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Original Articles

Anti-metastatic mechanism of mountain cultivated wild ginseng in human cancer cell line

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ABSTRACT

Objective : Ginseng is one of most widely used herbal medicine. Ginseng showed anti-metastasis activities. However, its molecular mechanisms of action are unknown. So we want to report the wild ginseng repress which plays key roles in neoplastic epithelial-mesenchymal transition process.

Methods : Treatment of the human colorectal carcinoma LOVO cells and human gastric carcinoma SNU601 cells with the increased concentrations of cultivated wild ginseng extracts resulted in a gradual decrease in the *AXIN2* gene expression.

Results : Metastasis-suppressor genes, *maspin* and *nm23* was not affected by the treatment of ginseng extracts in LOVO cells. Moreover, the mountain cultivated wild ginseng or mountain wild ginseng are similar in their inhibitory effects on the expression of *AXIN2* gene, but are substantially stronger than cultivated ginseng.

Conclusion : We described the novel mechanism of wild ginseng-induced anti-metastasis activity by repressing the expression of *AXIN2* gene that plays key roles in epithelial-mesenchymal transition process.

I. Introduction

Panax ginseng is one of the most popular natural tonics that have been used in oriental countries. Ginseng showed anti-tumor activities in slow growing tumors but not in rapidly growing tumors[1]. Ginseng also inhibited tumor angiogenesis and metastasis[2,3]. In an epidemiological study, the intake of ginseng reduced the incidence of human cancer[4]. However, its mechanisms of action have not been elucidated yet.

The canonical Wnt signaling pathway has a dominant role in regulating epithelial-mesenchymal cell interactions critical for morphogenesis[5,6]. In this signaling system, Wnt ligands bind to a coreceptor complex consisting of a seven-transmembrane-domain, Frizzled receptor and one of the LDL receptor-related proteins (LRPs), LRP5 or LRP6[7]. A signaling cascade is then engaged that leads to the formation of a bipartite complex between the adhesion molecule, β-catenin, and transcription factors of the TCF family. In turn, the β-catenin-T-cell

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factor(TCF) complex activates expression of a cohort of target genes hat impact on cellular function. Although the TCF complex as an important role in regulating normal cell function, increasing evidence indicates that canonical Wnt signaling can be disrupted in various cancerous states[8,9].

Accumulating evidence indicates that hyperactive Wnt signaling occurs in association with the progression of human cancer. As a consequence of engaging the canonical Wnt pathway, a TCF transcriptional complex is generated, which has been postulated to trigger the epithelial-mesenchymal transition (EMT), which convert epithelial cells into migratory mesenchymal cells[10]. Recent study has demonstrated that canonical Wnt signaling engages tumor cell dedifferentiation and tissue-invasive activity through an *AXIN2*-dependent pathway that stabilizes the Snail1 zinc-transcription factor, a key regulator of normal and neoplastic epithelial-mesenchymal transition programmes[11]. Axin2 regulates EMT by acting as a nucleocytoplasmic chaperone for GSK3β, the dominant kinase responsible for controlling Snail1 protein turnover and activity. Thus, a β-catenin-TCF regulated Axin2-GSK3β-Snail1 axis plays important role in cancer-associated EMT programmes.

Based on the grown environment and the cultivate method, the commercial trade ginseng is classified into three grades of ginseng, cultivated ginseng(CG), mountain cultivated wild ginseng(MCWG) and mountain wild ginseng(WG). The CG is cultivated artificially in forms and contributes the major quantity of ginseng in the current market. The WG grows in the natural environments, vegetating in the deep mountains and MCWG can be considered as mimicry WG which is seeded and grown up in the forest and mountain.

In this study, we examined the effect of MCWG on the transcription of the human *AXIN2* gene that plays key roles in neoplastic EMT process.

