

Hepatic Gene Expression Associated With Macrophage and Oxidative Stress of Simple Steatosis and Non-Alcoholic Steatohepatitis Model Rats Using DNA Microarray Analysis

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Abstract

Aim: To clarify the mechanism governing progression of Non-Alcoholic Steatohepatitis (NASH), we examined hepatic gene expression associated with macrophage and oxidative stress/inflammation, which plays an important role in the progression of Non-Alcoholic Fatty Liver Disease (NAFLD) in simple steatosis (SS) model and NASH model rats.

Methods: Four-month-old male Spontaneously Hypertensive Hyperlipidemic Rats (SHHR) and Sprague-Dawley (SD) rats were each divided into two groups: SD rats received a high-fat diet and 30% sucrose solution (HFDS) as SS model rats and SHHR received the HFDS as NASH model rats. Microarray analysis was performed on the liver of these rats at eight months of age to select those gene sets, e.g., "genes correlated with progression of NAFLD" and "genes expressed exclusively in NASH", which are related to macrophage or oxidative stress/inflammation.

Results: Thirteen genes were selected from the microarray analysis data. Four genes were associated with macrophage: acid phosphatase 5, tartrate-resistant (Acp5), a member of the RAS oncogene family (Rab8a), scavenger receptor class B, member 2 (Scarb2) and CD36 molecule (Cd36). Nine genes were associated with oxidative stress/inflammation: translocator protein (Tspo), prostaglandin I₂ synthase (Ptgis), tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9), glutathione S-transferase alpha 5 (Gsta5), regucalcin (Rgn), glutathione S-transferase kappa 1 (Gstk1), disabled homolog 2, mitogen-responsive phosphoprotein (Dab2), glutathione S-transferase mu 5 (Gstm5) and flavin-containing monooxygenase 5 (Fmo5). Acp5, Tspo, Ptgis, Tnfrsf9, Gsta5 (up-regulated) and Rab8a, Rgn, Gstk1 (down-regulated) were included in genes correlated with progression of NAFLD. Scarb2, Cd36, Dab2, Gstm5 (up-regulated) and Fmo5 (down-regulated) were included in genes expressed in only NASH model rats.

Conclusion: We hypothesized that scavenger receptor class B and glutathione S-transferase play an important role in the progression from simple NAFLD to NASH. Our results afford beneficial data regarding therapeutic targets of progression of NAFLD/NASH.

Keywords: Spontaneously hypertensive hyperlipidemic rats (SHHR) (SHHR); Non-alcoholic steatohepatitis (NASH); Non-alcoholic fatty liver disease (NAFLD); Microarray

Introduction

Non-alcoholic Fatty Liver Disease (NAFLD) is defined as the accumulation of lipid, primarily triacylglycerols, in individuals who do not consume large quantities of alcohol. NAFLD encompasses a spectrum of disease ranging from Simple Steatosis (SS) to inflammatory steatohepatitis with increasing levels of fibrosis and ultimately cirrhosis [1]. NAFLD, which is strongly correlated with obesity and insulin resistance, is recognized as representing the hepatic manifestation of the metabolic syndrome [2]. Non-Alcoholic Steatohepatitis (NASH) is a progressive form of NAFLD that is diagnosed on the basis of histopathological features [3]. The prevalence of the metabolic syndrome, which is rapidly increasing due to hyperalimentation and a sedentary lifestyle [4], is reflected by the increasing prevalence of NAFLD and associated complications [5]. NAFLD can be quite unobtrusive; in contrast, NASH is a serious condition, with nearly one quarter of affected patients developing cirrhosis, which, in turn, increases the risk of subsequent progression to hepatocellular carcinoma [6]. Despite recent advances in the elucidation of the complex metabolic and inflammatory pathways involved in NAFLD, the pathogenesis of steatosis and progression to steatohepatitis and fibrosis/cirrhosis remains incompletely understood [2,7].

