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cDNA Microarray Analysis of Transcriptional Response to Hyperin in Human Gastric Cancer Cells

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Abstract Antioxidants either scavenge superoxide and free radicals or stimulate the detoxification mechanisms within cells, resulting in increased detoxification of free radicals formation. Hyperin, isolated from the stem of *Uncaria rhynchophylla*, prevented oxygen radical formation and inhibited lipid oxidation. The effective concentrations were 31.3 μM for a radical scavenging assay and 2.2 μM for a microsome assay. cDNA microarray analysis to determine which genes were modulated by hyperin found that 50 genes were upregulated and 37 genes were downregulated in SNU-668 human gastric cancer cells. Among these genes, thirteen genes that were significantly affected by hyperin were verified by RT-PCR for their effect of genetic reprogramming.

Key words: Antioxidant, human gastric cancer cell, hyperin, microarray, RT-PCR

Reactive oxygen species including superoxide, peroxyl, alkoxyl, hydroxyl, and nitric oxide are potent toxins capable of modifying proteins, lipids, and DNA. Such oxidative modifications are considered to play a crucial role in aging and in several degenerative diseases such as cancer, heart disease, immune system deterioration, cataracts, and cognitive dysfunction [1, 8, 11]. The various antioxidants either scavenge superoxide and free radicals or stimulate the detoxification mechanisms within cells, resulting in increased detoxification of free radicals [17].

There has been considerable interest on various compounds as potential antioxidants in living systems. Vitamins C, E and carotenoids have been studied intensively as antioxidants. Besides these antioxidant vitamins, plant polyphenols including phenols, phenolic acids, flavonoids, tannins, and lignans are an important class of defense antioxidants. Among them, flavonoids that act as antioxidants have been extensively studied in the past, and important structure-activity relationships of the antioxidant activity have been established [5, 13, 18]. However, genes modulated by antioxidants are largely unknown. With the advent of cDNA microarray technologies, it is now possible to monitor gene expression levels on a genomic scale.

In the present work, we investigated the antioxidant activity of traditional herbal medicines used in Korea, to search for new active antioxidants from natural resources and to evaluate their effectiveness for gastric cancer cells. In addition, cDNA microarray analysis was used to identify genes modulated by antioxidants in human gastric cancer cells. Gene expression profile in human gastric cancer cells stimulated by antioxidant treatment was compared with that of normal cells. The results of the cDNA microarray analyses were verified by investigating the mRNA level of the selected genes using the RT-PCR technique.

Materials and Methods

Plant Materials and Preparation of Samples

Crude medicinal preparations were commercially available and purchased from Jechon Herbal Inc. (Jechon, Korea) and Dongbang Co. (Seoul, Korea). Crude sample was extracted with 300 ml of 80% methanol under reflux for 8 h, then the extract was evaporated under reduced pressure and stored.

Extraction, Isolation, and Identification

Dried stem of *Uncaria rhynchophylla* (3 kg, cultivated at Jechon in Korea) was sliced and then extracted three times with 80% methanol at room temperature for 24 h. The

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extract was evaporated under reduced pressure and partitioned between ethyl acetate and water. Ethyl acetate extracts after removal of the solvent were chromatographed on a silica gel column. Gradient elution with n-hexane-ethyl acetate with increasing proportions of ethyl acetate generated ten subfractions. RP-18 column chromatography of subfraction 5, eluted with water and methanol (1:1), afforded a pure compound. UV and IR spectra were obtained on a Shimadzu UV-Visible spectrophotometer UV-1601 (Kyoto, Japan) and Perkin Elmer Model 599B (Boston, U.S.A.), respectively. Mass spectra were taken with a JEOL JMS-AX505WA. (Tokyo, Japan) ^{1}H and ^{13}C-NMR spectra were obtained on a JEOL JNM-LA400 (Tokyo, Japan).

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity

Antioxidant solution in methanol (0.1 ml) was added to 3.9 ml of 6×10^{-5} M DPPH methanol solution. The exact initial DPPH concentration in the reaction medium was calculated from a calibration curve. The decrease in absorbance was determined at 515 nm at 0 min and every 30 min until the reaction reached a plateau (about 5 h). Anti-radical activity was expressed as a percentage and the EC_{50}, i.e. the antioxidant concentration necessary to decrease the initial amount of DPPH by 50%.

