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The Anti-Inflammatory Effect of IH-901 in HT-29 Cells

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Abstract

20-*O*-(β-D-Glucopyranosyl)-20 (*S*)-protopanaxadiol (IH-901) is one of the major metabolites of ginsenosides from *Panax ginseng*, and is suggested that IH-901 has been associated with various pharmacological and physiological activities. In this study, we demonstrate that IH-901 induced anti-inflammation in HT-29 human colon adenocarcinoma cells. Our results showed that IH-901 inhibited cell proliferation of HT-29 in a time- and dose-dependent manner. We also found that IH-901 was significantly decreased expression of iNOS compared with non-treated. We observed effect of IH-901 related with inflammatory genes using by cDNA microarray. We were known that the 34 inflammatory genes such as E2F, CDK6, TNF-α, and PKC were down-regulated. Thus, these results suggest that IH-901 may have a potential preventive factor to improving cancer induced by chronic inflammation.

Keywords: IH-901, Inducible Nitric Oxide Synthase (iNOS), Nitric Oxide (NO), Inflammation, HT-29 human colon adenocarcinoma cells

Panax ginseng C.A. *Meyer* (ginseng) has been used as a traditional medicine in Asian Countries for more than 2,000 years, and is now used numerous countries for the treatment of various diseases. The major components of ginseng are ginsenosides which have steroid-like structure[1,2], and are divided into three types according to their aglycones; protopanaxadiol, protopanaxatriol and oleanoic acid ginsenosides. Recently, it was reported that ginsenosides of protopanaxadiol-type such as Rb_1, Rb_2 and Rc are transformed to 20-*O*-*β*-D-glucopyranosyl-20(*S*)-protopanaxadiol (IH-901) by intestinal bacteria in human and rat[3]. IH-901 is discovered as one of the major metabolites in urine and blood after administration of ginseng extract[3]. It is non-toxic, and suppresses uptake of glucose by tumor cells, exhibits anti-metastatic effects and reverses multidrug resistance in tumor cells[4]. Lee, S. J. *et al*.[5], IH-901 induces apoptosis and suppresses growth of leukemia cells by activation of caspase-3 protease, which occurs via mitochondrial cytochrome C release independently of Bcl-2 modulation.

Nitric Oxide (NO) is synthesized from the oxidation of guanino group of L-arginine by diverse NADPH-dependent enzymes called nitric oxide synthase (NOS). The three difference isoforms of NOS are neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are generally expressed neurons and endothelial cells depended on calcium and calmodulin, whereas iNOS is induced by inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-1 and lipopolysaccharide (LPS) instead of calcium. Recent some studies suggested that increased expression and activation of iNOS are associated with development of colon cancer[6,7]. For example, Ambs, S. *et al*.[6], suggested that iNOS expressed low level of activity in the colon normal tissue contiguous to the neoplastic lesions, but they found very high level in the colon adenocarcinoma. According to study of Rao, C. V. *et al*.[19], 20(*S*)-protopanaxadiol inhibited iNOS induced by azoxymethane (AOM) in colonic aberrant crypt foci. Also, it is known that over -production NO by iNOS promotes inflammation, cancer progress, tumor angiogenesis and activation of proto-oncogene, and inhibits apoptosis[8,9,18].

It has been studied that ginsenosides down-regulated expression of iNOS and COX-2 and suppressed NO level[15]. The main focus of this study was investigated anti-inflammatory effect of IH-901 using by HT-29 human colon adenocarcinoma cells.

Inhibition of Proliferation on IH-901 in HT-29 Cells

We investigated proliferation inhibitory effect of IH-901 in HT-29 human colon adenocarcinoma cells using by MTT assay. HT-29 was treated with IH-901 at various concentrations from 20 to 320 μM and incubated 12 hrs. As shown in Figure 1, the results showed that IH-901 inhibits significantly cell growth in a dose- and time-dependent manner.

