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Effects of Japanese herbal crude drug, combined extract of Sasa albo-marginata leaves, Japanese red pine leaves and ginseng roots on gene expression in hypercholesterolemic rat liver

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Abstract

Diet-induced hypercholesterolemia is a major risk factor of adult disease. A commonly used class of drugs for the treatment of hypercholesterolemia is statins that decrease plasma cholesterol levels by inhibiting the cellular activity of 3-hydroxy-3-methyl-glutaryl-CoA reductase, a rate-limiting enzyme of cholesterol biosynthesis. Since the use of statins result in several side effects, it is alternatively beneficial to prevent hypercholesterolemia by habitual intake of natural medicines or crude drugs. In this study, we aimed to clarify the effects of the combined extract of Sasa albo-marginata leaves (*Sasa kurinensis* Makino), Japanse red pine leaves (*Pinus densiflora* Sieb. et Zucc) and ginseng roots (*Panax ginseng* C. A. Meyer) (SJG) on cholesterol homeostasis by using genome-wide expression analysis in the liver of diet-induced hypercholesterolemic rats. For a period of 3 weeks, experimental animal groups were given food and water as follows: standard diet and water, standard diet and 50% (v/v) SJG, high-cholesterol diet (HCD) and water, and HCD and 50% SJG. We then performed genome-wide expression analyses using microarray to obtain a gene expression profile of the liver. Venn diagram was drawn to extract cholesterol-regulated genes whose expressions were altered by SJG. We also performed principal component analysis to visualize the microarray data. We found that the expression of genes involved in cholesterol metabolism, such as P450 7A1 and 8B1 were statistically increased by treatment of SJG in the presence of cholesterol. Although SJG upregulated the expression of P450 7A1, plasma cholesterol levels were not improved by SJG. Further studies are needed to clarify the effects of SJG on cholesterol homeostasis.

Key words cholesterol, CYP7A1, CYP8B1, expression, microarray.

Abbreviations HCD, high-cholesterol diet; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LDLR, low-density lipoprotein receptor; CYP7A1, P450 7A1; CYP8B1, P450 8B1; CYP51, P450 51; PCA, principal component analysis; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; FXR, farnesoid X receptor; BSEP, bile salt export pump; MRP2, multidrug resistance protein 2; ANOVA, analysis of variance.

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Introduction

The prevalence of cardiovascular disease and myocardial infarction has recently risen. Arteriosclerosis is a major cause of myocardial infarction, with atherosclerosis representing the most common form of the condition. Elevated plasma cholesterol levels, especially low-density lipoprotein (LDL) cholesterol level, have been regarded as an important risk factor for the development of atherosclerosis. Indeed, many young people suffering from acute myocardial infarction have preexisting hypercholesterolemia. Nonetheless, the relationship between cholesterol levels and myocardial infarction remains unclear.

Statins are the most widely used drugs for lowering plasma cholesterol levels. These agents selectively inhibit the activity of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol biosynthesis, resulting in decreased cellular cholesterol levels and increased expression of LDL receptors (LDLR) on the cell membrane. Increased LDLR protein expression may potentially lower plasma cholesterol by promoting positive absorption of plasma cholesterol into the cell. Although statins represent powerful tools to lower plasma cholesterol, this medicine has several side effects such as muscle cramping, soreness, fatigue, weakness, and skeletal myopathy.[1,2] Thus, it is beneficial to prevent diet-induced hypercholesterolemia by improvement of life style or with mild natural medicine (s) and crude drug(s) obtained from plants. Recently, many groups have reported that several plant polyphenols can reduce the risk of arteriosclerosis.[3-7] The Sasa albo-marginata extracts, which are commonly used as components of natural medicines, were reported to lower cytokine levels and improve insulin resistance.[8,9] These extracts were also reported to contain several antioxidants.[10] However, whether Sasa albo-marginata extracts can prevent development of cardiovascular disease is unclear.

The Japanese herbal crude drug, combined extract of Sasa albo-marginata leaves (*Sasa kurinensis* Makino), Japanese red pine leaves (*Pinus densiflora* Sieb. et Zucc.) and ginseng roots (*Panax ginseng* C. A. Meyer) (SJG), is a liquid preparation. It is often taken as a diluted solution in water and is reported to have antidepressant and anxiolytic effects in mice.[11,12] In the present study, we aimed to examine the effects of SJG on cholesterol homeostasis and to clarify the molecular mechanism of its effects. We performed genome-wide expression analysis in the liver of diet-induced hypercholesterolemic rats by using the microarray technique and found that administration of SJG increases expression of the cholesterol-metabolizing enzymes P450 7A1 (CYP7A1) and 8B1 (CYP8B1) in the presence of cholesterol.