Few rat models lend themselves to the evaluation of atherosclerosis; as a result, Spontaneously Hypertensive Hyperlipidemic Rats (SHHRs) were developed as a stable model of early vascular degeneration [8], SHHRs display persistently high systolic blood pressure (above 150 mmHg) and plasma total cholesterol levels exceeding 150 mg/dL. In addition, vascular intimal lesions and lipid deposits have been observed under endothelial cells in the aorta of SHHRs, but not in spontaneously hyperlipidemic rats or controls [9]. Moreover, invasive changes occur in the subendothelium of SHHR when nitric oxide production is inactivated, followed by a high fat diet and sucrose water treatment (HFDS) [10]; moreover, a previous study noted visceral fat accumulation and increased oxidative stress in SHHR-HFDS [11,12].

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We detected hepatocyte ballooning and steatosis with HFDS feeding for 9-13 months. Severe fibrosis and cell inflammation around the central vein were observed in SHHR-HFDS but were only slightly detectable in SD-HFDS [13]; thus, SD-HFDS and SHHR-HFDS were employed as rat models of simple steatosis and NASH, respectively.

Numerous researchers have applied microarray analysis in order to clarify the underlying mechanism governing NAFLD; however, these investigations noted only the genes correlated with lipid metabolism. The metabolic abnormalities and gene expressions of liver in terms of the pathological progression of early stage NASH remain unclear.

Microarray analysis was performed in the current study to clarify the pathogenetic mechanisms governing early stage NASH and to evaluate SD-HFDS and SHHR-HFDS as models of simple steatosis and NASH, respectively.

Materials and Methods

Animals and samples

Four-month-old male SHHRs and SD rats were divided into two groups each: a control group, which received a regular diet (ND; CE2; CLEA Japan Inc., Tokyo, Japan), and a HFDS-fed group. The regular diet consisted of 8.9% water, 25.4% protein, 4.4% fat, 4.1% fiber, 6.9% carbohydrate and 50.3% nitrogen-free extracts (caloric value: 342.2 kcal/100 g). The high-fat diet (HFD) consisted of 8.2% water, 23.4% protein, 11.0% fat, 3.8% fiber, 6.3% carbohydrate and 46.3% nitrogen-free extracts (caloric value: 378.0 kcal/100 g). The regular diet was available to all groups ad libitum until the age of four months; subsequently, the two HFDS groups received the high-fat diet with 30% sucrose solution ad libitum for four months. Thus, rats of 8 months of age were obtained. This study utilized SHHRs characterized by systolic blood pressure over 150 mmHg, as determined by the tailcuff method (PS-100; Riken Kaihatsu, Tokyo, Japan). The rats were housed in a semi-barrier system under controlled room temperature (23 ± 1°C), humidity (55 ± 5%) and lighting (lights on from 6 AM to 6 PM). All experiments were

conducted according to the “Guiding Principles for the Care and Use of Laboratory Animals” of Showa University [12].

Statistical analysis

Data were analyzed employing the Mann-Whitney U-test. Correlations were calculated with the Pearson's product moment correlation coefficient. All data are expressed as mean ± S.E.M. p<0.05 was considered significant.

Preparation and biochemical determination of plasma samples

Blood samples, which were obtained from the inferior vena cava under pentobarbital anesthesia (35 mg/kg, intraperitoneal administration), were mixed with 3.2% sodium citrate solution in a volume ratio of 9:1. After 15 minutes of centrifugation at 3,000 rpm, the supernatant, as citrated plasma, was analyzed. Rats were sacrificed by decapitation, after which the liver and visceral fat (VisF) were isolated and weighed under the pentobarbital anesthesia.

Plasma levels of total cholesterol and total triglycerides were determined employing commercially available kits (Cholesterol E-test and Triglyceride E-test, respectively; Wako Pure Chemical Industries Ltd., Tokyo, Japan). Plasma levels of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) were determined with the Transaminase CII-test (Wako) [11]. Oxidative stress was measured in rat plasma utilizing the d-ROMs test (Free Radical Elective Evaluator; Wismerll Co. Ltd., Tokyo, Japan) by gently mixing 20 µl of plasma sample and 1 mL of buffer solution in a cuvette, prior to the addition of 10 µL of the chromogenic substrate. After mixing, the cuvette was incubated immediately in the thermostatic block of the analyzer for 5 minutes at 37°C; subsequently, the absorbance at 505 nm was recorded. Measurements are expressed as Carr units, with 1 Carr corresponding to 0.8 mg/L H₂O₂ [12].