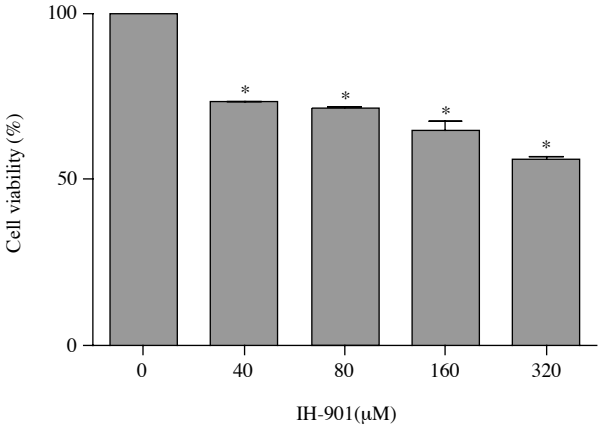

Figure 1. Effect of IH-901 on proliferation in HT-29 human colon carcinoma cells. HT-29 cells (1.5×10^4 cells/well) were treated with various concentration (40, 80, 160 and 320 μM) of IH-901 in serum-free media and incubated for 12 hrs. The cell viability was determined by MTT assay. The data were presented as experiments, and shown as percentage changed control to non-treated control. $P < 0.001$ compared to non-treated cells.

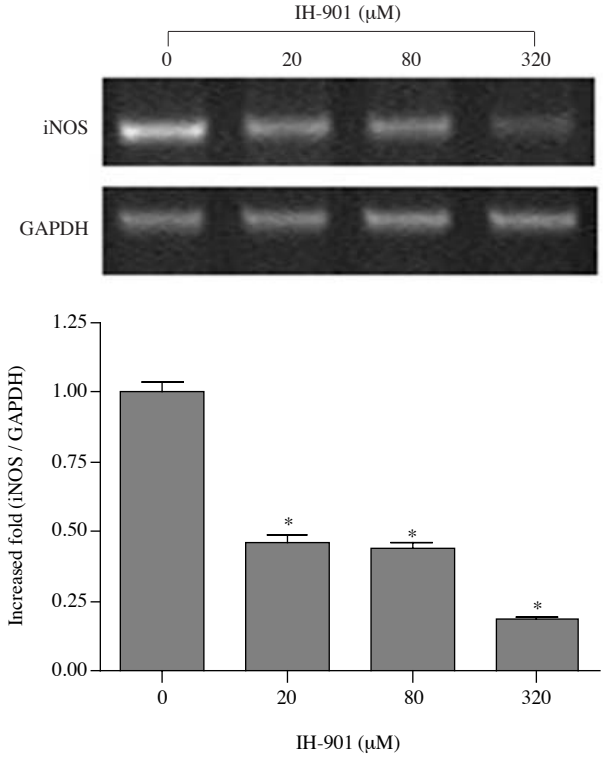

Figure 2. Effect of IH-901 on expression of iNOS in HT-29 cells. To observe inhibition of iNOS expression by IH-901, HT-29 cells (2.5×10^5 cells/well) were treated with IH-901 (20, 80 and 320 μM), and incubated for 12 hrs. mRNA levels of iNOS and GAPDH were measured by RT-PCR. The bar graph represents the result of densitometric analysis of iNOS mRNA normalized to GAPDH. Data are expressed as the means ± SD from three separate experiments. $*P < 0.001$ compared to non-treated cells.

Inhibition of iNOS mRNA on IH-901 in HT-29 Cells

To measure expression of iNOS mRNA, HT-29 cells were treated with IH-901 at 20, 80 and 320 μM for 12 hrs. After treatment, we investigated the expression of iNOS by RT-PCR. As shown in Figure 2, expression of iNOS in non-treated cells was increased, whereas expression of iNOS mRNA in response to IH-901 was significantly decreased in a dose-dependent manner. Compared to control, level of iNOS mRNA treated with in IH-901 at 20, 80 and 320 μM was decreased by 53%, 57% and 81%, respectively (Figure 2).

Gene Expression Profiling in HT-29 Cells

To find gene associated with effect of IH-901 in HT -29 cells, we investigated using by cDNA microarray. Gene expression profiles of interest were up-regulated and down-regulated in experimental group compared with non-treated group. The up- and down-regulated genes are listed in Table 1 and 2. Gene expression Profiles were showed up-regulated genes of 26 including major histocompatibility complex (MHC) class I, MHC class II and interleukin 2 receptor (Table 1). Also, Table 2 was demonstrated that 34 genes such as tumor necrosis factor (TNF), cAMP responsive element binding protein (CREB), and protein kinase C were down-regulated. Z scores analysis of ginseng- and control groups was performed and individual gene was plotted. To examine the relationships between ginseng- and control group, we used a hierarchical clustering and revealed up- and down-regulated genes on the basis of similar expression patterns, and the data is presented in a matrix format (Figure 3 and 4). The expression level of genes was visualized in color related with its median expression level across all samples. Red represented up-regulated expression, green represents down-regulated expression and block represented the median expression level.

