesion of HCT-116 cells to EA.hy926 human endothelial cells were investigated, and the expression levels of integrin-1, E-selectin and ICAM-1 in EA.hy926 human endothelial cells were examined. To determine the effects of NGR1 on intravasation and extravasation, the cell shape and transepithelial electrical resistance (TEER) of EA.hy926 cells were evaluated. The results of this study will help to elucidate the effects of NGR1 on migration, invasion and adhesion of human CRC cells via regulation of metastasis-associated molecules.

## Materials and methods

*Reagents*. Notoginsenoside R1 (NGR1) (3β,6α,12β)-20-(β-D-glucopyranosyloxy)-3,12-dihydroxydammar-24-en-6-yl 2-*O*-β-D-xylopyranosyl-β-D-glucopyranoside) was purchased from Tianjin Zhongxin Pharmaceutical Co. (Tianjin, China). Antisera against integrin-1, E-selectin and ICAM-1 were purchased from Abcam (Cambridge, MA, USA). Antisera against MMP-2 and MMP-9 were obtained from GeneTex, Inc. (Hsinchu, Taiwan). In addition, 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was purchased from Life Technologies GmbH (Darmstadt, Germany). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

*Cell cultures and treatment*. The human CRC cell line HCT-116 was used as a model for CRC intravasation, migration and adhesion (18), and the human umbilical vein endothelial cell line EA.hy926 was used as an extravasation model for cancer cell adhesion to endothelial cells (19). HCT-116 and EA.hy926 cells were purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37˚C in a humidified 5% $CO_2$ atmosphere.

For various biochemical analyses, the cells were plated and incubated for 24 h before treatment with 75, 150, 300 or 500 $\mu$M NGR1 for the indicated times. NGR1 was diluted in ethanol, and cells treated with ethanol alone served as the control group.

*Cell viability analysis*. Cell viability was evaluated using the 3-(4,5-dimethyazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described by Denizot and Lang (20). HCT-116 cells were incubated in 3-cm plates ($1x10^6$ cells) for 24 h and treated with 75, 150, 300, or 500 $\mu$M NGR1 for 12, 24 or 48 h. MTT (5 mg/ml) was then added, and the optical density (OD) was measured at 570 nm.

*Wound healing assay*. To determine whether the migration of HCT-116 cells was suppressed by NGR1, a monolayer wound healing assay was conducted. The protocol for the wound healing assay was a modified version of that described by Ang *et al* (21). HCT-116 cells were cultured in 3-cm plates ($1x10^6$ cells) for 24 h, and a micropipette tip was then used to make a uniform scratch in the center of the monolayer. After washing with phosphate-buffered saline (PBS), 75, 150 or 300 $\mu$M NGR1 was added for 0, 12, 24, or 48 h. HCT-116 cell morphology was subsequently observed under an inverted fluorescence microscope (Olympus IX51 microscope; Olympus, Tokyo, Japan), and the width of the wound area was measured using ImageJ software to determine the cell migration distance (22).

*Invasion assay*. An invasion assay was performed to assess intravasation and extravasation abilities. Matrigel solution (50 $\mu$l) was added to the wells of a 24-well Transwell plate, which was then incubated at 37˚C for 30 min. In the upper chamber, HCT-116 cells were resuspended in serum-free RPMI-1640 medium ($5x10^4$ cells/3-cm plate) in the absence or presence of NGR1 (75, 150 or 300 $\mu$M). RPMI-1640 medium (500 $\mu$l) containing 10% FBS was added to the lower chamber. After incubation for 24 h, the invading cells that migrated to the lower surface of the filter membrane were stained with 0.2% crystal violet for 15 min. The number of invading cells on the lower surface of the membrane was determined under an inverted fluorescence microscope (Olympus IX51; Olympus) using NIH ImageJ software (22).

*Adhesion assay*. The adhesion assay protocol used was a modification of that described by Braut-Boucher *et al* (23). EA.hy926 cells ($1x10^6$ cells) were cultured in 3-cm plates and exposed to 75, 150 or 300 $\mu$M NGR1 for 6 h. The cells were then washed with PBS and co-cultured for 1 h with HCT-116 cells labelled with 10 $\mu$M BCECF. After washing with PBS, the morphology of the BCECF-stained HCT-116 cells was evaluated under an

inverted fluorescence microscope (Olympus IX51; Olympus). The BCECF-stained cells were collected and measured fluorometrically using an ELISA reader at an OD of 580 nm.

*Scanning electron microscopy (SEM) examination*. SEM was used to examine morphological changes in cell shape and to examine protruding surface structures (24). HCT-116 cells were treated with 1 $\mu$g/ml LPS, an invasion inducer (25), or 300 $\mu$M NGR1 combined with 1 $\mu$g/ml LPS for 24 h. The cells were then collected, washed with PBS, and fixed for 30 min in 10% glutaraldehyde solution (Sigma Chemical Co., St. Louis, MO, USA; 25%). Dehydration was achieved in a graded ethanol series (2x15 min in 50 vol% ethanol, 2x15 min in 70 vol% ethanol, 2x15 min in 80 vol% ethanol, 2x15 min in 90 vol% ethanol, 2x20 min, and 60 min overnight in absolute ethanol). After dehydration, the cells were prepared on gold substrates and platinum-coated conductive substrates (JEOL JFC-1600; Jeol, Ltd., Tokyo, Japan). SEM images were obtained using a JEOL JSM-7000F SEM microscope at an accelerating voltage of 5 kV.