Materials and Methods

Reagents: All chemicals used in this study were of the highest grade available and were purchased from Wako (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Kanto (Tokyo, Japan). The herbal crude drug SJG was prepared by Wakanyaku Medical Institute, Ltd. (Maebashi, Japan). SJG is composed of a water extract of Sasa albo-marginata leaves (*Sasa kurinensis* Makino) and ethanol extracts of Japanese red pine leaves (*Pinus densiflora* Sieb. et Zucc.) and ginseng roots (*Panax ginseng* C. A. Meyer) in the ratio 8:1:1.[12] Sasa albo-marginata extract contains several active compounds, such as luteolin, tricin, and tricin-glycosides.[10] Pine leaf extract contains proanthocyanidins[13] and kaempferols.[14] Additionally, ginseng extract is known to contain ginsenosides.[15] SJG was supplied as liquid preparation and diluted in tap water to 50% (v/v). Several rats were given 50% SJG instead of water.

HPLC analysis: The chromatographic profiles of SJG were determined using HPLC with a photodiode array system using a Waters 600 pump and controller, 2996 photodiode array detector, and 2487 dual λ absorbance detector (Waters, Milford, MA, USA). SJG and standard compounds were separated in a reverse-phase C18 analytical column (Mightysil RP-18, 250 mm × 4.6 mm, 5 μm particle diameter; Kanto Chemical, Tokyo, Japan). The column temperature was 40℃. The mobile phase consisted of 30:70 (v/v) acetonitrile-water. The UV detection range was set from 195 nm to 400 nm. The flow rate was 1.0 ml/min and the injection volume was 20 μl of 90% acetonitrile extracts of SJG.

Animals and treatment: Sixteen male Wistar rats, aged

13 weeks, were placed in a room with controlled lighting (12 hours light/dark cycle), temperature (23 ± 1℃) and humidity (55 ± 10%). All rats were given food and tap water or 50% SJG ad libitum through experiment. We divided equally the rats into four groups (Group 1, 2, 3, and 4). After 1 week habituation, all rats were fed a standard diet (protein 23%, fat 5%, and nitrogen-free extract 55%; MF, Oriental Yeast Co., Tokyo, Japan) and given water (Groups 1 and 3) or 50% SJG (Groups 2 and 4) for 12 weeks. The rats were then given food and water as follows: standard diet and water (Group 1), standard diet and 50% SJG (Group 2), high-cholesterol diet (HCD) and water (Group 3), and HCD and 50% SJG (Group 4) for three weeks. The experimental schedule and conditions of food and water are summarized in Fig. 1 and Table 1, respectively. The HCD was standard diet supplemented with 0.5% cholesterol and 0.5% cholic acid. After HCD or standard diet feeding for three weeks, the rats were anesthetized with diethyl ether and sacrificed. The plasma were collected for measurement of cholesterol levels and the livers were collected for RNA extraction. Throughout the study, body weights were measured. Animal experiments were approved by the Animal Research Committee of Wakanyaku Medical Institute, Ltd. (Permission Number: WA-2011-03), and performed in accordance with the Guidelines for Care and Use of Laboratory Animals at Wakanyaku Medical Institute, Ltd. and the Guidelines for Proper Conduct of Animal Experiments from Science Council of Japan.

Plasma biochemical parameters: Blood samples were taken from postcaval vein with vacuum blood collection tube containing heparin. Plasma levels of total cholesterol, free cholesterol, and cholesteryl ester were determined by using L-type Wako cholesterol, and L-type Wako free cholesterol, and L-type Wako cholesterol and L-type Wako free cholesterol, respectively (Wako). Plasma levels of high-density lipoprotein (HDL) cholesterol, and LDL cholesterol were measured by using Cholestest N HDL, and Cholestest LDL (Sekisui Medical Co., Ltd, Tokyo, Japan).

