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Nutrigenomic Studies of Effects of *Chlorella* on Subjects with High-Risk Factors for Lifestyle-Related Disease

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ABSTRACT In order to clarify the physiological effects of *Chlorella* intake on subjects with high-risk factors for lifestyle-related diseases, we conducted *Chlorella* ingestion tests on 17 subjects with high-risk factors for lifestyle-related diseases and 17 healthy subjects over a 16-week period, including a 4-week post-observation period. We conducted blood biochemical tests and analyzed gene expression profile in whole blood cells in the peripheral blood before and after *Chlorella* intake. We confirmed that in both groups, *Chlorella* intake resulted in noticeable reductions in body fat percentage, serum total cholesterol, and fasting blood glucose levels. Through gene expression analysis, we found that gene expression profiles varied with *Chlorella* intake and identified many genes that exhibited behavior such that after the completion of the intake period, expression levels returned to pre-intake expression ones. Among these were genes related to signal transduction molecules, metabolic enzymes, receptors, transporters, and cytokines. A difference in expression level was found between the two groups at the start of the tests, and we were able to identify genes with noticeable variance in expression level resulting from *Chlorella* intake in the high-risk factor group. These included genes involved in fat metabolism and insulin signaling pathways, which suggests that these pathways could be physiologically affected by *Chlorella* intake. There were clear variations in the expression profiles of genes directly related to uptake of glucose resulting from *Chlorella* intake, indicating that the activation of insulin signaling pathways could be the reason for the hypoglycemic effects of *Chlorella*.

KEY WORDS: • ***hypocholesterolemic effect*** • ***hypoglycemic effect*** • ***insulin*** • ***insulin signaling pathway*** • ***gene expression*** • ***microarray*** • ***peripheral blood***

INTRODUCTION

*C*HLORELLA HAS BEEN CONSUMED by humans as a food supplement for generations because it is rich in essential nutrients, including high-quality protein, vitamins, minerals, and essential amino acids. In recent years, it has gained attention as a health food because it offers an outstanding balance of nutritional elements. Notably, it has been reported that *Chlorella* demonstrates physiological effects such as immune activation,[1] growth promotion,[2] and improvement in stress-related ulcers.[3] Most of this prior research, however, used rats, mice, and other laboratory animals; almost no large-scale clinical research has been undertaken to evaluate its physiological effects in humans.

The physiological effects of specific foods have long been known empirically, but because foods are composed of diverse components, it has been difficult to identify the components that are effective in bringing about desirable physiological effects. At the same time, there has been little research conducted on the mechanisms of these physiological effects, because of the lack of effective testing and analytical methods that take into account multiple aspects of these physiological effects. Amid a growing demand for health foods in recent years, there have been calls for proof of the effectiveness of these foods in humans, and for clarification of the mechanisms involved. The term "evidence-based food" is representative of this trend.[4]

Since the announcement in 2004 that the entire human genome sequence had been decoded, attention has been focused on a new research field called "nutrigenomics," which attempts to examine the utility of food products based on genomic information.[5] Specifically, a DNA microarray that can examine expression level of several thousand to several tens of thousands of genes in a single assay has come to be used extensively as a new tool for investigating diverse reactions in living organisms. This microarray technology is extremely well suited to comprehensive examinations of complex reactions in living organisms in which multiple components bring about complex effects, as in the case when food is ingested.

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In this research, we analyzed the gene expression profiles in all peripheral blood cells following *Chlorella* intake in 17 subjects with high risk factors for lifestyle-related diseases (diabetes or hyperlipemia) and 17 healthy subjects. We also gathered biochemical test data from these subjects.

As a result of these tests, we confirmed that in both groups, *Chlorella* intake resulted in noticeable reductions in body fat percentage, total blood serum cholesterol, and fasting blood glucose levels. Through gene expression analysis, we found that gene expression varied with *Chlorella* intake and identified many genes whose expression returned to pre-intake expression levels after the completion of the intake period. In the results for insulin signaling pathways in particular, variations were observed in the gene clusters directly related to active uptake of glucose, which provides evidence for the blood glucose reduction effects of *Chlorella*.

MATERIALS AND METHODS

Subjects

The subjects were healthy Japanese males 20 years of age or older. On three occasions—6 weeks, 4 weeks, and 2 weeks—before the start of the ingestion tests, the subjects were examined by a physician and also received physical examinations, clinical examinations, and glucose tolerance tests. Based on the conditions outlined below, 34 subjects were selected, with 17 assigned to each of two groups: the high-risk factor (for lifestyle-related diseases) group (referred to here as the "D Group") and the normal healthy subject group (referred to here as the "N Group").

Healthy subject group (N Group). At all three periods before beginning the tests, fasting blood glucose, total blood serum cholesterol, and concentration of triglycerides in the blood were within normal limits. These subjects were also judged as having normal glucose tolerance.

High-risk factor group (D Group). Subjects who at all three periods before beginning the tests (a) exhibited borderline high fasting blood glucose and total blood serum cholesterol and high triglycerides in blood and who were also judged as having low glucose tolerance or (b) demonstrated borderline high fasting blood glucose, were judged as having low glucose tolerance, and were also judged to have total blood serum cholesterol and serum triglycerides that deviated slightly from normal limits. Attributes for subjects in the N Group and D Group are summarized in Table 1. Serum was used for biochemical tests. Body fat percentage was evaluated by measuring bioelectrical impedance.