*Measurement of TEER*. TEER is a quantitative measurement of the barrier integrity of a monolayer that is used to examine cell-cell integrity and permeability (26). For TEER measurements, EA.hy926 cells ($5x10^4$ cells/well) were cultured for 24 h in RPMI-1640 medium containing 75, 150 or 300 $\mu$M NGR1. TEER values were obtained by subtracting the TEER measurement for the cell culture dish groove from the measurement obtained in the presence of a cell layer. These measurements were collected using a Millicell-ERS voltohmmeter (Millipore-Continental Water Systems, Bedford, MA, USA).

*Analysis of expression of regulatory proteins involved in migration, adhesion and invasion*. Approximately $5x10^5$ HCT-116 cells/3-cm plate were used for MMP-2, MMP-9, and integrin-1 expression analyses; $5x10^5$ EA.hy926 cells/3-cm plate were used for E-selectin and ICAM-1 expression analyses. The cells were incubated in a 12-well plate with 75, 150 or 300 $\mu$M NGR1 for 24 h, washed twice in cold PBS and harvested using 200 $\mu$l of lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), and 20 $\mu$g/ml aprotinin at pH 7.4. The levels of cellular protein were determined following the method described by Lowry *et al* (27).

For each sample, 10-20 mg of cellular protein was applied to 10% sodium dodecyl sulphate (SDS) polyacrylamide gels (28). After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (29). The membranes were then incubated with an anti-MMP-2, anti-MMP-9, anti-integrin-1, anti-E-selectin or anti-ICAM-1 antibody at 37˚C for 1 h and subsequently with a peroxidase-conjugated secondary antibody. Bands were visualized using hydrogen peroxide/tetrahydrochloride diaminobenzidine or an enhanced chemiluminescence detection kit (Amersham Life Science, Buckinghamshire, UK) and quantified using an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA).

*Statistical analysis*. Data were analysed using the statistical analysis software SPSS for Windows, version 20.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were employed to evaluate the significance of differences between two mean values. A P-value of <0.05 or 0.01 was considered to indicate a statistically significant result.

## Results

*NGR1 suppresses the viability of human CRC cells*. According to the MTT assay results, HCT-116 cell viability after treatment with 75, 150 or 300 $\mu$M NGR1 did not differ significantly from that of the control during the 48-h incubation period (data not shown). However, the viability of cells treated with 500 $\mu$M NGR1 for 48 h was significantly reduced (58±7.26%) compared with the control cells (100%) ($p<0.05$). Therefore, we used an NGR1 concentration of 75, 150 or 300 $\mu$M for all subsequent experiments.

*NGR1 inhibits the migratory and invasive abilities of human CRC cells*. The effects of NGR1 on the migratory abilities are presented in Fig. 1. When HCT-116 cells were incubated with various concentrations of NGR1 for 12, 24 or 48 h, the wound healing areas varied as shown in Fig. 1A. The results showed that the HCT-116 cells treated with 75, 150 or 300 $\mu$M NGR1 for 24 or 48 h exhibited reduced wound closure rates compared with the control group (17-21 and 24-34%, respectively) (Fig. 1B). These results indicate that NGR1 significantly suppressed HCT-116 cell migration. The results of the Transwell Matrigel invasion assay indicated that when HCT-116 cells were incubated in various concentrations of NGR1 for 24 h (Fig. 1C), the level of HCT-116 cell invasion was significantly decreased by 64±8, 61±14 or 51±19% after treatment with 75, 150 or 300 $\mu$M NGR1 for 24 h, respectively, compared with the control group (100%) ($p<0.01$) (Fig. 1D). This result suggests that NGR1 inhibits the inter-colon migratory and invasive abilities of colon cancer cells.

The effects of NGR1 on the levels of MMP-2 and MMP-9 are presented in Fig. 2. Immunoblotting assays showing the expression of MMP-2 and MMP-9 in HCT-116 cells after NGR1 treatment are presented in Fig. 2A. NGR1 did not regulate MMP-2 expression in the HCT-116 cells (Fig. 2B). However, when HCT-116 cells were treated with 150 or 300 $\mu$M NGR1 for 24 h, the MMP-9 protein expression was significantly decreased by 35 and 68%, respectively, compared with the control group (100%) ($p<0.01$) (Fig. 2C). These results indicate that NGR1 regulates migration and intravasation by regulating MMP-9 expression.

*NGR1 reduces adhesion of human CRC cells*. Fluorescence microscopic examination revealed that treatment of EA.hy926 cells with various concentrations of NGR1 decreased the number of adherent cells detected after co-culture with the HCT-116 cells (Fig. 3A). After EA.hy926 cells were incubated with 150 or 300 $\mu$M NGR1 for 24 h (Fig. 3B), cell adhesion was significantly decreased by 18 to 20% in a dose-dependent manner. Thus, the adhesion levels of these cells were significantly reduced compared with that of the control group (100%) ($p<0.01$) after 24 h. These results demonstrated that NGR1 decreased the ability of HCT-116 cells to adhere to EA.hy926 cells and may be able to reduce cancer cell adhesion to a metastasis target organ.