Discussion

Ginsenosides are major metabolite of *Panax Ginseng* C.A. *Meyer*, and these have been used in traditional medicine for treatment of many disorders. Also, it is known that ginsenosides have biological and pharmacological effects such as anti-inflammation, anti-cancer, anti-oxidation, anti-stress and immunomodulation[11]. IH-901 is one of novel ginsenosides metabolite formed by intestinal bacteria after oral

Table 1. Up-regulated gene expression in HT-29 cells.

Up-regulated gene	Abb	Z ratio
Apoptosis		
deleted in colorectal cancer	DCC	1.51
deoxyribonuclease ‖ , lysosomal	DNASE2	1.52
adenosine A2a receptor	ADOSRA2A	3.40
Cell cycle		
budding uninhibited by benzimidazoles 1 (yeast homolog), beta; MAD3-like protein kinase mRNA	BUB1B	2.18
Immune		
interleukin 11	IL-11	1.99
major histocompatibility complex, class I, A	HLA-A	2.18
major histocompatibility complex, class II, DQ beta 1	HLA-DOB1	2.52
interleukin 2 receptor, alpha	IL2RA	2.75
ferritin, heavy polypeptide 1	FTH1	3.33
Signal Transduction		
chemokine (C-C motif) receptor 5	CCR5	2.22
thyroid stimulating hormone receptor	TSHR	3.65
Transcription		
protein phosphatase 1, regulatory subunit 10	PPP1R10	1.55
similar to latent transforming growth factor beta binding protein 1	TGFB1	1.65
CCAAT/enhancer binding protein (C/EBP), delta	CEBPD	1.86
excision repair cross-complementing rodent repair deficiency, complementation group 2	ERCC2	1.89
TEA domain family member 4; RTEF1; TEF-3	TEAD4	1.89
nuclear transcription factor Y, alpha; Hap2	NFYA	3.11
POU domain, class 6, transcription factor 1	POU6F1	4.09
Others		
prodynorphin	PDYN	1.52
ubiquitin specific protease 6 (Tre-2 oncogene)	USP6	1.78
SH3-domain binding protein 2	SH3BP2	1.84
phosphogluconate dehydrogenase	PGD	1.85
T-cell receptor, alpha (V, D, J, C)	TRA@	1.87
similar to protein tyrosine phosphatase, non-receptor type 13	PTPN13	1.88
damage-specific DNA binding protein 1 (127 kD)	DDB1	2.04
tyrosine kinase 2	TK2	2.14

administration of ginseng extract, and it has been supposed that IH-901 is important form of protopanaxadiol saponin absorbed from the intestine[3,15]. Several researchers reported that IH-901 has different pharmacological activities such as inhibition of proliferation, metastasis and invasion and induction of apoptosis in cancer[16-20]. For example, IH-901 induced apoptotic cell death through down-regularion of apoptosis related protein such as c-Myc and Cyclin D1, and inhibited proliferation in tumor cell[20].

The purpose of this study is to determine effect of IH-901 related with inflammatory response in HT-29 human colon adenocarcinoma cells.

In our experimental present study, we also observed inhibitory effect of IH-901 on the growth of HT-29 human colon adenocarcinoma cells using MTT assay. We had shown that IH-901 was decreased proliferation of cancer cell in a time- and dose-dependent manner (Figure 1).

iNOS, pro-inflammation enzyme, is involved in regulation of physiological and pathological processes responsible for inflammation and cancer[10]. Many studies reported that increased levels of iNOS have been discovered in various cancers and inhibition of iNOS induces apoptotic cell death in human cancer cells[16]. Also, over-production of NO by iNOS induces tumorigenic process including DNA damage, lipid peroxidation and promotes neovascularization in some cancer[16,17].