Materials tested

The *Chlorella* used in these tests was "Sun Chlorella A" tablets (Sun Chlorella Corp., Kyoto, Japan), which contains dried *Chlorella* powder (more than 95.5%) as the active ingredient and lecithin (less than 4.5%) as a bulking agent. The *Chlorella* powder contained in the tablet was prepared by crushing the cell wall in a DYNO®-Mill (WAB, Inc., Basel, Switzerland) and spray-drying. The subjects took 20 tablets each morning and evening (total, 40 tablets/day) after meals with either cold or hot water.

Test design

This research protocol was approved by the Testing Committee at Miyawaki Orthopedic Hospital (Hokkaido, Japan). Before the tests began, the subjects received a written explanation and consent form from the physicians responsible for the tests. After receiving an explanation of the purpose and value of the tests as well as the methods, expected effects, and potential risks, etc., the subjects themselves confirmed their understanding of the details explained and then provided written consent indicating that they were participating of their own free will.

TABLE 1. CHARACTERISTICS OF HEALTHY AND HIGH-RISK SUBJECT GROUPS

Characteristic	*Healthy subject group (N Group)*	*High-risk factor group (D Group)*
Age (years)	34.3 ± 3.2	59.2 ± 1.9
Height (cm)	170.7 ± 1.6	164.5 ± 1.3
Body weight (kg)	64.4 ± 2.2	66.1 ± 1.9
Body fat percentage	19.8 ± 1.5	24.2 ± 1.1
Body mass index (kg/m^2)	21.8 ± 2.2	24.4 ± 2.1
Cholesterol (mg/dL)		
Total	173.8 ± 18.0	218.4 ± 38.7
HDL	59.5 ± 12.0	61.0 ± 11.7
LDL	101.2 ± 18.5	127.4 ± 37.0
Triglycerides (mg/dL)	59.3 ± 21.4	124.4 ± 76.6
Fasting blood sugar (mg/dL)	82.4 ± 7.2	111.4 ± 30.1
Hemoglobin A1c (%)	4.5 ± 0.2	5.3 ± 0.8
Immunoreactive insulin (mU/mL)	5.0 ± 3.1	6.1 ± 4.0

Data are mean ± SD values.

The tests were conducted in an open test format. The intake period lasted for 12 weeks; blood biochemical tests were conducted every 4 weeks, and again 4 weeks after the completion of the intake period.

During the test period, one subject from the N Group dropped out because of stomach pains, but no other subjects dropped out during the testing period. Data for statistical analysis were thus gathered for 17 subjects in the D Group and 16 subjects in the N Group. None of the subjects in either group reported any complications that could be considered to indicate harmful side effects during any of the four physical examinations conducted during the testing period.

Gene expression analysis

The blood sampling and RNA extraction for gene expression analysis was conducted using a PAXgene™ Blood RNA kit (manufactured by PreAnalytiX GmbH, Hombrechtikon, Switzerland), and a model 2100 Bioanalyzer (manufactured by Agilent Technologies, Palo Alto, CA) was used to confirm that there was no breakdown in the extracted RNA (rRNA profile used as a reference). RNA amplification reactions were checked based on the *in vitro* transcription method using primers with T7 promoter sequence. At this time, cRNA was synthesized through the uptake of dUTP with an aminoallyl group. The cRNA with Cy5 la-