Morphological study

The fresh left lobe of liver was harvested from all rats and stored in

	N	BW(g)	LW(g)	Vis Fat(g)	LW/100gBW	Vis Fat/100gBW	d-ROMS
SD-ND	8	574.1 ± 8.5	14.7 ± 0.3	17.3 ± 0.3	2.6 ± 0.0	3.0 ± 0.1	281.8 ± 8.7
SD-HFDS	8	590 ± 12.1	38.2 ± 1.4	19.3 ± 1.4	6.5 ± 0.1	3.3 ± 0.2	482.0 ± 35.0
SHHR-ND	6	565 ± 7.7	16.3 ± 0.5	14.7 ± 1.1	2.9 ± 0.1	2.6 ± 0.2	360.7 ± 13.0
SHHR-HFDS	6	671.2 ± 14.9	48.3 ± 0.5	50.4 ± 3.8	7.2 ± 0.1	7.5 ± 0.4	616.8 ± 23.4
		*3*5*6	*1*2*3 *4*5*6	*2*3 *4*5*6	*1*2*3 *4*5*6	*2*3 *4*5*6	*1*2*3 *4*5*6

Body weight (BW), visceral fat (VisF), plasma glucose (Glu), hemoglobin A1c (HbA1c), total cholesterol (TC), total triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and diacron reactive oxidative metabolites (d-ROMs) in Sprague-Dawley rats receiving normal diet (SD-ND) and SD-high-fat diet with sucrose (SD-HFDS) as well as in spontaneously hypertensive hyperlipidemic rats (SHHR-ND and SHHR-HFDS) at 8 months of age. The results are presented as mean ± S.E.M.

Student t test unpaired p<0.05 for *1, SD-ND vs. SD-HFDS; *2, SD-ND vs. SHHR-ND; *3, SD-ND vs. SHHR-HFDS; *4, SD-HFDS vs. SHHR-ND; *5, SD-HFDS vs. SHHR-HFDS; *6, SHHR-ND vs. SHHR-HFDS

Table 1a: Biochemical characteristics of SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS at 8 months of age.

	N	Glu(mg/dl)	HbA1c	T-Cho(mg/dl)	TG(mg/dl)	AST	ALT
SD-ND	8	150.9 ± 7.9	2.7 ± 0.1	65.8 ± 4.9	77.0 ± 8.2	141.9 ± 15.3	53.9 ± 8.9
SD-HFDS	8	162.6 ± 5.1	2.8 ± 0.1	109.9 ± 10.3	72.8 ± 5.4	264.1 ± 46.9	71.1 ± 10.0
SHHR-ND	6	135.8 ± 8.9	2.6 ± 0.1	140.2 ± 9.9	125.0 ± 9.2	140.3 ± 9.2	47.5 ± 4.8
SHHR-HFDS	6	162.8 ± 3.6	2.9 ± 0.1	457.3 ± 42.5	84.7 ± 6.6	170.7 ± 12.0	62.2 ± 18.0
		*4*6	*1*2	*3*5*6	*2*4*6	*1*4	

Body weight (BW), visceral fat (VisF), plasma glucose (Glu), hemoglobin A1c (HbA1c), total cholesterol (TC), total triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and diacron reactive oxidative metabolites (d-ROMs) in Sprague-Dawley rats receiving normal diet (SD-ND) and SD-high-fat diet with sucrose (SD-HFDS) as well as in spontaneously hypertensive hyperlipidemic rats (SHHR-ND and SHHR-HFDS) at 8 months of age. The results are presented as mean ± S.E.M. Student t test unpaired p<0.05 for *1, SD-ND vs. SD-HFDS; *2, SD-ND vs. SHHR-ND; *3, SD-ND vs. SHHR-HFDS; *4, SD-HFDS vs. SHHR-ND; *5, SD-HFDS vs. SHHR-HFDS; *6, SHHR-ND vs. SHHR-HFDS

Table 1b: Biochemical characteristics of SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS at 8 months of age.

saline on ice, after which it was dissected from the surrounding tissues and fixed in 10% neutral buffered formalin (pH 7.4; Wako). Sections of the liver were stained with Hematoxylin-Eosin (HE) and Masson Trichrome (MT).