RNA isolation and microarray analysis: Total RNA was extracted from frozen rat livers using a High Pure RNA Isolation Kit (Roche, Mannheim, Germany) and

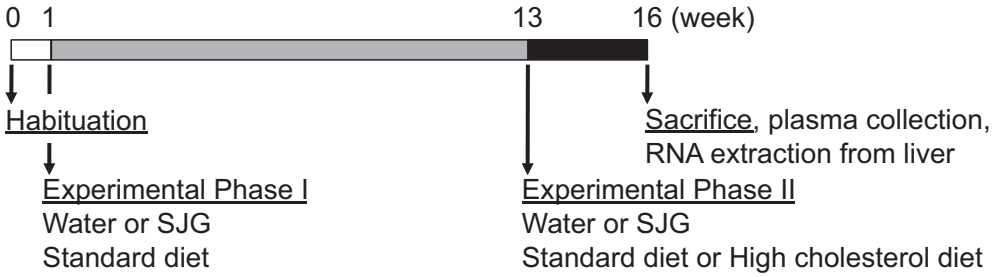

Fig. 1 Experimental schedule to evaluate the effects of SJG on diet-induced hypercholesterolemia. All rats were acclimatized for a week and fed a standard diet through habituation and experimental phase I. After habituation, rats were divided into four groups. Group 1 and group 3 received water throughout the experimental phase, while groups 2 and 4 received 50% SJG. In experimental phase II, groups 3 and 4 were fed a high-cholesterol diet.

Table 1 Experimental conditions, body weight, and cholesterol levels in plasma or liver.

Group	Experimental PhaseI		Experimental Phase II		body weight (g)	Plasma (mg/dl)					Liver (mg/100g)
	drink	food	drink	food	(at sacrifice)	Total cholesterol	LDL cholesterol	HDL cholesterol	Free cholesterol	cholesteryl esters	Total cholesterol
1	Water	Standard	Water	Standard	387.8 ± 7.6 ^{a}	66.0 ± 0.8 ^{a}	5.8 ± 0.2 ^{a}	17.6 ± 0.4 ^{a}	9.8 ± 0.4 ^{a}	56.2 ± 0.6 ^{a}	506.8 ± 9.8 ^{a}
2	SJG	Standard	SJG	Standard	391.9 ± 5.4 ^{a}	63.8 ± 3.0 ^{a}	6.0 ± 0.4 ^{a}	17.8 ± 0.4 ^{ac}	9.4 ± 0.7 ^{ac}	54.4 ± 2.3 ^{a}	488.0 ± 8.8 ^{a}
3	Water	Standard	Water	Cholesterol	381.9 ± 10.1 ^{a}	93.8 ± 5.1 ^{b}	18.4 ± 1.7 ^{b}	22.0 ± 0.5 ^{b}	14.0 ± 0.8 ^{b}	79.8 ± 4.3 ^{b}	3582.5 ± 271.0 ^{b}
4	SJG	Standard	SJG	Cholesterol	386.0 ± 10.4 ^{a}	91.2 ± 5.2 ^{b}	21.4 ± 2.0 ^{b}	19.2 ± 0.6 ^{c}	12.8 ± 0.7 ^{bc}	78.4 ± 4.5 ^{b}	4119.3 ± 298.5 ^{b}

Each value is the mean ± S. E. of four indivisuals. Different letters (a, b, and c) denote a statistically significant difference in means ($p < 0.05$; one-way ANOVA with Bonferroni's multiple-comparison post-tests for multiple comparisons).

subjected to oligonucleotide array (Agilent Microarray Design ID 028279; Agilent, Santa Clara, CA, USA) analysis by using a single-color labeling system (Low Input Quick Amp Labeling Kit for one color; Agilent). We performed *t* tests to compare signals between two groups and a Venn diagram was drawn to extract cholesterol-regulated genes whose expressions were altered by SJG with Subio platform software. We also performed a principal component analysis (PCA) to visualize the gene expression profile for each sample by using the significantly selected gene set as analyzing parameter with Subio platform software.

Quantitative real-time polymerase chain reaction (PCR): After the RNA samples were treated with reverse transcriptase (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, CA, USA), specific RNA transcripts were quantified by quantitative real-time PCR by using Power SYBR Green Master Mix and an ABI Prism 7000 real-time PCR system (Applied Biosystems). To standardize each experiment, the amount of each gene transcript was expressed as a percentage of RNA for the ubiquitously distributed transcription factor YY1 in the same sample. The primer sequences used to amplify each gene included: CYP7A1, forward 5'-TTGGCGGCTGAGAGTCATC-3', reverse 5'-TCGCTAGGGCGCATCAGT-3'; CYP8B1, forward 5'-ACGCAGAAAGTGCTAGACTTCGT-3', reverse 5'-TGGTACCCAAACACCTTGAACA-3'; and YY1, forward 5'-CCAAGCAACTGGCAGAATTT-3', reverse 5'-CCTTTATGAGGGCAAGCTATTG-3'.

Statistical analysis: The real-time PCR data are presented as means ± S.E. Differences between the groups were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison post-tests to evaluate the significance of the differences. A value of $p < 0.05$ was considered statistically significant.