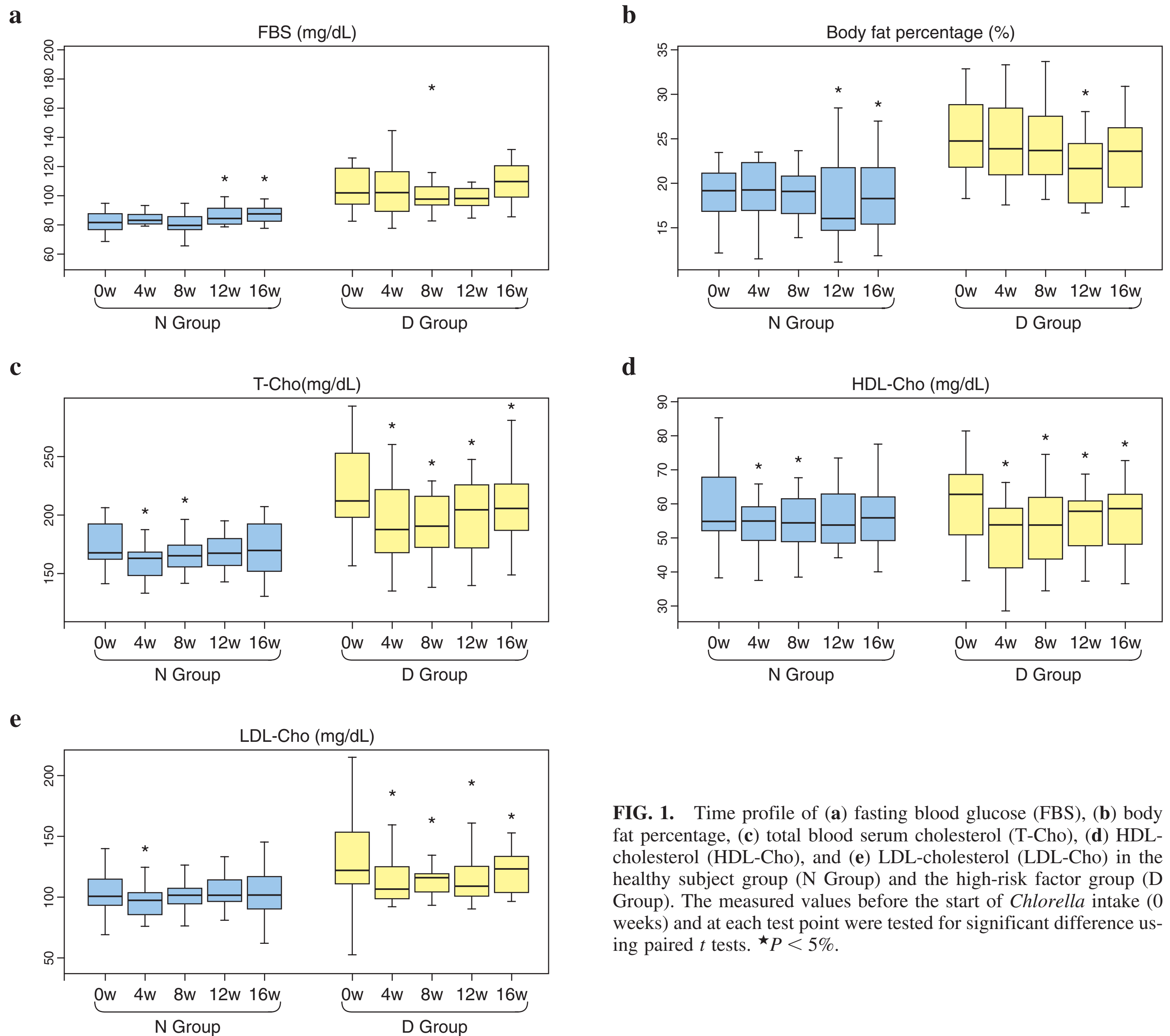

FIG. 1. Time profile of (**a**) fasting blood glucose (FBS), (**b**) body fat percentage, (**c**) total blood serum cholesterol (T-Cho), (**d**) HDL-cholesterol (HDL-Cho), and (**e**) LDL-cholesterol (LDL-Cho) in the healthy subject group (N Group) and the high-risk factor group (D Group). The measured values before the start of *Chlorella* intake (0 weeks) and at each test point were tested for significant difference using paired *t* tests. $^{\star}P < 5\%$.

beling was synthesized by applying coupling reactions to cRNA and Cy5 with a succinimide group (manufactured by GE Healthcare, Chalfont St. Giles, UK).

As the control sample for expression analysis, we used total RNA from commercially available human white blood cells (from Clontech Laboratories, Palo Alto), and Cy3 was used as a fluorochrome. In addition, we synthesized cRNA with the same labeling as the above blood-derived sample and used this cRNA as a common comparative reference sample for all blood-derived samples.

For the microarray, we used a custom microarray with additional loading of diabetes-related genes and other genes on a human drug response DNA chip (manufactured by Hitachi Ltd., Saitama, Japan) (number of genes loaded, 1,873). After the labeled cRNA is mounted on the microarray, it is subject to competitive hybridization at 45°C for 17 hours. Once hybridization is complete, the unit is washed and dried, and fluorescent images are captured using a scanner (ScanArray5000, GSI Lumonics, Billerica, MA). Through numerical processing, we then derived the variable ratio for expression intensities between the samples and the reference samples for each of the relevant genes. The global normalization method was used for normalization of Cy5 and Cy3.

For expression data analysis, we used GeneSpring (Agilent) and the R program package (R project; see http://www.r-project.org/).

RESULTS

Blood analysis

Figure 1 shows changes over time in fasting blood glucose, body fat percentage, total blood serum cholesterol, high-density lipoprotein (HDL)-cholesterol, and low-density lipoprotein (LDL)-cholesterol. Each of the measured values shows decreasing trends after *Chlorella* intake and increasing trends after completion of the intake test period (12 weeks). The measured values before the start of intake (0 weeks) and at each test point were tested for significance using paired *t* tests. Using a significance level of 5%, both the N Group and the D Group demonstrated significant differences in body fat percentage and total blood serum cholesterol from weeks 4 to 12. Particularly in the case of the D Group, decreases in HDL-cholesterol and LDL-cholesterol showed clearly significant differences at all measurement points after the start of *Chlorella* intake. Blood glucose levels were also significantly decreased in the D Group after 8 weeks of chlorella consumption. In all of these measured values, a temporary decreasing trend could be seen after the start of *Chlorella* intake; after the completion of the intake period, these values returned to the levels before intake began. This indicates that the variations in blood parameters were brought about as a result of *Chlorella* intake. On the other hand, no clear variations in volumes of triglycerides in the blood were observed before and after the start of *Chlorella* intake in either the healthy subject group or the high-risk factor group. Furthermore, no trends toward increased concentration of insulin in the blood could be seen as a result of *Chlorella* intake.