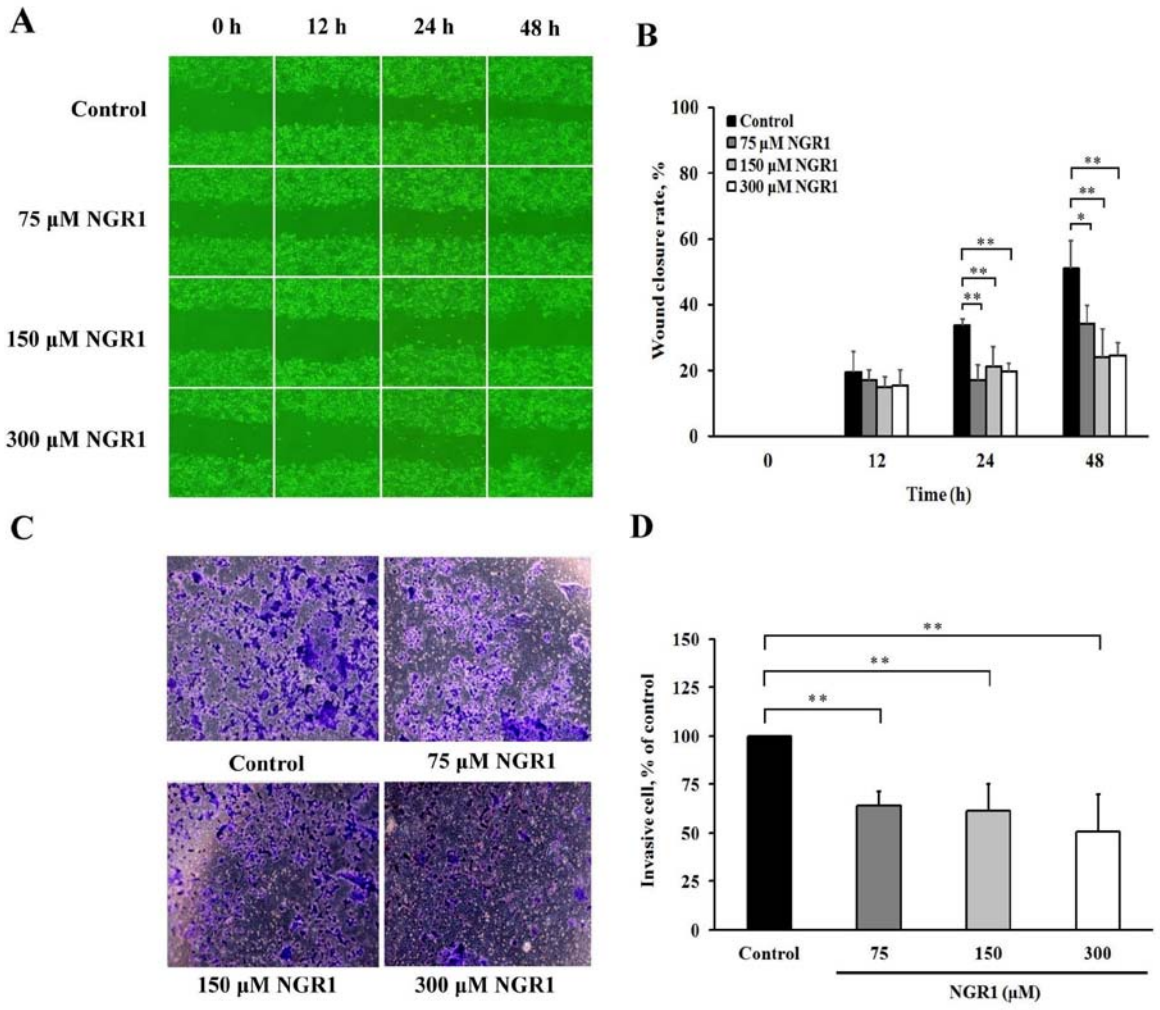


Figure 1. Effects of NGR1 on HCT-116 cell migration and invasion. HCT-116 cells ($5x10^5$ cells/3-cm plate) were treated with 75, 150, or 300 $\mu$M NGR1 for 12, 24 or 48 h. Ethanol-treated cells served as the control. (A) Effect of NGR1 on HCT-116 cell migration *ex vivo*. Movement of HCT-116 cells into the wound was demonstrated through a wound healing assay. (B) Quantitative analysis of the wound closure rate. (C) Effect of NGR1 treatment for 24 h on HCT-116 cell invasion through the Matrigel membrane from the upper chamber to the lower chamber. (D) Quantitative analysis of invasion rates. Values are presented as the means ± SD (n=3). $^{*}$P<0.05 or $^{**}$P<0.01 indicates a significant difference. NGR1, notoginsenoside R1.