II. Materials and methods

1. Preparation of ginseng extracts

Cultivated ginsengs(CG) used in this experiment were 5 years of age(Fig. 1-a). Mountain cultivated wild ginseng(MCWG) used in this experiment were of 10 years old(seeded in 1999) grown at ChonBangNongSan in Choongnam, Korea, (Fig. 1-b). Wild ginseng(WG) used in this experiment were collected from Changbai Mt. in July, 2007. They are about 20-40 cm long, dried weighs about 10-20g with the approximate age of 20~50 years(Fig. 1-c).

Selected dried ginseng, CG, MCWG and WG(5g) were added with 80% ethyl alcohol 500㎖ and refluxed for 3hours, 3times to obtain extraction. Then this extraction was suspended in a distilled water for using experimental studies.

2. Cell culture and Cell lines

Human cancer cell lines were obtained from the Korean Cell Line Bank (KCLB, Korea) and were maintained in the recommended medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25μg/μl amphotericin B at 37℃ under a humidified 5% CO_2 atmosphere.

3. RNA extraction

RNA was extracted from various ginsengs treated cells using Easy-spin RNA Extraction kit (iNtRON, Biotech, Korea) according to the manufacturer's instructions.

4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

The reverse transcription reaction was performed on 2μg of total RNA using a SuperScript II First-

Strand Synthesis system (Invitrogen) with random primer. PCR was carried out in solution containing 1X PCR buffer (TaKaRa, Japan), 200μM each deoxynucleotide triphosphate, 2.5pmol of each primer, 1 unit of ExTaq polymerase (TaKaRa, Japan). The housekeeping gene β-actin served as an internal control to confirm the success of the reverse transcription reaction. The PCR products were subjected to 2% agaroseg elelectrophoresis.

5. Real-time RT-PCR

Volume of the reaction mixture was made up to 20μl. Real-time quantitative PCR was performed using SYBR GreenER qPCR SuperMix reagents (Invitrogen) and a Bio-Rad iCycler. Relative transcript quantities were calculated using the $\Delta\Delta$Ct method with β-actin as the endogenous reference gene amplified from the samples(Table 1.).

III. Results

1. Effect of mountain cultivated wild ginseng on the expression of AXIN2 Gene

Recent study[11] has demonstrated that canonical Wnt signaling engages tumor cell dedifferentiation and tissue-invasive activity through an *AXIN2*-dependent pathway(Fig. 2). Thus, the effect of cultivated wild ginseng on the expression of the *AXIN2* gene was examined as follows. MCWG extract was added at the time of plating, and after 3 days *AXIN2* gene expression was determined using RT-PCR. Treatment of the LOVO cells and human gastric carcinoma SNU 601 cells with the increased concentrations of mountain cultivated wild ginseng extracts resulted in a gradual decrease in the *AXIN2* gene expression(Fig. 3).

Quantitative real-time PCR results confirmed a dose-dependent decrease in *AXIN2* gene expression[3],which decreased 4-fold after treatment with 100μg/ml of cultivated wild ginseng extracts (Fig. 4). Taken together, theses results suggest that the cultivated wild ginseng repress the specific expression of the *AXIN2* gene.

2. A comparison analysis of three types of ginseng on the expression of AXIN2 gene

Both of MCWG and WG are considered superior to CG. We therefore effects of these three types of ginseng in the expression of *AXIN2* gene. Treatment with either MCWG extracts or WG for 3 days induced a decrease in the mRNA levels of *AXIN2* gene in human colorectal carcinoma LOVO cells. However, CG treatment of the LOVO cells had no significant effects on the transcription of the *AXIN2* gene(Fig. 5). These results suggest that the MCWG or WG are similar in their inhibitory effects on the expression of *AXIN2*, which are key regulators of neoplastic EMT programmes, but are substantially stronger than CG.

3. Effect of WG on the expression of metastasis-suppressor genes

Maspin (mammary serine protease inhibitor, 42 kDa) is a metastasis-suppressor gene first described by Zou et al in 1994 by subtraction of cultured breast cancer cell and normal breast tissue mRNAs[12]. Maspin has been thought to inhibit carcinoma invasion, metastasis, and angiogenesis. In addition, it has been reported that maspin induced apoptosis of neoplastic cells[13] and expression of maspin are suppressed as the carcinoma progresses in breast carcinoma and prostatic carcinoma[14,15].