Based on the above data, we have summarized the effects of *Chlorella* intake as follows. With regard to fat metabolism, although no significant changes could be seen in the concentration of triglycerides in the blood, both body fat percentage and total serum cholesterol were decreased in both the N and D Groups. With regard to glucose metabolism, however, *Chlorella* lowered blood glucose in the D Group. Since no clear increases in insulin concentrations in the blood were observed, the decrease in serum glucose concentrations may be due to improved insulin sensitivity induced by *Chlorella* intake.

Gene expression analysis

In both N and D Groups, we identified the genes whose mRNA expression levels varied owing to *Chlorella* intake by comparing expression intensities at 0 weeks (before the start of intake tests) and 4 weeks and between 0 weeks and 12 weeks. Moreover, the genes whose mRNA expression levels exhibited behavior consistent with the blood chemistry data, namely, gene expression profile varied with *Chlorella* intake and then returned nearly to pre-intake expression levels, were extracted by comparison between expression change of 4 weeks versus 0 weeks and that of 16 weeks versus 0 weeks based on *t* test. For these identifications, we used *t* tests with the False Discovery Rate = 0.05 as the level of significance.[6] Next, the genes superimposed in these two kinds of identified genes were extracted in both the N and D Groups. A total of 129 genes were chosen, 66 of which are associated with canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) database and are listed in Table 2. Many kinds of genes involved in signal transduction, metabolism, receptors, transporters, and cytokines were included. Moreover, this result suggested that many kinds of pathways involved in the insulin signaling pathway and immunological function may be influenced by *Chlorella* intake.

In the D Group, significant differences resulting from *Chlorella* intake were observed for fasting blood glucose, body fat percentage, and total serum cholesterol. In order to identify the genes associated with these physiological effects, we extracted genes that demonstrated differences in expression level between the D Group and the N Group before the start of the *Chlorella* intake tests and that also varied as a result of *Chlorella* intake. Table 3 lists the 18 genes thus identified. When we referred to the KEGG database to determine which pathways the identified genes were associated with, we found two genes (*protein tyrosine phosphatase 1B* and *growth factor receptor-bound protein 2*) that are associated with the insulin signaling pathway. We then investigated the changes in expressions for the genes among the loaded genes that were associated with the insulin signaling pathway. The results of this investigation are shown

Table 2. Genes Whose Expression Levels Varied with *Chlorella* Intake and Returned to Pre-Intake Expression Values

Target accession number	*Gene symbol*	*Gene name*	*Pathway name*
NM_000208.1	*INSR*	Insulin receptor	Adherens junction, insulin signaling pathway, type 2 diabetes mellitus, dentatorubropallidoluysian atrophy
Z69881.1	*ATP2A3*	ATPase, Ca^{2+} transporting, ubiquitous	Calcium signaling pathway
J03132.1	*ICAM1*	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	Cell adhesion molecules
Y00062.1	*PTPRC*	Protein tyrosine phosphatase, receptor type, C	Cell adhesion molecules, T cell receptor signaling pathway
NM_002592.1	*PCNA*	Proliferating cell nuclear antigen	Cell cycle
M24898.1	*NR1D1*	Nuclear receptor subfamily 1, group D, member 1	Circadian rhythm
D89479.1	*SULT1B1*	Sulfotransferase family, cytosolic, 1B, member 1	Cysteine metabolism
D49950.1	*IL18*	Interleukin 18 (interferon-gamma-inducing factor)	Cytokine–cytokine receptor interaction
U37518.1	*TNFSF10*	Tumor necrosis factor (ligand) superfamily, member 10	Cytokine–cytokine receptor interaction, apoptosis
U01134.1	*FLT1*	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	Cytokine–cytokine receptor interaction, focal adhesion
M32977.1	*VEGF*	Vascular endothelial growth factor	Cytokine–cytokine receptor interaction, focal adhesion
M65290.1	*IL2B*	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	Cytokine–cytokine receptor interaction, Toll-like receptor signaling pathway, Jak-STAT signaling pathway
D29013.1	*POLB*	Polymerase (DNA directed), beta	DNA polymerase
U96132.1	*HADH2*	Hydroxyacyl-coenzyme A dehydrogenase, type II	Fatty acid elongation in mitochondria, fatty acid metabolism, valine, leucine, and isoleucine degradation, lysine degradation, tryptophan metabolism, butanoate metabolism, caprolactam degradation
NM_021187.1	*CYP4F11*	Cytochrome P450, family 4, subfamily F, polypeptide 11	Fatty acid metabolism, gamma-hexachlorocyclohexane degradation, tryptophan metabolism
Y12863.1	*FIGF*	c-*fos*-induced growth factor (vascular endothelial growth factor D)	Focal adhesion
NM_030773.2	*TUBB1*	Tubulin, beta 1	Gap junction
J03746.1	*MGST1*	Microsomal glutathione *S*-transferase 1	Glutathione metabolism
NM_030821.3	*PLA2G12A*	Phospholipase A2, group XIIA	Glycerophospholipid metabolism, prostaglandin and leukotriene metabolism, MAPK signaling pathway
V00572.1	*PGK1*	Phosphoglycerate kinase 1	Glycolysis/gluconeogenesis, carbon fixation
U79143.1	*PIK3CA*	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	Inositol phosphate metabolsim, phosphatidylinositol signaling system, apoptosis, focal adhesion, Toll-like receptor signaling pathway, Jak-STAT signaling pathway, T cell receptor signaling athway, B cell receptor signaling pathway, regulation of actin cytoskeleton, insulin signaling pathway, type 2 diabetes mellitus
U92436.1	*PTEN*	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	Inositol phosphate metabolism, phosphatidylinositol signaling system, focal adhesion, tight junction
NM_005399.3	*PRKAB2*	Protein kinase, AMP-activated, beta 2 noncatalytic subunit	Insulin signaling pathway, adipocytokine signaling pathway
AB003791.1	*CHST1*	Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	Keratan sulfate biosynthesis