Previous studies had reported that ginsenosides have effect of anti-inflammation. Oh, G. S. *et al.*[10], studied that protopanaxatriol blocked activation of LPS induced iNOS and COX-2 in RAW 264.7 murine machrophage cells via gluococorticoid receptor-independent pathway. Also, Lee, S. J. *et al.*[23], demonstrated that IH-901 inhibited activation of iNOS and production of NO by promotion of oxigenase-1 (HO-1) expression. Therefore, to investigate anti-inflam-

Table 2. Down-regulated gene expression in HT-29 cells.

Down-regulated gene	Abb	Z ratio
Anti-apoptosis		
Presenilin 1	PSEN1	−1.67
myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	−1.78
interleukin 10	IL-10	−2.55
synuclein, alpha (non A4 component of amyloid precursor)	SNCA	−3.24
Cell cycle		
cyclin B1	CCNB1	−1.66
cyclin D2	CCND2	−1.82
cyclin-dependent kinase 6	CDK6	−2.57
E2F transcription factor 3	E2F3	−4.26
Immune		
tumor necrosis factor, alpha-induced protein 1	TNFAIP1	−2.88
similar to mitogen-activated protein kinase kinase kinase kinase 2	MAP4K2	−3.72
Transcription		
interferon regulatory factor 1	IRF1	−1.69
sterol regulatory element binding transcription factor 1	SREBTF-1	−3.82
MAX protein; helix-loop-helix zipper protein	MAX	−5.13
Signal Transduction		
mitogen-activated protein kinase 10	MAPK10	−1.60
mitogen-activated protein kinase 11	MAPK11	−1.72
anti-Mullerian hormone receptor, type II	AMHR2	−1.80
cAMP responsive element binding protein-like 1	CREBP1	−2.37
small inducible cytokine A5; RANTES	CCL5	−2.73
regulator of G-protein signaling 1	RGS1	−3.28
Others		
ribosomal protein S25	RPS254	−1.50
milk fat globule-EGF factor 8 protein; breast epithelial antigen BA46 mRNA	MFGE8	−1.54
glutathione synthetase	GSS	−1.71
insulin-like growth factor binding protein 3	IGFBP3	−1.80
phosphodiesterase I/nucleotide pyrophosphatase 2 (autotaxin)	ENPP2	−1.95
surfactant, pulmonary-associated protein C	SFTPC	−1.96
serine protease inhibitor, Kunitz type, 2	SPINT2	−1.99
integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	−2.05
protein kinase C	PRKC	−2.11
defensin, alpha 6, Paneth cell-specific	DEFA	−2.81
integrin, beta 8	ITGB8	−3.31
P glycoprotein 1/multiple drug resistance 1; MDR1	ABCB1	−3.72

matory effect of IH-901, we monitored expression of iNOS in HT-29 cells. RT-PCR analysis exposed that IH-901 inhibited expression of iNOS (Figure 2).

Also, we observed gene expression profiles related with inhibitory effect of IH-901 on colon cancer using by cDNA microarray. In this study, several genes were up- or down-regulated, and these genes were arranged according to their functions (Table 1 and 2). These genes were categorized according to their functions; apoptosis, cell cycle, immune, signal transduction, transcription and others. In DNA microarray results, we are known that genes connected with inflammation such as Tumor Necrosis Factor, Alpha-Induced Protein 1 (TNFAIP1), interleukin 10, E2F transcription factor 3 (E2F3), MAX and cAMP Responsive Element Binding Protein-like (CREPBP1) were down-regulated in HT-29 cells, and genes related with anti-inflammation such as deleted colorectal cancer (DCC), major histocompatibility complex, class I-A (HLA-A), T-cell receptor alpha (V, D, J, C) (TCA@) and interleukin 2 receptor alpha (IL2RA) were up-regulation.

Our results demonstrated that IH-901 significantly inhibited proliferation in HT-29 human colon adenocarcinoma cells. Also, IH-901 possesses anti-inflammatory capacity, resulting in down-regulation of iNOS expression and NO production in HT-29 cells. And, we were known that IH-901 inhibited pro-inflammatory factors based on results of cDNA microarray. In conclusion, we suggested that effect of IH-901 may be important in the understanding of a novel mechanism for the anti-inflammatory action.