The protein levels of integrin-1 in HCT-116 cells, and E-selectin and ICAM-1 in the EA.hy926 cells were analysed (Fig. 4). The expression levels of integrin-1, E-selectin and ICAM-1 are presented in Fig. 4A. Treatment of HCT-116 cells with 75, 150 or 300 $\mu$M NGR1 for 24 h significantly decreased integrin-1 protein levels by 10 to 27% compared with the control group (100%) (Fig. 4B). As shown in Fig. 4C, after treatment with 75, 150 or 300 $\mu$M NGR1 for 24 h, the levels of E-selectin protein in the EA.hy926 cells were 85±5, 78±7 and 78±4%, respectively, compared with the control values (100%); and these values were significantly lower than levels in the control group ($p<0.01$). The levels of ICAM-1 protein in cells incubated with 75, 150 or 300 $\mu$M NGR1 were significantly reduced (86±3, 68±3 and 34±2%, respectively) (Fig. 4D) compared with these levels in control cells (100%). These results indicate that expression of these adhesion molecules was suppressed by NGR1 and NGR1 may exert an important anti-adhesion effect during metastasis.

*NGR1 inhibits extravasation of human CRC cells.* To investigate the effects of NGR1 on extravasation, changes in HCT-116 cell shape were examined using SEM technology. The LPS-treated HCT-116 cells exhibited a flattened and partially collapsed shape compared with the shape of the control cells, whereas HCT-116 cells treated with LPS combined with 300 $\mu$M NGR1 exhibited a less flattened shape compared with the cells treated with LPS alone (Fig. 5A). The effect of NGR1 on the TEER of EA.hy926 cells was also examined. After treatment with 75, 150 or 300 $\mu$M NGR1, the TEER values were 113±3, 128±14 and 134±16%, respectively (Fig. 5B), representing significant increases compared with the control group (100%) ($p<0.05$). This result shows that NGR1 may increase inter-epithelial cell TEER, thereby decreasing cell permeability and inhibiting the invasive ability of endothelial cells.

## Discussion

This study demonstrated that notoginsenoside R1 (NGR1), an important component of *P. notoginseng*, significantly inhibited HCT-116 cell metastasis by suppressing migration, invasion and adhesion through regulation of MMP-9, integrin-1, E-selectin and ICAM-1 expression. In addition, our previous findings