RNA extraction and microarray hybridization

A sample of each liver specimen was stored at -80°C until the microarray analysis.

Total RNA was purified using an RNeasy Kit (Qiagen, Germany) per the manufacturer's manual. Cy3-labeled cRNA was obtained from 200 ng total RNA with the Agilent Low Input Quick Amp Labeling Kit. Cy3-labeled cRNA was hybridized to Whole Rat genome Oligo Microarray ver 3.0 (4x44 k) according to the manufacturer's hybridization protocol. After the washing step, microarray slides were analyzed with an Agilent Microarray scanner B version; the default settings were applied for all parameters. Microarray expression data were obtained utilizing Agilent Feature Extraction software ver 10.5.1; the default settings were applied for all parameters. The raw data and associated sample interpretation were loaded and processed by GeneSpring ver11 (Tomy Digital Biology). Four experiments were performed; data were expressed as mean values.

Results

Comparison of the four groups

Tables 1a and 1b displays body weight and Liver Weight (LW) as well as the levels of VisF, plasma glucose, hemoglobin A1C, total cholesterol, total triglycerides, AST, ALT and d-ROMs in SD-ND, SD-HFDS, SHHR-ND and SHHR-HFDS. In both the SD and SHHR groups, VisF and LW were elevated significantly or tended to be elevated following ingestion of HFDS. VisF and LW in SHHR-ND and SHHR-HFDS increased markedly in comparison with SD-ND and SD-HFDS, respectively. The level of oxidative stress (d-ROMs) demonstrated meaningful elevation after ingestion of HFDS. The level of d-ROMs in SHHR-ND and SHHR-HFDS increased significantly in comparison with SD-ND and SD-HFDS, respectively. Plasma hemoglobin A1C, AST and ALT levels were unchanged among the SD and SHHR groups.

Morphological study

Figures 1 and 2 presents the results of the morphological study

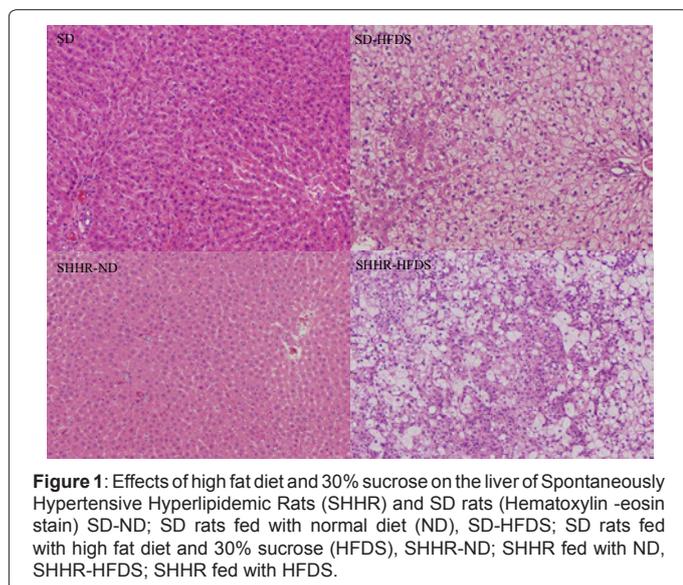


Figure 1: Effects of high fat diet and 30% sucrose on the liver of Spontaneously Hypertensive Hyperlipidemic Rats (SHHR) and SD rats (Hematoxylin -eosin stain) SD-ND; SD rats fed with normal diet (ND), SD-HFDS; SD rats fed with high fat diet and 30% sucrose (HFDS), SHHR-ND; SHHR fed with ND, SHHR-HFDS; SHHR fed with HFDS.