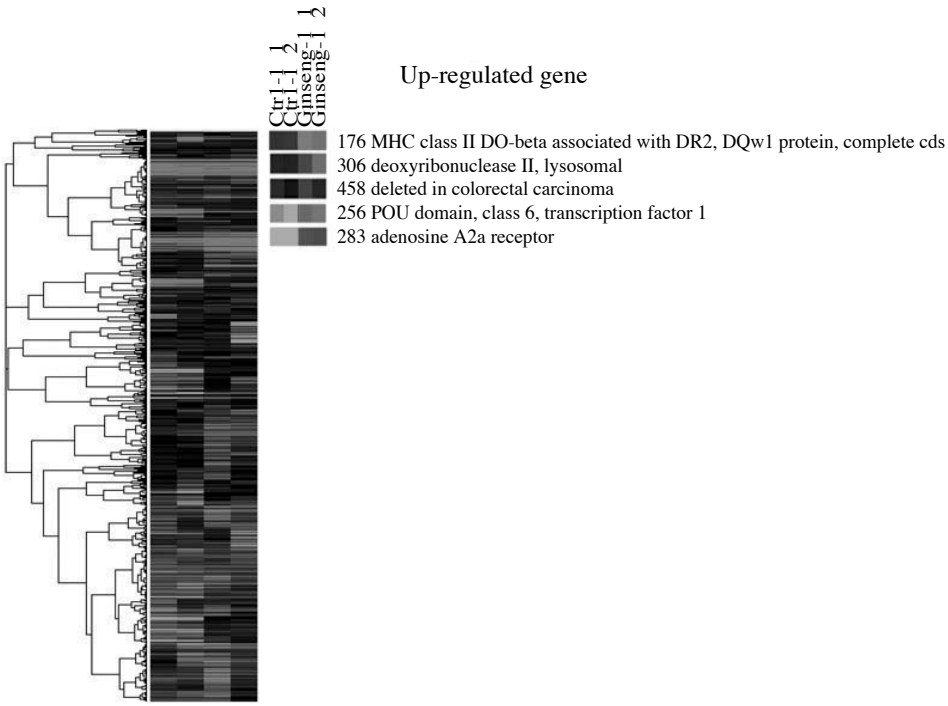

Figure 3. Clustertgram of up-regulate genes in IH-901 group. HT-29 cells (1.5×10^4 cells/wells) were treated with concentration at 20 μM of IH -901 for 12 hrs. Microarray data from control group and experimental group were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate program available as shareware form Michael Eisen's lab.

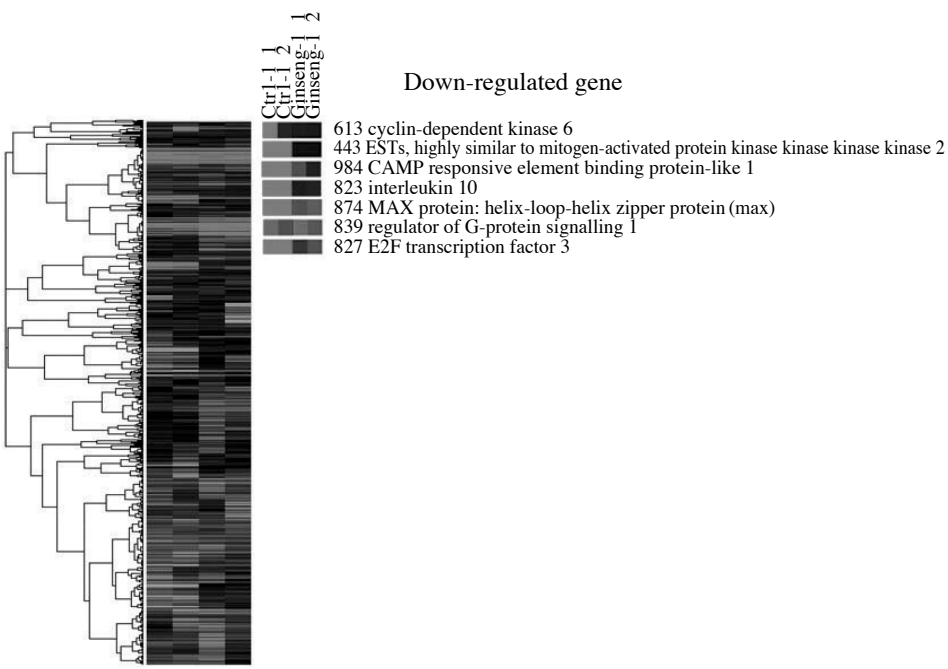

Figure 4. Clustertgram of down-regulate genes in IH-901 group. HT-29 cells (1.5×10^4 cells/wells) were treated with concentration at 20 μM of IH-901 for 12 hrs. Microarray data from control group and experimental group were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate program available as shareware form Michael Eisen's lab.