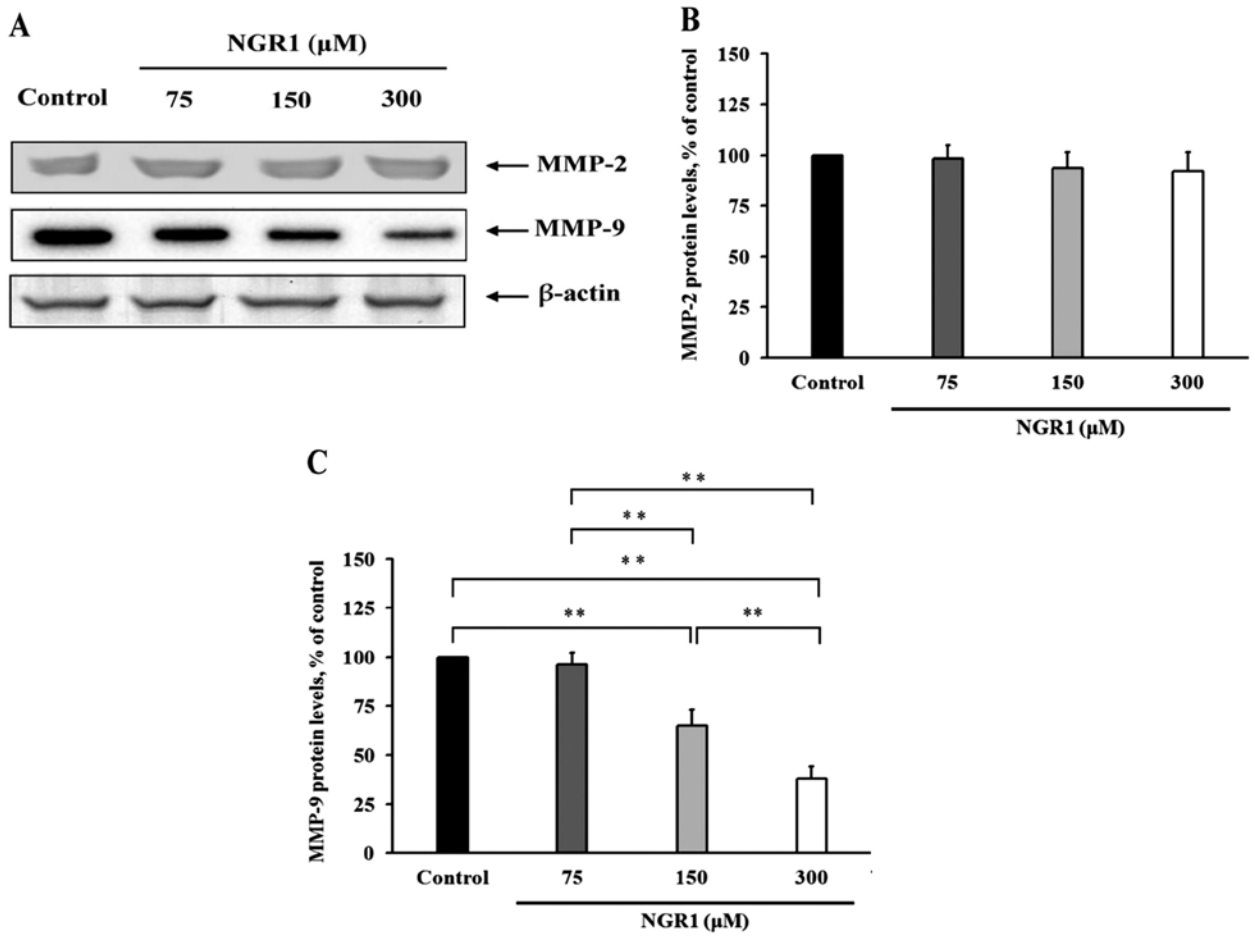


Figure 2. Effects of NGR1 on the levels of MMP-2 and MMP-9 proteins in the HCT-116 cells. HCT-116 cells ($5x10^5$ cells/3-cm plate) were treated with 75, 150, or 300 $\mu$M NGR1 for 24 h. Ethanol-treated cells served as the control. (A) Immunoblotting assays were used to analyze the levels of MMP-2 and MMP-9 in the HCT-116 cells. Protein levels of (B) MMP-2 and (C) MMP-9 were quantified by densitometry, and the levels in the control group were considered as 100%. Values are presented as the means ± SD (n=3). $^{**}$P<0.01 indicates a significant difference. NGR1, notoginsenoside R1; MMP-9, matrix metalloproteinase.

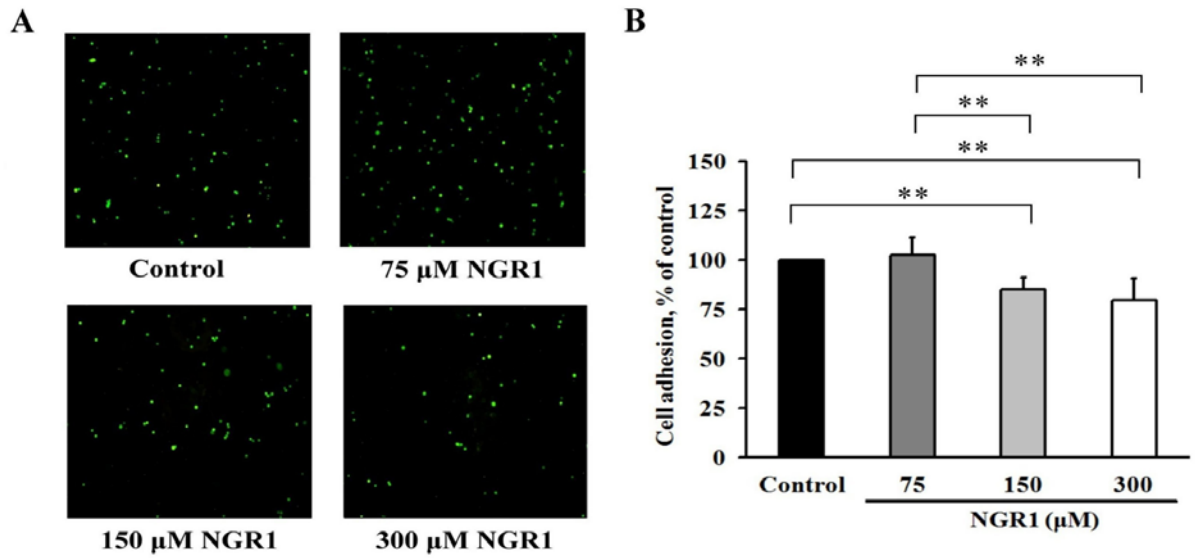


Figure 3. Effect of NGR1 on adhesion in HCT-116 cells. HCT-116 cells ($5x10^5$ cells/3-cm plate) were treated with 75, 150, or 300 $\mu$M NGR1 for 24 h. Ethanol-treated cells served as the control. HCT-116 and EA.hy926 cells were co-treated for 1 h. (A) After the HCT-116 cells were stained with BCECF, the adhesion of HCT-116 cells to EA.hy926 cells was observed under a fluorescence microscope. (B) HCT-116 cell staining was observed using a fluorescence ELISA reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Values are presented as the means ± SD (n=3). $^{**}$P<0.01 indicates a significant difference. NGR1, notoginsenoside R1.