Lipid Peroxidation in Rat Liver Microsomes

Microsomes were prepared from rat liver by homogenization with 5 volumes of ice-cold 250 mM sucrose containing 5 mM Hepes and 0.5 mM EDTA (pH 7.5) [10]. The microsomes were preincubated in a shaking waterbath at 37°C for 10 min with varying concentrations (0.01–100 μM) of the compound to be tested, followed by lipid peroxidation with 100 μM Fe^{2+} and 500 μM ascorbate. After 60 min incubation, the inhibitory effect on lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances [22]. Briefly, 500 μl of microsomal fraction was added to 500 μl of 20% trichloroacetic acid to stop the reaction, and 500 μl of 0.74% thiobarbituric acid was then added. The mixture was heated in a boiling waterbath for 15 min. After centrifugation, 200 μl of the supernatant was transferred to a tube, and absorbance was measured at 535 nm and compared with standards prepared from the acid hydrolysis of malonaldehyde tetraethylacetyl (Sigma, St. Louis, U.S.A.). Inhibition of lipid peroxidation was expressed as the effective concentration to inhibit 50% (EC_{50}), calculated from the inhibition curve.

Cell Culture and Total RNA Isolation

Human gastric cancer cell line, SNU668, was obtained from Seoul National University Cell Bank (Seoul, Korea) and grown in RPMI 1640 medium containing 10% fetal bovine serum. SNU-668 was treated with hyperin (1 μg/ml) for 16 h. Isolation of total RNA from SNU-668 was carried out by using Trizol agent, according to the manufacturer's instruction manual (Life Technologies, Gaithersburg, U.S.A.).

Microarray Analysis

The labeling, hybridization, and washing of respective cDNAs in cDNA microarray were performed according to the instruction manual provided by the manufacturer (PerkinElmer, Boston, U.S.A.). The control sample (hyperin untreated) and test sample (hyperin treated) were labeled with cy-5 (Red) and with cy-3 (Green), respectively. A set of cDNA microarray slides containing a duplicate set of 2,400 human cDNA spots were used in each analysis of expression profiles. For complete gene description and grid orientation on NEN Micromax cDNA microarray (Boston, U.S.A.), the reader should refer to the NEN web site (www.nen.com.). Fluorescent images of hybridized microarrays were obtained using a gene PIX 4000 axon

Table 1. Primers used in this study.

Genes	Sequences (5'→3')	
Tumor antigen L6	F: TATGGGAAGTGTGCACGATG	R: AAGCCACATATGCCTCCAAG
Putative serine/threonine protein	F: GCAGAAGGACAGGACAAAGC	R: GGCACTCTAACGCTCGTTTC
Protein kinase related to ERK	F: ATGGCTGAGAAGGGTGACTGC	R: GGTACCACTTTGTTACCAACCC
Sigma receptor	F: CTTCCAGCGCGAAGAGATAG	R: GCCAAAGAGGTAGGTGGTGA
Muscle α-actin	F: ATGGTCGGTATGGGTCAGAA	R: TGGCAGCTCGTAGCTCTTTT
Keratin 6 isoform K6e	F: CCAAGGCAGACACTCTCACA	R: CTGGTACTCCTTCAGCAGCC
Iduronate 2 sulfatase	F: CAGCCTCCTCTTCCAGAATG	R: ATGTCCATCCAGGGGTTGTA
Erythrocyte membrane protein	F: GATTTTGGTGGCGTTCTCAT	R: CCCTGGATGCATTCATTTCT
HMG-Y protein isoform	F: AGGGAAGCAAAAACAAGGGT	R: CAAACCAAAAGCCCAGAGAG
Macrophage colony stimulating factor	F: ACCCCAGTTGTCAAGGACAG	R: GGTGATGAGATCCTGGGAGA
Glucose transporter	F: ATGAAGGAAGAGAGTCGGCA	R: TGAAGAGTTCAGCCACGATG
Drg1 protein	F: CTCTGTTCACGTCACGCTGT	R: ATTGGTCGCTCAATCTCCAG
Na-K-Cl cotransporter	F: AGATGTCCATCGATCAAGCC	R: TGAGTTGCAGTCTTGCCATC
GAPDH	F: GGTGAAGGTCGGAGTCAA	R: GATACCAAAGTTGTCATGG

microarray scanner (Axon Instruments, Foster City, U.S.A.) and were analyzed with the Gene PIX program. Single spots or areas with blemishes were excluded from the analysis. The normalization of cDNA concentrations of normal and untreated control samples was carried out by the cDNA microarray hybridization of slides containing house-keeping genes. The expression ratio between treated and untreated control samples was determined by using Gene spring software (Silicon Genetics, Redwood City, U.S.A.).