(*continued*)

TABLE 2. GENES WHOSE EXPRESSION LEVELS VARIED WITH *CHLORELLA* INTAKE AND RETURNED TO PRE-INTAKE EXPRESSION VALUES (CONT'D)

Target accession number	*Gene symbol*	*Gene name*	*Pathway name*
U65928.1	*COPS5*	COP9 constitutive photomorphogenic homolog subunit 5 (*Arabidopsis*)	Lysine degradation, biotin metabolism
U28014.1	*CASP4*	Caspase 4, apoptosis-related cysteine protease	MAPK signaling pathway
X03541.1	*NTRK1*	Neurotrophic tyrosine kinase, receptor, type 1	MAPK signaling pathway, apoptosis
U24153.1	*PAK2*	p21 (CDKN1A)-activated kinase 2	MAPK signaling pathway, axon guidance, focal adhesion, T cell receptor signaling pathway, regulation of actin cytoskeleton
X63717.1	*FAS*	Fas (tumor necrosis factor receptor superfamily, member 6)	MAPK signaling pathway, cytokine–cytokine receptor interaction, apoptosis
L13858.1	*SOS2*	*son of sevenless* homolog 2 (*Drosophila*)	MAPK signaling pathway, dorsoventral axis formation, focal adhesion, gap junction, Jak-STAT signaling pathway, T cell receptor signaling pathway, regulation of actin cytoskeleton, insulin signaling pathway
M22995.1	*RAP1A*	RAP1A, member of the RAS oncogene family	MAPK signaling pathway, focal adhesion
X79483.1	*MAPK12*	MAPK 12	MAPK signaling pathway, Toll-like receptor signaling pathway
AF004709.1	*MAPK13*	MAPK 13	MAPK signaling pathway, Toll-like receptor signaling pathway
NM_000686.3	*AGTR2*	Angiotensin II receptor, type 2	Neuroactive ligand–receptor interaction
M18737.1	*GZMA*	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	Neuroactive ligand–receptor interaction
BC007720.1	*HTR1D*	*5-Hydroxytryptamine* (serotonin) receptor 1D	Neuroactive ligand–receptor interaction
AF000546.1	*P2RY5*	Purinergic receptor P2Y, G-protein coupled, 5	Neuroactive ligand–receptor interaction
U13699.1	*CASP1*	Caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	Neurodegenerative disorders, MAPK signaling pathway, Huntington's disease, dentatorubropallidoluysian atrophy
NM_006312.1	*NCOR2*	Nuclear receptor co-repressor 2	Notch signaling pathway
NM_004718.2	*COX7A2L*	Cytochrome *c* oxidase subunit VIIa polypeptide 2-like	Oxidative phosphorylation
NM_004374.2	*COX6C*	Cytochrome *c* oxidase subunit VIc	Oxidative phosphorylation
NM_001865.2	*COX7A2*	Cytochrome *c* oxidase subunit VIIa polypeptide 2 (liver)	Oxidative phosphorylation
L35249.1	*ATP6V1B2*	ATPase, H^+ transporting, lysosomal 56/58 kDa, V1 subunit B, isoform 2	Oxidative phosphorylation, ATP synthesis
Y15286.1	*ATP6V0E*	ATPase, H^+ transporting, lysosomal 9 kDa, V0 subunit e	Oxidative phosphorylation, ATP synthesis, cholera
M62762.1	*ATP6V0C*	ATPase, H^+ transporting, lysosomal 16 kDa, V0 subunit c	Oxidative phosphorylation, ATP synthesis, cholera
J05682.1	*ATP6V1C1*	ATPase, H^+ transporting, lysosomal 42 kDa, V1 subunit C, isoform 1	Oxidative phosphorylation, ATP synthesis, cholera
NM_024330.1	*SLC27A3*	Solute carrier family 27 (fatty acid transporter), member 3	Propanoate metabolism, ethylbenzene degradation, limonene and pinene degradation, caprolactam degradation, alkaloid biosynthesis II
NM_003739.4	*AKR1C3*	Aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	Prostaglandin and leukotriene metabolism
NM_000895.1	*LTA4H*	Leukotriene A4 hydrolase	Prostaglandin and leuikotriene metabolism
NM_001116.1	*ADCY9*	Adenylate cyclase 9	Purine metabolism, calcium signaling pathway, gap junction cholera
AF025840.1	*POLE2*	Polymerase (DNA directed), epsilon 2 (p59 subunit)	Purine metabolism, pyrimidine metabolism, DNA polymerase