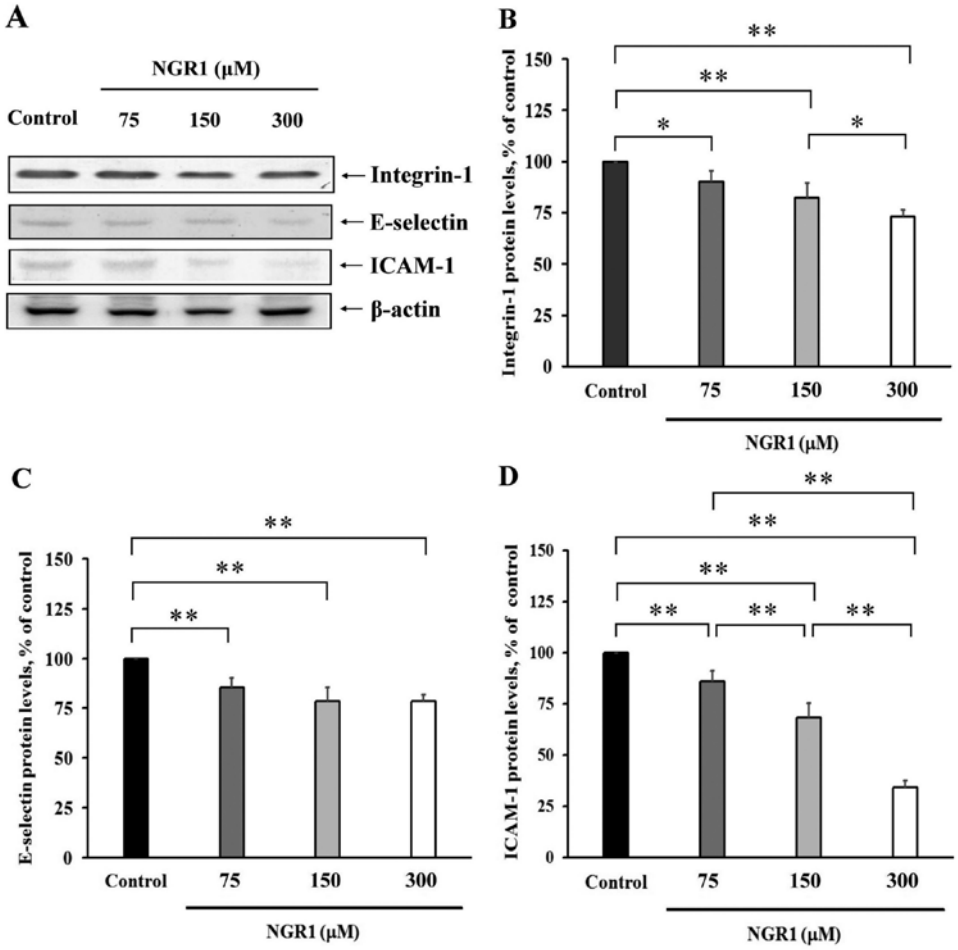


Figure 4. Effect of NGR1 on integrin-1, E-selectin and ICAM-1 expression. HCT-116 cells ($5x10^5$ cells/3-cm plate) were treated with 75, 150, or 300 $\mu$M NGR1 for 24 h. Ethanol-treated cells served as the control. HCT-116 and EA.hy926 cells were co-treated for 1 h. (A) Immunoblotting assays were used to analyze the levels of integrin-1, E-selectin and ICAM-1 in the HCT-116 cells. Protein levels of (B) integrin-1, (C) E-selectin and (D) ICAM-1 were quantified by densitometry, and the levels in the control group were considered as 100%. Values are presented as the means ± SD (n=3). $^*$P<0.05 or $^{**}$P<0.01 indicates a significant difference. NGR1, notoginsenoside R1; ICAM-1, intercellular adhesion molecule 1.

indicated that a *P. notoginseng* ethanol extract significantly suppressed metastasis of CRC (9). We conclude that NGR1 may be an important component of *P. notoginseng*, exerting an anti-metastasis effect. This finding is important in regard to the use of *P. notoginseng* and NGR1 as anti-metastatic agents, as 40 to 70% of colon cancers metastasize to the liver, and the prognosis is quite poor once metastasis has occurred (2). Indeed, anti-metastasis has become an important field of study for cancer prevention and therapy, as have investigations of the use of *P. notoginseng* or other active principle components.

*P. notoginseng* contains ~12% total saponins. The three major types of saponins are ginsenosides (e.g., Rb1, Rg1, Rg3), notoginsenosides (e.g., R1, R2, R3) and jiaogulan glucosides (e.g., RVI) (30-33). The structures of both notoginsenosides and ginsenosides exhibit a tetracyclic gonane steroid core. Gonane, the simplest steroid, and its metabolites typically act as signaling molecules that are associated with various physiological effects (31). These saponins are also active principle components of *P. notoginseng*, and NGR1 is the most abundant of the notoginsenosides (33). Previous anticancer studies have indicated that NGR1 suppresses human colon cancer cell proliferation (16,17,34). However, we are the first to report that NGR1 significantly suppressed CRC metastasis. Among saponins with a gonane structure, ginsenoside Rb1 was found to exhibit anti-metastatic and anti-angiogenic effects by suppressing the formation of endothelial tube-like structures on human umbilical vein endothelial cells (HUVECs) (35). Ginsenoside Rg1 also suppressed transforming growth factor β1 (TGF β1)-induced invasion and migration in HepG2 liver cancer cells (36). In addition, by inhibiting MMP-13 expression, ginsenoside Rg3 significantly suppressed the migration, invasion, wound healing, and colony-forming abilities of B16F10 cells in a dose-dependent manner (37), and by inhibiting wound healing and MMP-9 expression via suppression of NF-κB phosphorylation and DNA binding activity, ginsenoside Rg1 suppressed phorbol myristate acetate-induced invasion and migration in MCF-7 breast cancer cells (38). The above studies indicate that saponins can reduce the activities of MMPs to inhibit cancer metastasis.