Nm23 (*nonmetastatic*)23 is a metastasis suppressor gene[16] and its over expression has been shown experimentally to inhibit the metastatic phenotype and/or promote differentiation in melanoma, breast carcinoma, and transformed neural cell lines[17]. The low expression of the protein has been linked to increased metastatic potential in human breast car-

cinoma, hepatoma, and gastric carcinoma[18,19].

As shown in Fig. 6, RT-PCR analysis revealed that the expression of two metastasis-suppressor genes, maspin and nm23 was not affected by the treatment of ginseng extracts in LOVO cells.

IV. Discussion

Metastasis is the major cause of death for cancer patients with solid tumors, due mainly to the ineffectiveness of current therapies once metastases begin to form. Metastasis is defined as the formation of progressively growing secondary tumor foci at sites discontinuous from the primary lesion[20]. In cancer, it is believed that epithelial tumor cells may be able to convert differentiated epithelial cancer cells into de-differentiated cells that possess more mesenchymal characteristics[21]. This epithelial-mesenchymal transition (EMT) phenotype in cancer has been associated with a decrease in tumor growth, increased resistance to apoptosis, increased motility and invasiveness, and enhanced metastatic ability[22]. These phenotypic transitions are reversible, and it is hypothesized that once tumor cells have reached their destination, they may transform back into an epithelial phenotype in order to facilitate tumor growth in the secondary site[23]. EMT is an important process in tumor development, and several studies suggest that the Wnt/β-catenin signal pathway may play an important role in EMT .

The present study focuses on identifying the mechanism that underlies the anti-metastasis activity of ginseng. The study shows, for the first time, that ginseng can repress the expression of *AXIN2* gene that play key roles in the downsream signaling of the neoplastic EMT process. These results showed us a novel mechanism of ginseng' s anti-metastasis activity and could provide a molecular link between ginseng intake and its inhibitory effects on metastasis.

V. Acknowledgement

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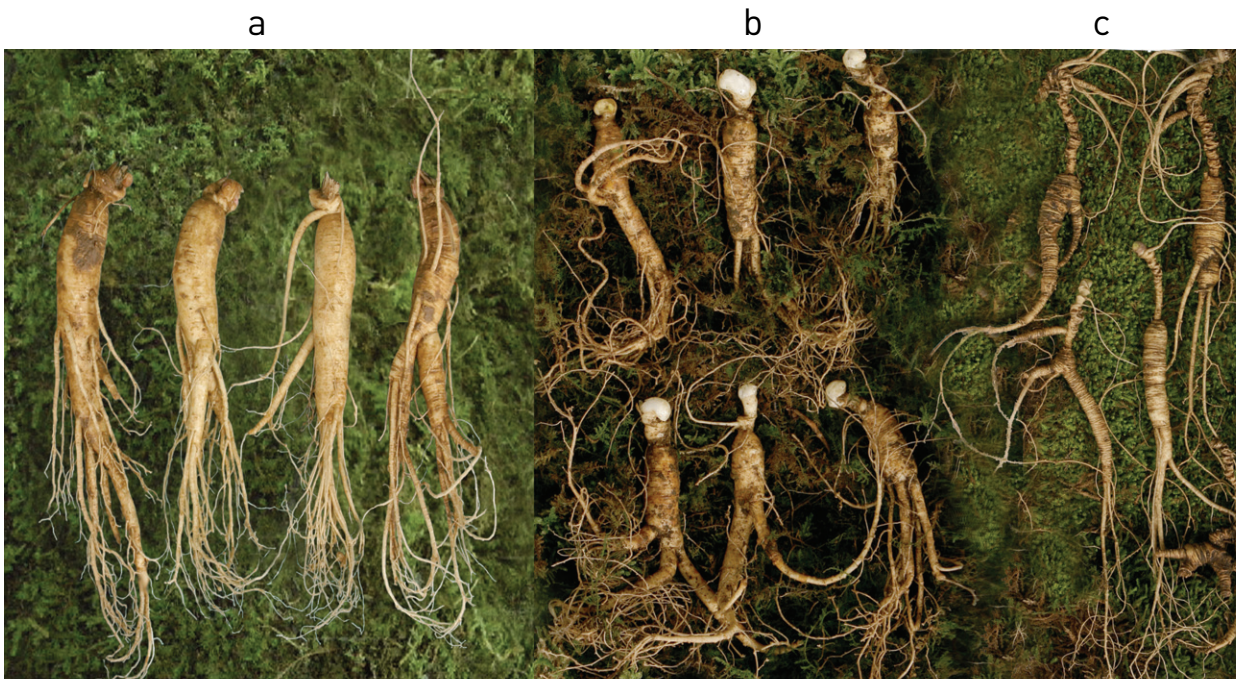