Methods

Reagents

IH-901 was a product of Ambo Institute (Seoul, South Korea). IH-901 was dissolved in Dimethyl sulfoxide (DMSO) to a concentration of 0.1%. 3-(4, 5-dimethylthiazol 1-yl)-2, 5-diphenyltetrazolium bromide (MTT), Sulfanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride and Phosphoric acid were purchased from Sigma Aldrich (Steinheim, Germany). iNOS and GAPDH were obtained from

Bioneer Corporation (Daejeon, South Korea). RPMI-1640, Fetal Bovine Serum (FBS) was from WelGENE (Daegu, South Korea).

Cell Culture

HT-29 human colon adenocarcinoma cells were cultured in the RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 2% 200 mM L-glutamine and 2% penicillin and streptomycin solution (10,000 units/mL penicillin and 10 mg/mL streptomycin). The cultures were maintained at 37˚C in humidified 5% CO_2.

MTT Assay

Cells were plated in 96-well plates at 1.0×10^4 cells/well in a 200 μL medium for cell viability measurement. 24 hrs later, cells were washed and changed the fresh medium, and they were treated with IH-901 at 40, 80, 160 and 320 μM for 12 hrs. After treatment, 10 μL of MTT stock solution (0.5 mg/mL) was added to each well and incubated at 37˚C for 3 hrs. The formazan product was dissolved in 200 μM DMSO, and shook for 10 min. After 10 min, it measured optical density using the Microplate Leader (Molecular Devices, Spectra Max, Union City, CA, USA) at 540 nm.

RNA Preparation

Cells were seeded in 6-well plate at 2.5×10^4 cells/well in a 2 mL medium for RT-PCR and cDNA microarray analysis. 24 hrs later, cells were washed and changed the fresh medium, and they were treated various concentration of IH-901 for 12 hrs. RNA isolated using 1 mL of TRIzol reagent (Invitrogen, CA, USA). The RNA pellets were dried and dissolved in Diethylpyrocarbonate (DEPC) treated water (Invitroge, CA, USA) and incubated at 55 to 60˚C for 30 minutes. The total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Nano Drop, DE, USA).

RT-PCR

After extraction and quantification of RNA, RT-PCR reaction was performed as described the manufactured. Briefly, complementary DNA (cDNA) was synthesized using 5 μg of total RNA at 37˚C for 60 min. For the iNOS gene (5′-ACAGGAGGGGTTAA-AGCTGC-3′, 5′-TTGTCTCCAAGGGACCAGG-3′) and GAPDH gene (5′-CGGAGTCAACGGATTTG-GTC-3′, 5′-AGCCTTCTCCATGGTGGTGA-3′) the temperature profile consisted of 45 sec denaturation at 94˚C, 45 sec annealing at 55˚C and 15 min extension at 72˚C. The iNOS PCR product was amplified for 35 cycles and GAPDH PCR product was amplified for 30 cycles. The resulting products were electrophoresis on a 1.5% agarose gel in 0.5 M TAE buffer, stained with ethidium bromide, and the GAPDH gene used as positive amplification control.

Human cDNA Microarray

A human cDNA Microarray was primarily derived from a commercially available master set of approximately 15,000 human verifies-sequences (Research genetics, Inc, AL, USA). The human cDNA clone set was sorted for a list of genes (1,152 elements) representing families correlated with differentiation, development, proliferation, transformation, cell cycle progression, immune response, transcription and transcription factors, oncogenes, and molecules related to cell growth and maintenance. PCR-amplified cDNAs were spotted on nylon membranes. The general methodology of arraying was based on the procedures by DeRisi, J. *et al.*[20].