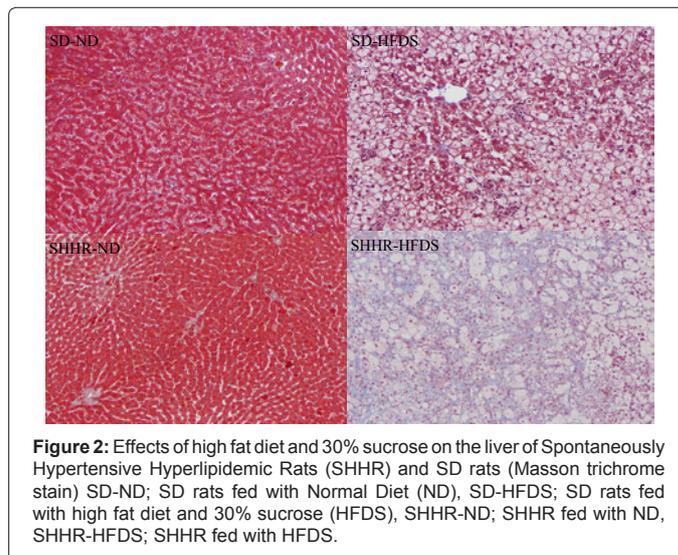


Figure 2: Effects of high fat diet and 30% sucrose on the liver of Spontaneously Hypertensive Hyperlipidemic Rats (SHHR) and SD rats (Masson trichrome stain) SD-ND; SD rats fed with Normal Diet (ND), SD-HFDS; SD rats fed with high fat diet and 30% sucrose (HFDS), SHHR-ND; SHHR fed with ND, SHHR-HFDS; SHHR fed with HFDS.

with a representative sample of liver from each group of SHHR and SD rats at eight months stained with HE and MT. Hepatocyte ballooning and steatosis are well recognized with HFDS feeding. Fibrosis and cell inflammation, which were observed in SHHR-HFDS, were slightly detectable in SD-HFDS.

DNA microarray analysis

On the basis of quality control, 8388 genes were detected. Genes were analyzed statistically employing Student T test unpaired (p-value computation; asymptotic, multiple testing correction; Benjamini-Hochberg, p-value cut off; <0.05) to select pathophysiologically important genes: "Genes correlated with progression of NAFLD", which met all criteria; p<0.05 for SD-ND vs. SD-HFDS, SHHR-ND vs. SHHR-HFDS, SD-ND vs. SHHR-ND, SD-HFDS vs. SHHR-HFDS. "Genes expressed exclusively in NASH", which met all criteria; p<0.05 for SD-ND vs. SD-HFDS, p<0.05 for SHHR-ND vs. SHHR-HFDS, SD-ND vs. SHHR-ND, SD-HFDS vs. SHHR-HFDS. Subsequently, those genes demonstrating identical regulation among all four criteria were obtained as up-regulated or down-regulated. Forty-four and 49 genes were correlated with up-regulation and down-regulation, respectively, in terms of NAFLD progression. Seventy-seven and 32 genes were correlated with up-regulation and down-regulation, respectively, in NASH. Finally, 13 genes were selected from the macrophage infiltration- and oxidative stress-related genes per microarray analysis data.

Discussion

Several earlier studies have established a relationship between obesity and lifestyle-related diseases. In particular, visceral obesity is directly correlated with the clustering of lifestyle-related diseases, leading to various ailments [14-16]. Our previous report demonstrated that visceral fat accumulation is closely associated with increased oxidative stress [12]. We disclosed that oxidative stress, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play an important role in NASH progression in the liver of NASH model rats fed HFDS for 9-13 months. We also determined that hepatocyte ballooning and steatosis are well recognized with HFDS feeding for 9-13 months; moreover, severe fibrosis and cell inflammation were readily apparent around the central vein in SHHR-HFDS but only slightly detectable in SD-HFDS [13]. This study examined hepatic gene expression profiles in the early stage of NASH in the liver of NASH model rats receiving the HFDS for 8 months.