Fig. 1 Cultivated ginseng(CG-a), mountain cultivated wild ginseng(MCWG-b) and mountainwild ginseng(WG-c) were used for ginseng extracts.

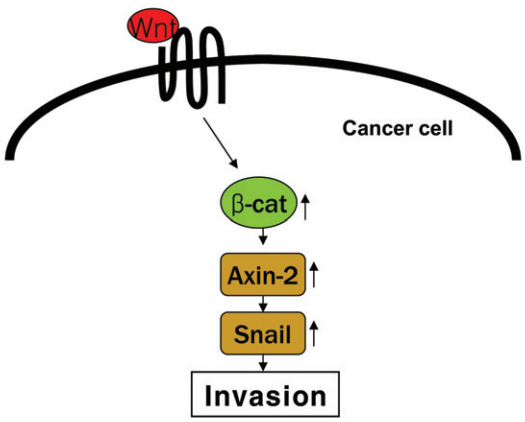

Fig. 2 Schematic diagram showing that β,-catenin, stabilized either by canonical Wnt signalling, triggers TCF-dependent signaling that induces Axin2 expression. In turn, Snail1 protein half-life is increased, thereby engaging a Snail1-dependent EMT process.

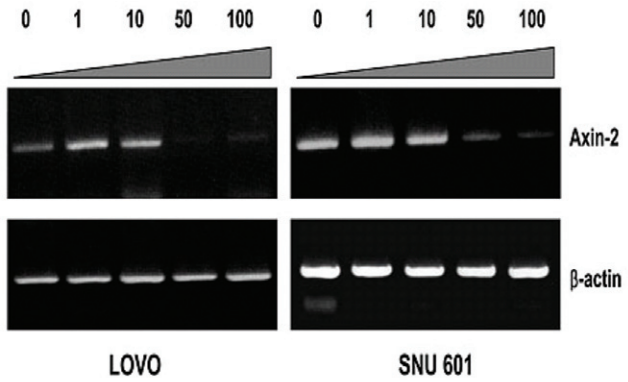

Fig. 3 Repression of *AXIN2* mRNA after treating LOVO and SNU601 cells with MCWG. *AXIN2* mRNA levels were analyzed by semi-quantitative RT-PCR. Human colorectal carcinoma LOVO cells and human gastric carcinoma SNU601 cells were cultured in media containing mountain cultivated wild ginseng extracts (0 -100 μg/ml) for 3 days, and the *AXIN2* mRNA level was evaluated by semi-quantitative RT-PCR using the *AXIN2*-specific primers. RT-PCR analysis revealed the expected 325 bp *AXIN2* band and β-actin being an internal control.