TABLE 2. GENES WHOSE EXPRESSION LEVELS VARIED WITH *CHLORELLA* INTAKE AND RETURNED TO PRE-INTAKE EXPRESSION VALUES (CONT'D)

Target accession number	*Gene symbol*	*Gene name*	*Pathway name*
Z47727.1	*POLR2K*	Polymerase (RNA) II (DNA directed) polypeptide K, 7.0 kDa	Purine metabolism, pyrimidine metabolism, RNA polymerase
X63563.1	*POLR2B*	Polymerase (RNA) II (DNA directed) polypeptide K, 140 kDa	Purine metabolism, pyrimidine metabolism, RNA polymerase
J04965.1	*POLR2E*	Polymerase (RNA) II (DNA directed) polypeptide K, 25 kDa	Purine metabolism, pyrimidine metabolism, RNA polymerase
U09178.1	*DPYD*	Dihydropyrimidine dehydrogenase	Pyrimidine metabolism, beta-alanine metabolism, pantothenate and coenzyme A biosynthesis
NM_000436.2	*OXCT1*	3-Oxoacid coenzyme A transferase 1	Synthesis and degradation of ketone bodies, valine, leucine, and isoleucine degradation, butanoate metabolism
U88878.1	*TLR2*	Toll-like receptor 2	Toll-like receptor signaling pathway
U88540.1	*TLR1*	Toll-like receptor 1	Toll-like receptor signaling pathway
NM_001752.2	*CAT*	Catalase	Tryptophan metabolism, methane metabolism, amyotrophic lateral sclerosis
L34587.1	*TCEB1*	Transcription elongation factor B (SIII), polypeptide 1 (15 kDa, elongin C)	Ubiquitin-mediated roteolysis
NM_005438.2	*FOSL1*	FOS-like antigen 1	Wnt signaling pathway
NM_030775.2	*WNT5B*	*wingless*-type mouse mammary tumor virus integration site family, member 5B	Wnt signaling pathway, hedgehog signaling pathway
L37042.1	*CSNK1A1*	Casein kinase 1, apha 1	Wnt signaling pathway, hedgehog signaling pathway, gap junction, circadian rhythm
NM_004422.1	*DVL2*	*dishevelled, dsh* homolog 2 (*Drosophila*)	Wnt signaling pathway, Notch signaling pathway
M74088.1	*APC*	Adenomatosis polyposis coli	Wnt signaling pathway, regulation of actin cytoskeleton

A total of 129 genes were extracted (see text) in this study. Among 129 genes, 66 genes that involved in canonical pathways summarized in KEGG are listed. Jak-STAT, Janus kinase–signal transducers and activators of transduction; MAPK, mitogen-activated protein kinase.

in Figure 2, which shows that after *Chlorella* intake, there is an increase in expression levels for genes related to the signal transduction routes linked to translocation of glucose transporter (GLUT4) below the insulin receptors (producing insulin receptor substrate, phosphoinositide-3-kinase, 3-phosphoinositide-dependent kinase-1, and v-akt murine thymoma viral oncogene homolog 3). Protein tyrosine phosphate-1B (PTP1B) acts to suppress signal transduction, but the expression level for PTP1B showed a tendency to decrease as a result of *Chlorella* intake. Based on the changes in gene expression levels, we can therefore surmise that insulin signaling pathways are activated by the intake of *Chlorella*. Recently, Cheng *et al.*[7] reported that in *in vitro* screening systems using monocytes from human peripheral blood, *Chlorella* inhibited the activation of PTP1B.

DISCUSSION

Cherng and Shih[8] reported changes in blood glucose concentrations resulting from administration of *Chlorella* in streptozotocin-induced diabetic mice. In that study, they reported that administration of *Chlorella* (100 mg/kg) steadily reduced both glucose concentrations in the blood and increased glucose values during glucose tolerance tests, but that no increases in insulin concentrations in the blood could be seen. Their results correspond closely with the results of the current research on humans. It has also been reported that in streptozotocin-diabetic mice, *Chlorella* intake increases glucose uptake in the liver and skeletal muscles.[9]

Dimitriadis *et al.*[10] reported that in monocyte *in vitro* systems separated from peripheral blood, insulin exposure brought about an increase in the uptake of glucose and a translocation of GLUT4 to the membrane surface. Estrada *et al.*[11] reported that exposing peripheral blood monocytes from healthy subjects and insulin-dependent diabetic patients to insulin-like growth factor-I immediately causes uptake of glucose and that although the relationship of insulin-like growth factor-I concentration and glucose uptake volumes was similar in both healthy subjects and diabetes patients, the cells obtained from diabetic patients demonstrated lower uptake volumes overall. These recent investigations also show that peripheral blood cells are an effec-

TABLE 3. LIST OF GENES THAT SHOWED SIGNIFICANT DIFFERENCES IN EXPRESSION LEVEL BETWEEN THE D GROUP AND THE N GROUP BEFORE THE START OF THE *CHLORELLA* INTAKE TESTS AND THAT ALSO VARIED AS A RESULT OF *CHLORELLA* INTAKE