MMPs can degrade collagen in the ECM and basement membrane components such as collagen type IV, elastin and fibronectin (39). MMP-2 and MMP-9 are major gelatinases that catalyse degradation of type IV collagen (39). Our previous study showed that a *P. notoginseng* ethanol extract inhibited HCT-116 cell migration by significantly decreasing MMP-9

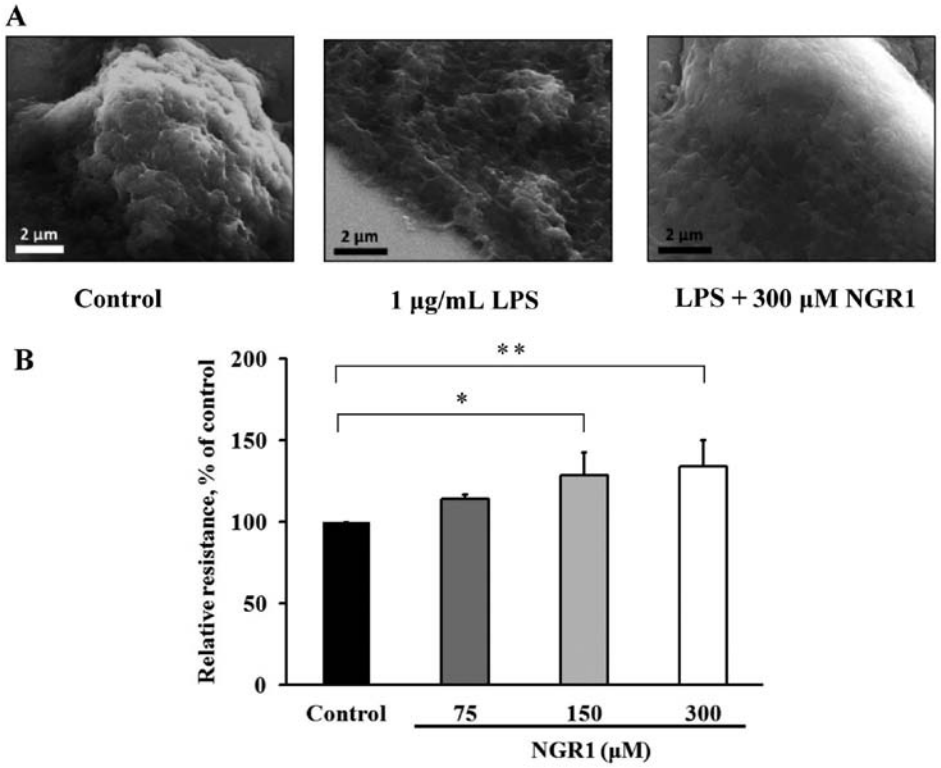


Figure 5. Effect of NGR1 on invasive morphology and TEER in HCT-116 cells. HCT-116 cells ($5x10^5$cells/3-cm plate) were treated with 75, 150, or 300 $\mu$M NGR1 for 24 h. Ethanol-treated cells served as the control. The invasion-induced group was treated with LPS (1 $\mu$g/ml), and the experimental group was treated with LPS combined with NGR1 (300 $\mu$M). (A) SEM images of cells exposed to LPS or LPS combined with NGR1. (B) Effect of NGR1 on TEER in EA.hy926 cells. Protein levels were quantified by densitometry, and the levels in the control group were considered as 100%. Values are presented as the means ± SD (n=3). $^{*}$P<0.05 or $^{**}$P<0.01 indicates a significant difference. NGR1, notoginsenoside R1; TEER, transepithelial electrical resistance; LPS, lipopolysaccharide; SEM, scanning electron microscopy.