Results

HPLC analysis of SJG: SJG is a mixture of extracts of Sasa albo-marginata leaves, pine leaves, and ginseng roots, which are known to contain tricin, *p*-coumaric acid and catechin, and ginsenosides, respectively. Accordingly, we performed HPLC analysis to examine the SJG components and found that it may contain several compounds such as *p*-coumaric acid, (+)-catechin, ginsenoside Rb1, and tricin (Fig. 2).

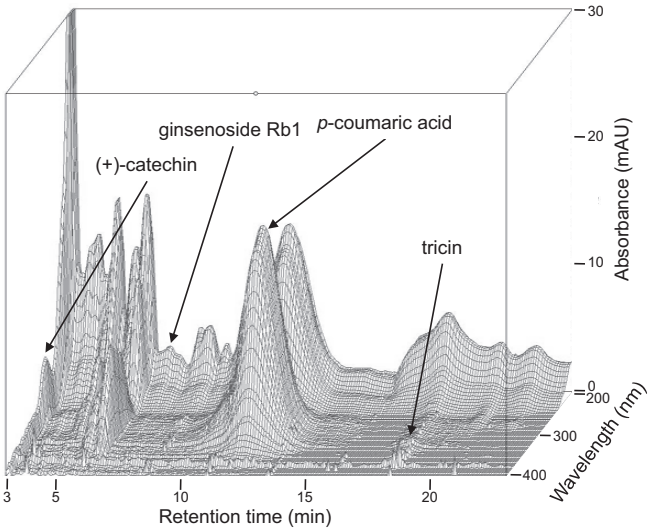

Fig. 2 Analytical HPLC chromatograms of SJG. Three-dimensional photodiode array chromatogram of the same lot of SJG used in this study is presented. Several standard compounds, such as *p*-coumaric acid, (+)-catechin, ginsenoside Rb1, and tricin were separated using the same conditions as SJG, and the peak for each compound is represented in the figure. The detailed HPLC conditions are described in the Materials and methods section.

Effects of SJG on body weight and cholesterol levels in plasma and liver: After 1-week habituation, the rats were divided into four groups. During experimental phase II (Fig. 1), the animals were treated with either water and standard diet (group 1), SJG and standard diet (group 2), water and HCD (group 3), or SJG and HCD (group 4). No significant differences in the body weight (Table 1) and the intake of food and water between groups were observed (data not shown). The plasma and liver cholesterol levels for each group are shown in Table 1. In plasma, elevated levels of total cholesterol, LDL cholesterol, HDL cholesterol, free cholesterol, and cholesteryl ester were observed as a result of HCD intake. The HCD-induced hypercholesterolemia was not improved by treatment with SJG (group 3 vs. group 4). Similarly, diet-induced elevated total cholesterol levels in the liver were not altered by treatment of SJG.

Effects of SJG on the genome-wide expression profile: We examined the effects of SJG on gene

expression profiles in the liver of HCD-induced hypercholesterolemic rats by using the microarray technique. First, we evaluated the expression of several genes related to the synthesis of cholesterol, such as HMGCR, CYP51, NAD(P)-dependent steroid dehydrogenase-like, 24-dehydrocholesterol reductase, squalene epoxidase, farnesyl diphosphate farnesyl transferase 1, transmembrane 7 superfamily member 2, hydroxysteroid (17-beta) dehydrogenase 7, and lanosterol synthase. We also evaluated the expression of sterol regulatory element binding transcription factor 2, which regulates the expression of cholesterol synthesizing enzymes, and LDLR. The transcripts of all genes were decreased by HCD and SJG had little or no effect on the altered expression of these genes (Fig. 3).

We then aimed to select the genes, which exhibited altered expression following intake of HCD and which were further modified by treatment of SJG. Thus, we used a Venn diagram to select the overlapped genes of three comparisons, group 1 vs. group 2 to exclude the drug potency in the absence of cholesterol, group 1 vs. group 3 to select the genes that respond to the cholesterol, and group 3 vs. group 4 to select the genes with expressions that were sensitive to SJG in the presence of cholesterol. As a result, we selected 44 overlapping genes (Fig. 4A). We conducted PCA to examine the validity of these 44 genes and to visualize the results of the