Target accession number	*Gene symbol*	*Gene name*	*Category*
NM_001116.1	*ADCY9*	Adenylate cyclase 9	Signal
NM_033375.3	*MYO1C*	Myosin IC	—
NM_000805.2	*GAST*	Gastrin	Receptor
NM_021187.1	*CYP4F11*	Cytochrome P450, family 4, subfamily F, polypeptide 11	P450
NM_022444.3	*SLC13A1*	Solute carrier family 13 (sodium/sulfate symporters), member 1	Transporter
NM_004718.2	*COX7A2L*	Cytochrome *c* oxidase subunit VIIa polypeptide 2-like	Metabolism
U32519.1	—	Ras-GTPase-activating protein SH3-domain-binding protein	Signal
NM_014585.3	*SLC40A1*	Solute carrier family 40 (iron-regulated transporter), member 1	Transporter
M96995.1	*GRB2*	Growth factor receptor-bound protein 2	Signal
NM_001114.1	*ADCY7*	Adenylate cyclase 7	Signal
U48251.1	*PRKCBP1*	Protein kinase C binding protein 1	Signal
U21858.1	*TAF9*	TAF9 RNA polymerase II, TATA box binding protein-associated factor, 32 kDa	Repair
U50062.1	*RIPK1*	Receptor (tumor necrosis factor receptor superfamily)-interacting serine-threonine kinase 1	Apoptosis
M92287.1	*CCND3*	Cyclin D3	Cell cycle
NM_014235.2	*UBL4*	Ubiquitin-like 4	—
AF038950.1	*ABCB7*	ATP-binding cassette, subfamily B (MDR/TAP), member 7	Transporter
NM_002827.2	*PTPN1*	Protein tyrosine phosphatase, non-receptor type 1	Signal
L25610.1	*CDKN1A*	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Cell cycle

tive target for studies of improved glucose uptake and insulin sensitivity. The current research has shown that one of the mechanisms of reduced blood glucose levels achieved by *Chlorella* intake is an activation of insulin signaling pathways resulting from changes in gene expression in the peripheral blood cells. This research also suggests, however, that changes in gene expression profile in the peripheral blood can be useful as a surrogate marker when investigating glucose metabolism. This marker is particularly effective in research targeting human beings.

The current research also showed that *Chlorella* intake in humans is useful in improving fat metabolism. Particularly in the case of the D Group, significant decreases in total blood serum cholesterol, HDL-cholesterol, and LDL-cholesterol were observed at all measurement points after the start of *Chlorella* intake.

Merchant and Andre[12] used a double blind test to study the effects of *Chlorella* intake on symptom improvements in a total of 55 patients suffering from fibromyalgia syndrome, hypertension, and ulcerative colitis. They confirmed that *Chlorella* intake lowers cholesterol in the blood, which is consistent with the outcome of the current research

Shibata *et al*.[13] reported that in rats raised on feed containing cholesterol, the administration of *Chlorella* decreases cholesterol concentrations in the blood and liver, but that there were no changes in neutral fat or phospholipid volumes and that excretion of neutral steroids was increased. They surmised that the cholesterol-lowering effects of *Chlorella* are brought about by increasing neutral steroid elimination in feces. Sano *et al*.[14] also reported that in rats with hyperlipemia created through excess administration of cholesterol, the administration of *Chlorella* increased steroid elimination in the feces. Connor *et al.*[15] reported that administering high-molecular-weight unsaturated fatty acids to humans increases the neutral steroid content of feces and also reduces blood cholesterol concentrations. Based on the fact that about 74% of the fatty acids contained in *Chlorella* are unsaturated fatty acids, we can infer that the presence of a physiological mechanism in which *Chlorella* intake causes the reductions in blood cholesterol as follows: *Chlorella* intake may increase neutral steroid elimination in the feces, causing a concomitant demand for cholesterol in the liver, which in turn reduces cholesterol concentrations in the blood.