RT-PCR

Primers used in this study are described in Table 1. Messenger RNA isolated from SNU-668 cells treated with hyperin was converted into cDNA by superscript reverse transcriptase (Life Technologies, Gaithersburg, U.S.A.), and later subjected to PCR [12].

Results and Discussion

Traditional Herbal Medicines Show the Oxygen Scavenging Activity

As shown in Table 2, 10 of the extracts tested showed the oxygen scavenging activity. Among them, the extract of *Uncaria rhynchophylla* showed the strongest antioxidant activity, which has not yet been reported until now. Therefore, we selected *Uncaria rhynchophylla* and purified it for its active component.

Hyperin Isolated from the Stem of *Uncaria rhynchophylla* Has Strong Antioxidant Activity

The methanol extract of the stem of *U. rhynchophylla* was fractionated with ethyl acetate and water. The active compound having radical scavenging activity was isolated by silica gel and RP-18 column chromatography, and the purified compound was identified as hyperin (Fig. 1) by comparing the spectral and physical data with those in the literatures [18].

The biological properties of hyperin were compared with those of commercial antioxidants (BHA, BHT, and α-tocopherol) (Table 3). In particular, the antioxidant activity was investigated *in vitro* by measuring the radical scavenging ability using the free radical DPPH. The protection against lipid peroxidation was assessed with rat liver microsomes. Hyperin showed the highest antioxidant activity with the DPPH and microsome assays. The most potent antioxidant from *U. rhynchophylla* was hyperin, which contains flavonol and four hydroxyl groups, including the catechol structure in the ring. This result showed that the catechol structure is essential for the antioxidant activities of phenolic glycosides [19]. Recently, phenolic glycosides isolated from various plants showed antioxidant activities [4, 16] and their important dietary roles as chemopreventive agents have been suggested. The noted beneficial effects of these bioactive compounds are now considered to be a plausible approach to suppress cancer development [9]. The mechanism by which antioxidants suppress generation of reactive oxygen species in cancer cells has not yet been addressed.

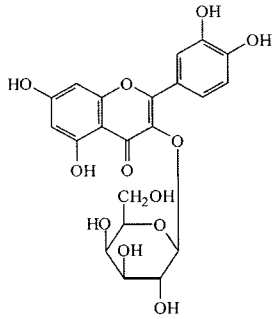

Fig. 1. Chemical structure of hyperin isolated from the stem of *U. rhynchophylla* Miq.

Table 2. Oxygen scavenging activity of traditional herbal medicines.

Scientific name	Used part	Inhibition (%)
Foeniculum vulgare	Seed	34
Melia azedarach	Kernel	26
Mucuna birdwoodiana	Stem	19
Rhaponticum uniflorum	Root	23
Rheum undulatum	Root	23
Stewartia koreana	Branchlet	33
Styrax japonicus	Stem	27
Terminalia chebula	Fruit	27
Uncaria rhynchophylla	Stem	21

Hyperin Influences Gene Expression in SNU-668 Human Gastric Cancer Cell

With the advent of microarray technologies, it is now possible to monitor gene expression on a genomic scale. To identify genes modulated by hyperin, cDNA microarray hybridization analysis was carried out using 2,400 human genes. Thus, the SNU-668 gastric cancer cell line was treated with 1 μg/ml hyperin for 16 h and mRNA was isolated.

Table 3. Antioxidant activities of hyperin and commercial antioxidants (BHA, BHT, and α-tocopherol).

Compound	Microsomes EC_{50} (μM)	DPPH EC_{50} (μM)
Hyperin	2.2±0.3	31.3±3.1
BHA	2.4±0.3	33.8±4.0
BHT	3.2±0.4	48.5±4.5
α-tocopherol	2.8±0.3	35.6±3.8

*The antioxidant activities are expressed by EC_{50} as described under Materials and Methods. Each value is the mean of at least three independent experiments±S.D.