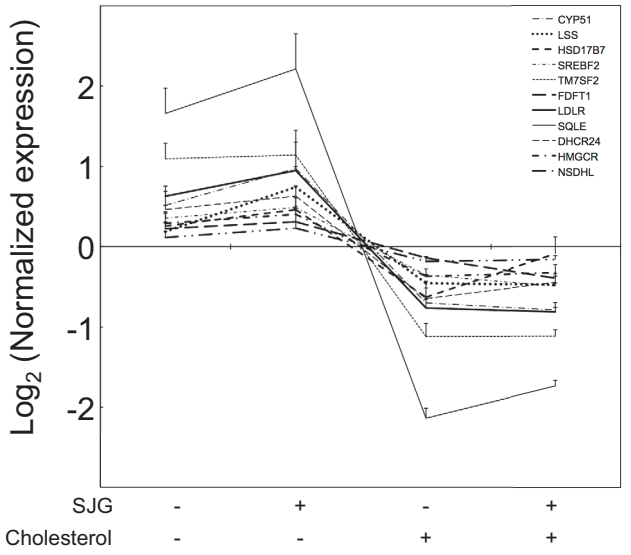

Fig. 3 Line graph of the expression levels of genes associated with cholesterol synthesis. Data from the microarray analysis are shown. The vertical axis represents the $\log_2$ normalized expression of cytochrome P450, family 51 (CYP51); NAD(P)-dependent steroid dehydrogenase-like (NSDHL); 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR); 24-dehydrocholesterol reductase (DHCR24); squalene epoxidase (SQLE); low density lipoprotein receptor (LDLR); farnesyl diphosphate farnesyl transferase 1 (FDFT1); transmembrane 7 superfamily member 2 (TM7SF2); sterol regulatory element binding transcription factor 2 (SREBF2); hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7); lanosterol synthase (LSS). Legends of each line are shown in the figure.

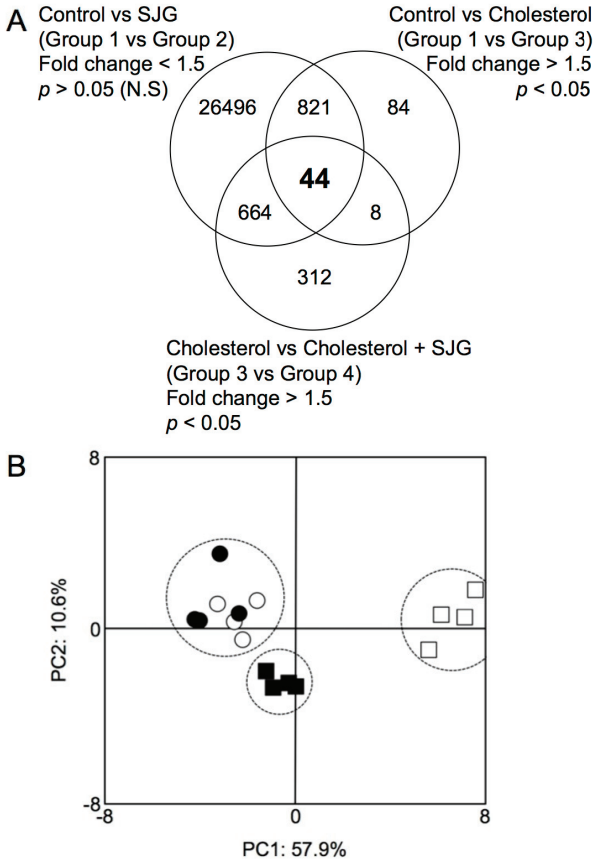

Fig. 4 The results of genome-wide expression analysis in the liver of diet-induced hypercholesterolemic rats. (A) Venn diagrams showing the number of genes altered by cholesterol or a combination of cholesterol and SJG. A total of 28,025 genes were not altered in response to SJG only. Among these genes, 865 (821 + 44) or 708 (664 + 44) genes were altered in response to cholesterol or a combination of cholesterol and SJG, respectively. Forty four commonly altered genes were selected as good parameters to distinguish each treatment group. (B) Principal component analysis (PCA) of gene expression in all samples using the significantly selected gene set as analyzing parameter. The microarray data were analyzed with PCA by using Subio platform software. The two-dimensional plot view of gene expression data is shown with respect to their correlation to the first two principal components. Approximately 68.5% variation was revealed in the samples in the first two principal components. Each sample used for microarray was plotted with the same symbol for each group (open circle, group 1, water and standard diet; solid circle, group 2, SJG and standard diet; open square, group 3, water and high-cholesterol diet; solid square, group 4, SJG and high-cholesterol diet).

microarray analysis. PCA is the statistical method to visualize high dimensional data. A set of observations of possibly correlated variables is converted into a set of values of uncorrelated variables, which is called principal components, by orthogonal transformation. The results were plotted on two-dimensional axes (principal components 1 and 2), and 68.5% (57.9% + 10.6%) sample variation was revealed in the two principal components (Fig. 4B). On the plot, three clusters consisting of group 1 and 2, group 3, and group 4 were observed. The group 4 cluster was plotted closer to the group 1 and 2 cluster than to the group 3 cluster. These results suggest that the selected 44 genes might be an appropriate marker to examine the effects of SJG on cholesterol homeostasis. Furthermore, expression of the selected genes was altered by HCD, an effect that may be recovered by treatment of SJG. The selected genes are listed in Table 2.