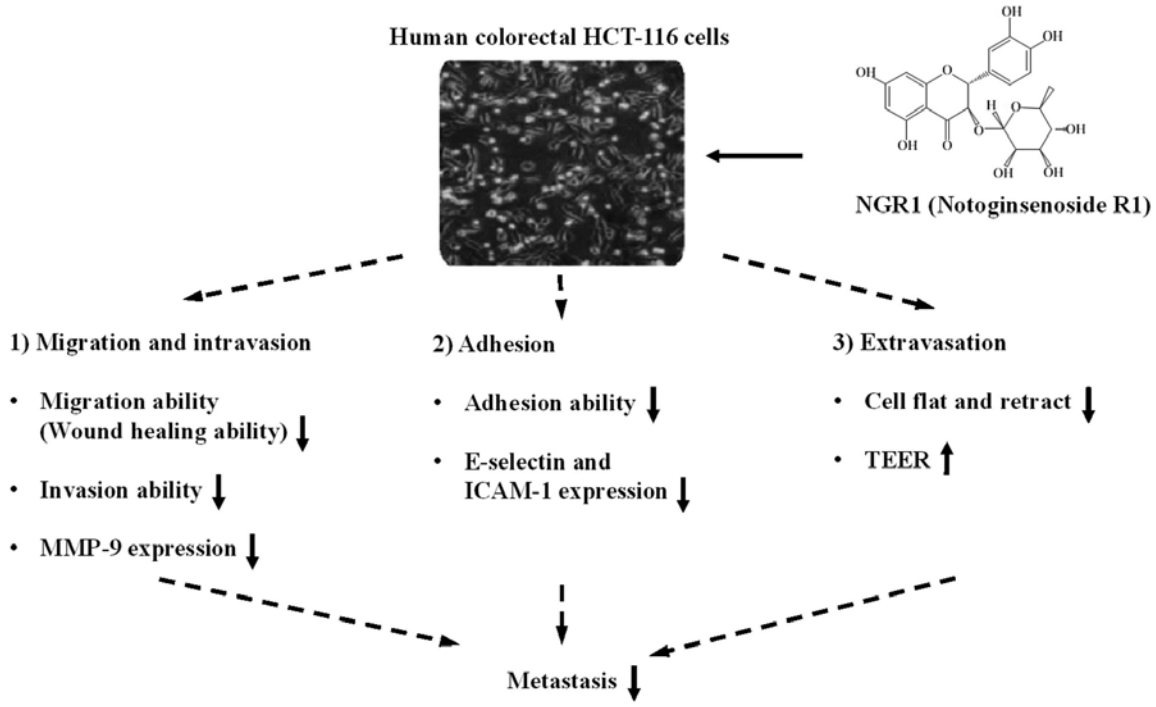


Figure 6. Possible mechanisms through which notoginsenoside R1 (NGR1) inhibits human colorectal cancer (CRC) HCT-116 cell metastasis.

but had no effect on MMP-2 expression (9). Ginsenoside Rg1 also suppressed PMA-induced MMP-9 expression but did not affect MMP-2 expression (38). In the present study, NGR1 was found to regulate MMP-9 expression in the HCT-116

cells. Levi *et al* demonstrated that MMP-2 and MMP-9 have different specificities for growth factor receptors in metastatic regulation (40). Nonetheless, the MMP signalling mechanism of NGR1 requires further study.

In the present study, NGR1 suppressed adhesion of a colon cancer cell line to an endothelial cell line of the blood vasculature of metastatic target organs by inhibiting integrin-1 levels in HCT-116 cells and reducing E-selectin and ICAM-1 levels in EA.hy926 cells. This important finding indicates the potential anti-metastatic effect of *P. notoginseng* and its active principle components. During the metastatic process, cancer cells leave the primary tumor site and enter the blood or lymphatic circulation via migration and intravasation. These circulating tumor cells migrate along the endothelial cell surface and invade metastatic target organs, which is referred to as adhesion and extravasation (41,42). Once the adhesion reaction is triggered, various adhesion molecules, such as integrin-1, E-selectin and ICAM-1, play key roles in regulating the adhesion of circulating tumor cells to endothelial cells (43,44). A previous study indicated that tumor necrosis factor-α (TNF-α) induced ICAM-1 expression in human A549 alveolar epithelial cells and adhesion of U937 cells to these cells reduced their metastatic ability (45). Bisdemethoxycurcumin, a major active compound of curcuminoids, significantly inhibited the adhesion, migration, invasion and metastasis of SKOV-3 cells by inhibiting MMP-9 and ICAM-1 expression (46). Among these adhesion-regulating molecules, integrins, a major class of cancer cell surface receptors of primary cancer cells, were responsible for the adhesion of cells to ECM proteins (47), and as demonstrated previously, E-selectin-mediated adhesion of tumor cells to the vascular endothelium is crucial for extravasation and metastasis (48). ICAM-1 plays a central role in cell-cell contacts, and cells overexpressing ICAM-1 may become targets for T cell infiltration. Indeed, ICAM-1 can enhance immune cell adhesion and migration (49). These integrins mediate strong adhesion between cancer cells and endothelial cells (50), and this process promotes the transmigration of cancer cells through the endothelium to metastatic sites (51,52). Our previous study showed that *P. notoginseng* exerts anti-metastasis effects in HCT-116 cells by decreasing adhesion activity through reduction in the levels of such adhesion molecules, including integrin-1, E-selectin and ICAM-1 (9). The suppressive effect of NGR1 on adhesion may be an important factor that contributes to the observed anti-migration effect.

In addition to their use in our analysis of the adhesion reaction, EA.hy926 cells were used in this study as a blood model to investigate the effects of NGR1 on extravasation in the vascular endothelial monolayer of the circulatory system. Extravasation is triggered when tumor cells adhere to endothelial cells and penetrate the endothelial cell layer. These metastatic cells bind to the monolayer cells, leading to disruption of endothelial cell-cell interactions and retraction of metastatic cells (52-54). The results of SEM examination indicated that HCT-116 cells became flattened and retracted after LPS induction. However, this flattening was significantly reduced by treatment with both NGR1 and LPS. Monolayer endothelium cell-cell permeability and integrity also play important roles in regulating the extravasation of tumor cells to endothelial cells (5,6). The TEER value is used as an indicator of the integrity of cellular barriers before they are evaluated for the transport of organisms (e.g., cells, microorganisms), drugs or chemicals, and cell-cell permeability and barrier integrity are vital for the physiological activities of tissues (55). Thus, TEER is a widely accepted quantitative technique for measuring the integrity of endothelial and epithelial monolayers in cell culture models. Our results indicate that NGR1 inhibits HCT-116 cell extravasation. EA.hy926 cells treated with 150 or 300 μg/ml NGR1 exhibited significantly reduced monolayer endothelial cell permeability, resulting in the inhibition of extravasation.

As shown in Fig. 6, our results illustrate a model in which NGR1 suppresses CRC cell metastasis. NGR1 significantly suppresses migration-regulated molecules (e.g., MMP-9), reduces adhesion trigger molecules (e.g., integrin-1, E-selectin and ICAM-1), and increases cell-cell permeability, resulting in inhibition of HCT-116 migration, invasion and adhesion. Hence, NGR1 may serve as a potential anti-metastatic agent.

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