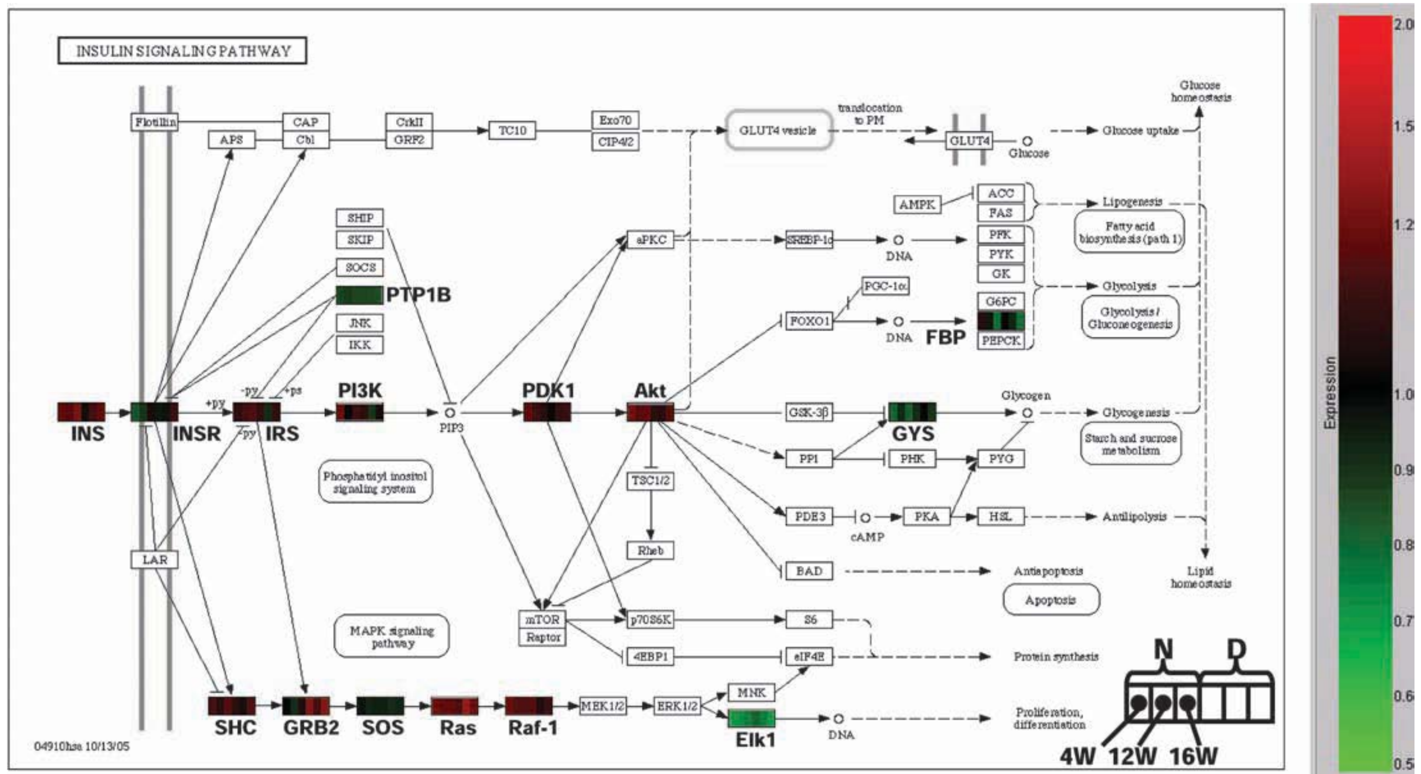

FIG. 2. Schematic diagram of the insulin signaling pathway based on the KEGG database (see text). The changes of expression level of genes associated with this pathway resulting from *Chlorella* intake at 4 weeks, 12 weeks, and 16 weeks compared to 0 week value are mapped in the heat map format; red and green mean up-regulation and down-regulation, respectively. At each gene six boxes are drawn; the three boxes on the left display change of expression level measured at 4 weeks, 12 weeks, and 16 weeks (from left to right) in the N Group, and the other three boxes on the right display the data in the D Group. INS, insulin; INSR, insulin receptor; IRS, insulin receptor substrate 1; PI3K, phosphoinositide-3-kinase; PTB1B, protein tyrosine phosphatase 1B; Akt, v-akt murine thymoma viral oncogene homolog 3; GYS, glycogen synthase 1; FBP, fructose-1,6-bisphosphatase 1; SHC, Src homology 2 domain containing transforming protein 2; GRB2, growth factor receptor-bound protein 2; SOS, *son of sevenless* homolog 1; Ras, v-Ha-ras Harvey rat sarcoma viral oncogene homolog; Raf, v-raf murine sarcoma 3611 viral oncogene homolog; Elk1, member of ETS oncogene family. Refer to the following URL for further explanations of other abbreviations: http://www.genome.ad.jp/dbget-bin/www_bget?pathway+hsa04910.

In recent years, the so-called Randle hypothesis[16] was proposed to describe the inhibition of glucose uptake by abnormalities in fat metabolism, stating that increases in fatty acids limit the oxidation and uptake of glucose. This hypothesis has been explained through extensive experimental results. Another approach has also been proposed in skeletal muscle cells whereby the uptake of free fatty acids is stimulated, deactivating the insulin signal transduction systems that transduce signals from insulin receptors to GLUT4.[17] In the current research as well, we can assume that *Chlorella* intake first improves fat metabolism, resulting in improved glucose uptake. Although cholesterol levels in the blood decrease, triglyceride concentrations do not necessarily drop, so the relationship between the improvement of fat metabolism and the decrease in blood glucose level is unclear at present. It will be necessary to conduct further studies, including *in vitro* experiments, to elucidate the mechanisms involved.

CONCLUSIONS

In order to clarify the physiological effects of *Chlorella* intake on subjects with high-risk factors for lifestyle-related diseases, we conducted blood biochemical tests on a high-risk factor group and a healthy subject group and analyzed gene expression profiles in peripheral blood cells before and after *Chlorella* intake. The results of these tests showed that *Chlorella* intake brings about improvements in both fat metabolism and glucose metabolism. The expression of genes involved in the insulin signaling pathway was also affected by *Chlorella* intake, especially those related to glucose uptake in tissue, providing support for the observation that *Chlorella* lower blood glucose levels. These results indicate that changes in gene expression in the peripheral blood can be useful as a surrogate marker for investigating the mechanisms of modulation of glucose sensitivity in humans. In the clarification of how functional and health foods can contribute to human health, the combination of the nutrige-

nomics research methods with conventional blood biochemical tests such as those used in this study is being used more widely.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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