Effects of SJG on the expression of CYP7A1 and 8B1: Bile acid synthesis is the most important pathway of cholesterol metabolism. The list of genes (Table 2) contains CYP8B1, which is required for the synthesis of cholic acid and determines the ratio of cholic acid to chenodeoxycholic acid. Thus, we examined gene expression of CYP8B1 and CYP7A1, the rate-limiting enzymes of cholesterol metabolism, by using quantitative real-time PCR (Fig. 5). We found that SJG significantly

Table 2 The list of cholesterol-regulated genes whose expressions were altered by SJG.

Agilent ProbeID	Genbank	UniGene	Symbol	Name
A_44_P427695	NM_001009683	Rn.2164	Tor3a	torsin family 3, member A
A_64_P050979				GTAATAAATTCTGGACATTTGTTATTCCTAAACAGGCATTTCACAGGCTCTACCCACTCT
A_44_P357870	NM_031559	Rn.2856	Cpt1a	carnitine palmitoyltransferase 1a, liver
A_64_P312191	XM_001078700	Rn.19449	Zfc3h1	zinc finger, C3H1-type containing, transcript variant 2
A_64_P003176				AGTCTAAATACAGAAAGGAGAACCACCATAATGGGAGAGACTCTACCAAAGGCAGACACC
A_44_P238424	NM_133515	Rn.122003	Akap5	A kinase (PRKA) anchor protein 5
A_64_P060302				ATTGTCCAGCCGCAGCTTCCTGTTAAGGTGGGATTCTGCCAAGGGGAAGCAGATGATGTG
A_64_P150519	NM_012944	Rn.10159	Drd4	dopamine receptor D4
A_64_P084836	XM_576721		LOC501308	hypothetical gene 2 supported by BC059164
A_64_P035261				TAAAAACAAGGATATCAGAAAGGTTTTGGATGGCATCCATGTGTCTGAGAGGGGAACCGT
A_64_P038847	X62889		FAS	FAS gene for fatty acid synthase
A_64_P147385				ATTTCCAAAGACCCTTTCCCGGGTGACAGCTTCTTCCAGGAAGTCCCCCAAGAACTGCCA
A_64_P122795	NM_001109546	Rn.199531	Vsx1	visual system homeobox 1
A_64_P076057	XM_001064969	Rn.225484	LOC685716	similar to OX-2 membrane glycoprotein precursor (MRC OX-2 antigen) (CD200 antigen)
A_64_P008874				AAGCCATCTGGGCCAAAGGAATAAGGAATGTTAAGGAATGTTCCATACCACATCGGAGTA
A_64_P013324	NM_001107865	Rn.33103	Tcf3	transcription factor 3
A_64_P045807	NM_001108562	Rn.17547	Cyb561d1	cytochrome b-561 domain containing 1
A_64_P080846				AAGCTAAATTTACTAGACTAAAGGTTTCACTATGCAACCCAGGCTATCCCGGAACTCTAT
A_44_P387780	NM_031241	Rn.23013	Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1
A_64_P112429	NM_001037549	Rn.155416	Defb5	defensin beta 5
A_64_P020506	NM_001106813	Rn.153173	Crtam	cytotoxic and regulatory T cell molecule
A_64_P153674	NM_001106949	Rn.105030	Mbnl3	muscleblind-like 3 (Drosophila)
A_44_P388801	NM_001000080	Rn.142343	Olr1583	olfactory receptor 1583
A_44_P791030	XM_575656	Rn.225874	Cecr6	cat eye syndrome chromosome region, candidate 6 homolog (human)
A_44_P461291	NM_001177817	Rn.220343	LOC100362040	Ac2-143-like
A_44_P421391	NM_198763	Rn.52251	Slc1a4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4
A_64_P004622				AAGAAGAAGAAGAAGAGAAGGAAAGAAAAAAGAAAAGAAAAGAAAGTTTATAAGCGCAAC
A_64_P139078	NM_017265	Rn.109394	Hsd3b6	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6
A_64_P034094	NM_001000121	Rn.103618	Olr35	olfactory receptor 35
A_44_P369825	NM_023956	Rn.209304	Gucy1a2	guanylate cyclase 1, soluble, alpha 2
A_64_P021104				ATATTAGGAAAATGTAAGGTTACAGAGACAGAGACAGAGACAGAGACAGAGAGAGAGAGA
A_44_P989658				AGCAGCTGTCTATTTCGGCCAGAAACTTGGAGCCCGCCCAGAAGCTGTCTACTGCAAGCA
A_44_P471270	XM_232762			similar to ribosomal protein L23a
A_64_P133502				TACCCTGGCATGCTGACTTAGAATGTTAATAAGGATCTGTTCGTCTTTCCAGCCCAACCT
A_64_P091128	NM_001109521	Rn.130189	LOC689065	hypothetical protein LOC689065
A_64_P133421	XM_002725600		Eid2b	EP300 interacting inhibitor of differentiation 2B
A_64_P078017				TGTACAAATTGATTCTGACATGACTCATGTACCAGGGGGTTTGTGTTCCAGGAACTCGGA
A_44_P832019	NM_001014235	Rn.140721	Phf11l	PHD finger protein 11-like
A_64_P114970	XM_001065185	Rn.201707		OX-2 membrane glycoprotein homolog
A_43_P16284	NM_001135249	Rn.198797	Rarg	retinoic acid receptor, gamma
A_44_P384389				AGTATTTGGAGATTCCCAGAAAGTACATCCCTGAAACAAAAATGATCTTCACTGGAATTA
A_64_P155039	XM_342445	Rn.214587	Slc25a12	solute carrier family 25 (mitochondrial carrier, Aralar), member 12
A_64_P120694				ATGAGCTGTAGTCTTGGCATGGGGTCACTGTCAGACATAACTTTTATTAGTCCTTGGGGT
A_64_P010052				CATATCAGGAGGCTCACAATAACCTGTAAGGGATCCAGCATCTTAATCTAGTTTAAAAAA