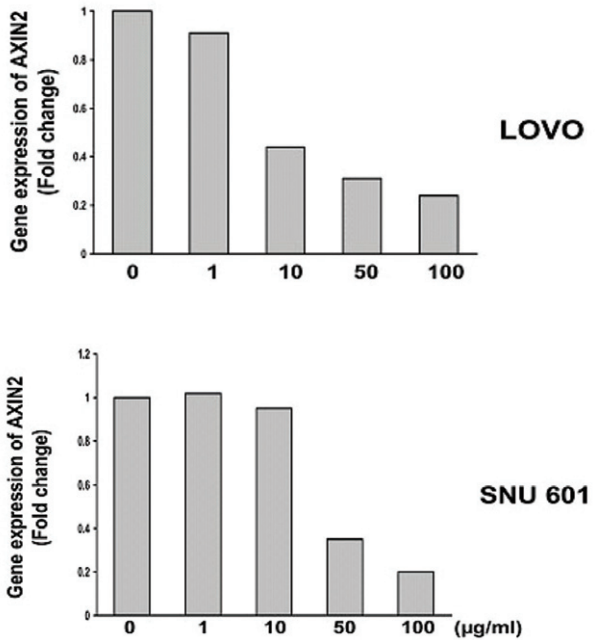

Fig. 4 Quantitative real-time RT-PCR analysis of *AXIN2* transcripts. Total RNA extracted from the cells (2μg) was reverse-transcribed to cDNA (40μl), and aliquots (1μl) were applied to real-time PCR(20μl) with each primer(0.4 mM). Values represented relative expression of *AXIN2* gene (calculated with threshold cycle number, C_T) of MCWG treated cells compared with that of non-treated control cells. Each value was adjusted with C_T of internal control(β-actin).

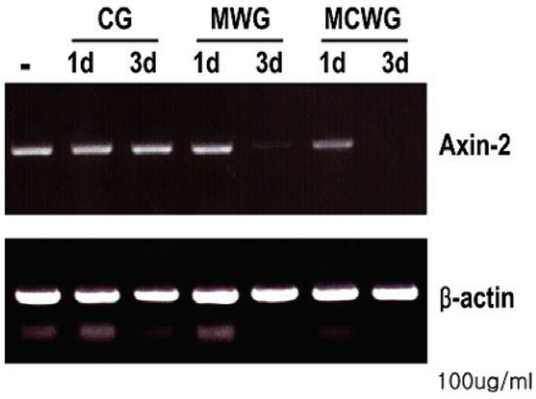

Fig. 5 RT-PCR analysis of *AXIN2* mRNA expression in human colorectal carcinoma LOVO cell lines treated with the CG extracts, MCWG extracts, or WG extracts(100μg/mℓ) for 3 days. Lane (-), negative control.

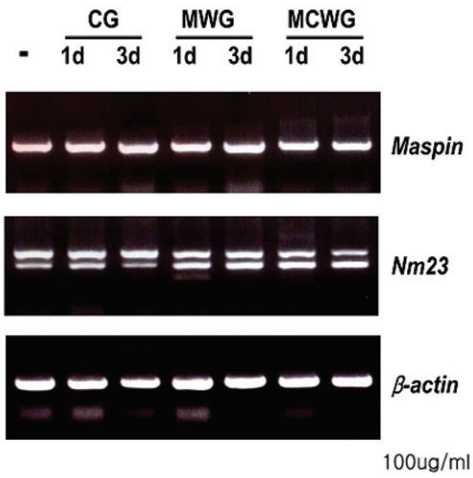

Fig. 6 Expression of mRNA for *maspin* and *nm23* on LOVO cells. Total RNA(2μg) from LOVO cells treated with the CG extracts, MCWG extracts, or WG extracts(100μg/mℓ) for 3 days was examined by RT-PCR analysis. Lane (-), negative control.

Table 1 Primer for RT-PCR

Gene	Accession No.	Primer sequence	Product size (bp)
β-actin	NM001101	F: CTCTTCCAGCCTTCCTTCCT R: AGGGCAGTGATCTCCTTCTG	179
AXIN2	NM004650	F:AGGCCAGTGAGTTGGTGTC R: AGT TGCTCACAGCCAAGACA	325
Nm23	NM002513	F: AGGAAGGGCTTCAAGTTGT R: TCCAGACAGGTGGATGTTCA	505
Maspin	NM002639	F: AGACATTCTCGCTTCCCTGA R: CACATGACCAGATGGGACAG	516