Table 4. Functional classification of genes modulated by antioxidant hyperin.

Categories	Up-regulated number	Down-regulated number
Cytokine and growth factors	0	2
DNA and RNA associated proteins	6	11
Domains	10	6
Electron transport proteins	3	1
Hormones	0	2
Hydrolase	3	2
Inhibitors	1	0
Isomerases	2	0
Ligases	0	0
Lyases	2	1
Other transport proteins	1	1
Oxidoreductases	1	3
Posttranslation modifications	12	2
Protein secretion and chaperons	0	0
Receptors	2	1
Structural proteins	0	3
Transferases	3	2
Others	4	0
Total	50	37

At this concentration, hyperin did not induce appreciable changes of growth of the cancer cells. Therefore, mRNA obtained was converted to cDNA, and cDNA was subjected to cDNA microarray hybridization analysis. It was found that a total of 50 genes were upregulated (>2 fold) and 37 genes were downregulated (<2 fold) by hyperin. Table 3 shows the functional classification of genes modulated by hyperin, and suggests that hyperin acted rather selectively, since about 2% of the genes were upregulated or downregulated. The results of the cDNA microarray analyses were verified by examining selected genes using the RT-PCR technique. Thirteen genes significantly affected by hyperin were selected and assayed by RT-PCR (Table 4).

Genes for human Na-K-Cl cotransporter, glucose transporter, and erythrocyte band 7 integral membrane protein (EPB 72) were induced by hyperin. Interestingly, these genes are membrane proteins involved in membrane transport. Iduronate-2-sulfatase, which is responsible for heparan sulfate and dermatan sulfate degradation [14], was also increased by 2.0-fold. Drg 1 protein, which is expressed during differentiation in colon lumen [20], and macrophage-specific colony-stimulating factor (CSF-1) were upregulated by hyperin. Interestingly, HMGI(Y), a nonhistone chromatin protein implicated in modulating nucleosome and DNA interactions [2], was upregulated by hyperin.

Hyperin also downregulated the sigma receptor, which has a protective effect against cytotoxicity elicited by nitric oxide [7]. The level of MAP kinase isoform p63, known to be implicated in the signal transduction pathway [6], was decreased. Structural genes for skeletal muscle alpha actin and keratin 6 isoform were decreased. Tumor antigen L6, which is highly expressed in lung, breast, colon, and ovarian carcinomas, was also decreased [15]. Serine/threonine protein kinase, which is involved in the functional link between the cellular hydration state and metabolic control, [21] was downregulated.

Changes of gene expression levels, observed by cDNA microarray analysis, were further confirmed by RT-PCR analysis. There was a close correlation between microarray data and RT-PCR analysis (Table 5). These combined results showed that hyperin affected genes involved in cell-differentiation, DNA/RNA metabolism, and membrane transport. In transformed cells, the interaction between reactive oxygen and cellular signaling pathways results in activation of transcription factor, modulation of gene expression, culminating in enhanced proliferation [3].

In this study, genes in gastric cancer cells modulated by hyperin are described. Gastric cancer remains one of the major causes of cancer death in the world. Microarray analysis gives a glimpse into the antioxidation mechanism. Nevertheless, lack of detailed knowledge on sufficient number of genes makes it difficult to understand the mechanism of antioxidation by hyperin. cDNA microarray analysis of gastric cancer cells treated with various antioxidants are currently being carried out. These studies should further reveal molecular mechanisms involved with antioxidants. These studies should help with the classification of these various antioxidants based on expression profiling.

Table 5. Repression (A) and induction (B) of genes in SNU-668 cells treated with hyperin from RT-PCR.

Gene name		RT-PCR	Microarray fold
	C H		
[A]		Tumor antigen L6	0.179
		Muscle α-actin	0.479
		Protein kinase related to ERK	0.42
		Sigma receptor	0.473
		Putative serine/threonine protein	0.433
		Keratin 6 isoform K6e	0.486
		GAPDH	
[B]		Erythrocyte membrane protein	2.013
		HMG-Y protein isoform	2.12
		Drgl protein	3.609
		Macrophage colony stimulating factor	2.159
		Glucose transporter	2.869
		Na-K-Cl cotransporter	6.17
		Iduronate 2-sulfatase	2.007

C denotes SNU-668 untreated and H denotes SNU-668 treated with hyperin (1 μg/ml).

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