The information of each genes (probe ID, genbank ID, unigene ID, symbol, and name) are represented. The sequence of probes which have no annotation is represented in "name" column.

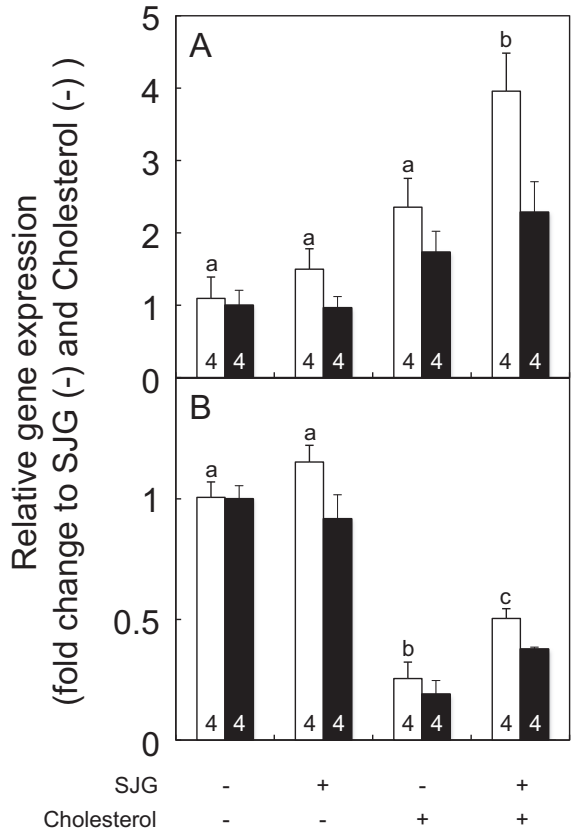

Fig. 5 Effect of SJG on high cholesterol diet-induced alterations in gene expression in the rat liver. Total RNA was extracted from the rat livers after experimental phase II. They were treated with water and standard diet (group 1), SJG and standard diet (group 2), water and high-cholesterol diet (group 3), SJG and high-cholesterol diet (group 4). We examined gene expression of CYP7A1 (A) and CYP8B1 (B). The data were analyzed using quantitative real-time PCR (open bar of each panel) or microarray (solid bar). The vertical axis represents the ratio of the quantity of target gene transcript for each sample to that of group 1 samples as a magnitude of induction (fold change) after normalization with YY1 gene, which is a ubiquitously distributed transcription factor (quantitative real-time PCR) or after global normalization (microarray). The number of replicates is shown in each bar. Each value is the mean ± S.E. Different letters (a, b, and c) denote a statistically significant difference in means ($p < 0.05$; one-way ANOVA with Bonferroni's multiple-comparison post-tests for multiple comparisons).

increased expression of CYP7A1 and CYP8B1 in the presence of cholesterol.