cDNA Radiolabeling

After quantification, 2-3 μg of total RNAs prepared from blood was used for each sample for adjustment of different diagnosis. To synthesize ^{33}P-labeled cDNAs, quantified RNA were labeled in a reverse transcription reaction containing 8 μL of 5X first standard PCR buffer (Invitrogen, Milano, Italy), 4 μL of 24-mer poly dT primer (Invitrogen, CA, USA), 4 μL of dNTP excluding dCTP (Invitrogen, CA, USA), 4 μL of 0.1 M Dithiothreitol (DTT) (Invitrogen, Milano, Italy), 1 μL of RNaseOUT (LIT), 1 μL of DEPC treated water and 5 μL of 3,000 Ci/mmol a-^{33}P dCTP to a final volume of 20 μL. Two μL of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen, CA, USA) was then added and the samples were incubated for 30 minutes at 42˚C, followed by the addition of 2 μL of M-MLV reverse transcriptase and another 30 minutes at 42˚C. 2.5 μL of 0.5 M Ethylene-Diamine-Tetra-Acetic Acid (EDTA) was added to chelate divalent cations. After the addition of 5 μL 0.1 M NaOH, the samples were incubated at 65˚C for 30 minutes to hydrolyze remaining RNA. Following the addition of 12.5 μL of 1 M Tris HCl (pH 8.0), the samples were purified using purification columns (Bio-rad, CA, USA). After purification, each sample was put in 4 mL of hybridization buffer and reacted with nylon membrane during 24 hrs.

Hybridization and Scanning

cDNA microarrays were pre-hybridized in hybridization buffer containing 4 mL Microhyb and 10 μL of 8 mg/mL poly dA (Invitrogen, CA, USA). Both Human Cot 1 and poly dA were denatured at 95˚C for 5 min prior to use. After 4 hrs of pre-hybridization

at 42°C, approximately 10^7 cpm/mL of heat-denatured (95°C, 5 minutes) probe was added, and incubation continued at 42°C for 17 hrs. Hybridized arrays were washed 3 times in 2X SCC and 0.1% SDS for 15 minutes at room temperature. The microarrays were exposed to phosphorimager screens for 1-5 days, and the screens were then scanned using a FLA-8000 (Fuji Photo Film Co., Japan) at 50 μm resolution.

Data Analysis

Microarray images were trimmed and rotated for further analysis using L-Processor system (Fuji Photo Film Co., Japan). Gene expression of each microarray was captures by the intensity of each spot produced by radioactive isotopes. Pixels per spot were counted by Arraygauge (Fuji Photo Co., Japan) and exported to Microsoft Excel (Microsoft, WA, USA).

Statistical Methods

Data analysis of microarray was used by Microsoft Excel (Microsoft, WA, USA). To normalize each membrane or each gene across membranes, we used global normalization basic methods, which calculate the mean or median of the signal intensities of each individual experimental data set and then calculate the mean of the means (or grand mean) for all of the included experiments. Each individual data set is then mathematically adjusted such that the mean of that data set equals the calculated grand mean. Raw intensity values obtained from the previous step were exported to EXCEL and normalized with Z transformation by subtracting with each average of gene intensity and dividing with each S. D. Raw intensity data for each experiment is $\log_{10}$ transformed and then used for the calculation of Z scores. Z score transformation statistics have been used in comparing experimental and control group gene expression differences by microarray[21]. Z value, Z difference and Z ratio are calculated according to the formula:

$$\text{Z value}_{(\text{gene1})} = \log_{10}[\text{raw intensity}_{(\text{gene1})}] - \log_{10}[\text{mean raw intensity}_{(\text{all genes})}] / \text{S. D. } \log_{10}[\text{raw intensity}_{(\text{all genes})}]$$

$$\text{Z difference}_{(\text{gene1})} = \text{Z}_{(\text{gene1, array1})} - \text{Z}_{(\text{gene2, array2})}$$

$$\text{Z ratio}_{(\text{gene1})} = \text{Z difference}_{(\text{gene1})} / \text{S.D.}_{(\text{Z difference all genes})}$$

Hierarchical clustering was determined using software programs developed at Stanford University[22]. And cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisens' laboratory (http://rana.lbl.gov). Clustering of changed in gene expression was determined by using public domain cluster based on pairwise complete-linkage cluster analysis. Gene expression raw data, log values and Z scores were averaged by using the mean ± S.D.