Discussion

In the present study, we performed a genome-wide gene expression analysis to examine the beneficial effects of SJG on diet-induced hypercholesterolemia. First, we analyzed the levels of cholesterol in the plasma and liver and found no effects of SJG on cholesterol levels (Table 1). Other groups have previously reported that plasma cholesterol levels in HCD-induced hypercholesterolemic rats reach about 200 mg/dl while control levels are 70-100 mg/dl.[16-19] In our results, plasma cholesterol levels of the HCD-fed groups (group 3 and 4) were about 90 mg/dl while those of the standard diet-fed groups were nearly equal to that reported in other reports (Table 1).[16-19] We suspect that the milder hypercholesterolemia observed in this study may have been due to the lower cholesterol concentration in the HCD that was used, compared with those used in other reports. Thus, it may be important to examine the effects of the drug(s) in animals with more severe hypercholesterolemia. Statins decrease plasma cholesterol levels by inhibition of HMGCR activity. This inhibition causes a decrease of cholesterol levels and an increase in LDLR mRNA in the liver. Increased LDLR density results in the positive absorption of plasma cholesterol, which decreases plasma cholesterol levels.[20] In the present study, cholesterol levels were elevated (Table 1) and the levels of LDLR mRNA were decreased (Fig. 3) in the liver of hypercholesterolemic rats, and SJG did not reduce cholesterol levels or increase LDLR mRNA. Thus, elevated plasma cholesterol levels may not be decreased in SJG-treated rats.

Several genes of interest, such as CYP8B1 and fatty acid synthase (FAS), were listed in the 44 genes selected by microarray (Table 2). The expression of CYP8B1 (Fig. 5B) and FAS (data not shown) was decreased in response to the HCD, an effect that was prevented by treatment with SJG. Numerous fatty acids work as an agonist of peroxisome proliferator-activated receptor (PPAR) α. Additionally, it is reported that PPARα decreases the expression of CYP7A1[21] and increases the expression of CYP8B1.[22] These results might suggest that PPARα plays a role in altering the expression of CYP7A1 and CYP8B1, as we observed in this study. Cholesterol-induced reduction of CYP8B1 was recovered by treatment of SJG (Fig. 5). CYP8B1 plays a role in production of primary bile acid cholic acid. The decrease in CYP8B1 expression might lead to a reduction of cholic acid and increased chenodeoxycholic acid, which is a powerful ligand of farnesoid X

receptor (FXR) that negatively regulates the expression of CYP7A1.[23] Thus, the prevention of cholesterol-induced reduction of CYP8B1 by SJG could effectively result in increased expression of CYP7A1. Furthermore, the expression of CYP7A1 is also regulated by other transcription factors such as pregnane X receptor, liver X receptor, liver receptor-homolog 1, hepatocyte nuclear factor 4, and small heterodimer partner.[24-28] Tumor necrosis factor and cytokines, such as interleukins, are also reported to control the expression of CYP7A1.[29,30] Because the expression of CYP7A1 is intricately regulated by several factors, further studies are needed to clarify the mechanisms of CYP7A1 upregulation by SJG in the presence of cholesterol. When considering effects on cholesterol homeostasis, we could not exclude the potential role of transporter(s) that play a role in excretion of cholesterol and bile acids and in resorption of bile acids in the enterohepatic circulation. In particular, the bile salt export pump (BSEP) and multidrug resistance protein 2 (MRP2), which are involved in the excretion of bile acids from liver, are positively regulated by FXR.[31-33] In our microarray results, the expressions of BSEP and MRP2 in the liver were induced by the intake of HCD, and the induction was not altered by the treatment of SJG (data not shown). The HCD-induced upregulation of CYP7A1 in the presence of cholesterol might result in the increase of bile acids and in the induction of these transporters. Further study is needed to evaluate the intestinal expression of these in rats with severe hypercholesterolemia.

In conclusion, we confirmed the usefulness of SJG for the upregulation of CYP7A1 in hypercholesteromic rats in this study. This upregulation may be beneficial in preventing several diseases. Indeed, transgenic mice, which overexpress CYP7A1, resist high-fat diet-induced obesity, fatty liver and insulin resistance.[34] Thus, SJG may potentially help to prevent ischemic heart disease.

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