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References

1. Tachikawa, E. *et al*. *In vitro* inhibition of adrenal catecholamine secretion by steroidal metabolites of ginseng saponins. *Biochem Pharmacol* **66**:2213-2221 (2003).
2. Lee, Y. *et al*. A ginsenoside-Rh1, a component of ginseng saponin, activates estrogen receptor in human breast carcinoma MCF-7 cells. *J Steroid Biochem Mol Biol* **84**:463-468 (2003).
3. Bae, E. A. *et al*. Ginsenosides Rg3 and Rh2 inhibit the activation of AP-1 and protein kinase A pathway in lipopolysaccharide/interferon-gamma stimulated BV-2 microglial cells. *Planta Med* **72**:627-633 (2006).
4. Jung, S. H. *et al*. Ginseng saponin metabolite suppresses phorbol ester-induced matrix metalloproteinase-9 expression through inhibition of activator protein-1 and mitogen-activated protein kinase signaling pathways in human astroglioma cells. *Int J Cancer* **118**:490-497 (2006).
5. Lee, S. J. *et al*. Induction of apoptosis by a novel intestinal metabolite of ginseng saponin via cytochrome c-mediated activation of caspase-3 protease. *Biochem Pharmacol* **60**:677-685 (2000).
6. Ambs, S. *et al*. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. *Cancer Res* **58**:334-341 (1998).
7. Cianchi, F. *et al*. Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis. *Am J Pathol* **162**:793-801 (2003).
8. Ambs, S. *et al*. Up-regulation of inducible nitric oxide

synthase expression in cancer-prone p53 knockout mice. *Proc Natl Acad Sci USA* **95**:8823-8828 (1998).
9. Li, J., Billiar, T. R., Talanian, R. V. & Kim, Y. M. Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem Biophys Res Commun* **240**:419-424 (1997).
10. Oh, G. S. *et al*. 20 (S)-Protopanaxatriol, one of ginsenoside metabolites, inhibits inducible nitric oxide synthase and cyclooxygenase-2 expressions through inactivation of nuclear factor-kappaB in RAW 264.7 macrophages stimulated with lipopolysaccharide. *Cance Lett* **205**:23-29 (2004).
11. Helms, S. Cancer prevention and therapeutics: Panax ginseng. *Altern Med Rev* **9**:259-274 (2004).
12. Oh, S. H. & Lee, B. H. A ginseng saponin metabolite-induced apoptosis in HepG2 cells involves a mitochondria-mediated pathway and its downstream caspase-8 activation and Bid cleavage. *Toxicol Appl Pharmacol* **194**:221-229 (2004).
13. Li, G. *et al*. Ginsenoside 20(S)-protopanaxadiol inhibits the proliferation and invasion of human fibrosarcoma HT1080 cells. *Basic Clin Pharmacol Toxicol* **98**:588-592 (2006).
14. Ming, Y. L. *et al*. Anti-proliferation and apoptosis induced by a novel intestinal metabolite of ginseng saponin in human hepatocellular carcinoma cells. *Cell Biol Int* **31**:1265-1273 (2007).
15. Wakabayashi, C. *et al*. An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. *Biochem Biophys Res Commun* **246**:725-730 (1998).
16. Thomsen, L. L. *et al*. Nitric oxide synthase activity in human gynecological cancer. *Cancer Res* **54**:1352-1354 (1994).
17. Zhang, R., Ma, A., Urbanski, S. J. & McCafferty, D. M. Induction of inducible nitric oxide synthase: a protective mechanism in colitis-induced adenocarcinoma. *Carcinogenesis* **28**:1122-1130 (2007).
18. Jenkins, D. C. *et al*. Roles of nitric oxide in tumor growth. *Moncada SProc Natl Acad Sci USA* **92**:4392-4396 (1995).
19. Rao, C. V., Kawamori, T., Hamid, R. & Reddy, B.S. Chemoprevention of colonic aberrant crypt foci by an inducible nitric oxide synthase-selective inhibitor. *Carcinogenesis* **20**:641-644 (1999).
20. DeRisi, J. *et al*. Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat Genet* **14**:457-460 (1996).
21. Cheadle, C., Vawter, M. P., Freed, W. J. & Becker, K. G. Analysis of microarray data using Z score transformation. *J Mol Diagn* **5**:73-81 (2003).
22. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95**:14863-14868 (1998).
23. Lee, S.J. *et al*. Induction of apoptosis by a novel intestinal metabolite of ginseng saponin via cytochrome c-mediated activation of caspase-3 protease. *Biochem Pharmacol* **60**:677-658 (2000).