The most accepted theory regarding the explanation of progression from simple steatosis to NASH is the “two-hit hypothesis” wherein fat accumulation is sufficient to induce progression to steatohepatitis, rendering the liver more susceptible to a “second hit” that, once imposed upon the steatotic liver, causes further aberrations that culminate in the development of NASH [17,18]. A key factor in this “second hit” is oxidative stress [13]. Biochemical characteristics of SHHR-HFDS indicate that SHHR-HFDS at 8 months of age may develop metabolic syndrome leading to NAFLD.

A second feature of this study was the statistical method utilized to clarify the pathophysiological mechanism governing progression from simple steatosis to NASH. Based on our previous report, e.g., severe fibrosis and cell inflammation around the central vein were readily apparent in SHHR-HFDS but only slightly detectable in SD-HFDS [13], two groups of genes were extracted: “Genes correlated with progression of NAFLD” and “Genes expressed exclusively in NASH”. The former category contained those genes changed in expression, correlated with liver steatosis, oxidative stress and visceral fat accumulation; in other words, “first-hit” induced the gene expressions and “second-hit” enhanced them. The latter category consisted of those genes induced not by “first-hit” but by “second-hit”, associated with the aforementioned background. Recently, it was reported that macrophage infiltration and oxidative stress are an initiating event in NASH [19,20]. Therefore, 13 genes were selected from the candidate genes per microarray analysis data and classified as macrophage-related or oxidative stress/inflammation-related genes (Tables 2a-2c). Four genes were associated with macrophage: acid phosphatase 5, tartrate-resistant (Acp5), a member of the RAS oncogene family (Rab8a), scavenger receptor class B, member 2 (Scarb2) and CD36 molecule (Cd36). Furthermore, nine genes were associated with oxidative stress/inflammation: translocator protein (Tspo), prostaglandin I2 synthase (Ptgis), tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9), glutathione S-transferase alpha 5 (Gsta5), regucalcin (Rgn), glutathione S-transferase kappa 1

	Genes correlated with progression of NAFLD		Genes expressed exclusively in NASH	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
macrophage	Acp5	Rab8a	Scarb2 Cd36	
oxidative stress inflammation	Tspo Ptgis Tnfrsf Gsta5	Rgn Gstk1	Dab2 Gstm5	Fmo5

Table 2a: Macrophage- and oxidative stress/inflammation-related genes acid phosphatase 5, tartrate resistant (Acp5), translocator protein (Tspo), prostaglandin I2 synthase (Ptgis), tumor necrosis factor receptor superfamily, member 9 (Tnfrsf), glutathione S-transferase alpha 5 (Gsta5), member RAS oncogene family (Rab8a), regucalcin (Rgn), glutathione S-transferase kappa 1 (Gstk1), scavenger receptor class B, member 2 (Scarb2), CD36 molecule (Cd36), disabled homolog 2, mitogen-responsive phosphoprotein (Dab2), glutathione S-transferase mu 5 (Gstm5), flavin-containing monooxygenase 5 (Fmo5).

Gene Symbol	SD-HFDS vs. SD-ND	SHHR-ND vs. SD-ND	SHHR-HFDS vs. SD-HFDS	SHHR-HFDS vs. SHHR-ND
Up-regulated genes				
Acp5	2.21	2.38	3.26	3.02
Tspo	2.29	2.17	2.11	2.22
Gsta5	4.00	6.34	3.44	1.84
Ptgis	1.90	4.84	13.50	5.30
Tnfrsf9	6.51	7.75	4.25	3.57
Down-regulated genes				
Rgn	-2.31	-3.32	-2.51	-1.75
Rab8a	-1.31	-1.31	-1.22	-1.22
Gstk1	-1.97	-1.64	-1.68	-2.02

Table 2b: Changed genes correlated with steatosis HFDS treatment significantly increased or decreased gene expression in SD and SHHR. Meaningful differences were observed between comparison groups. Each mean fold value is presented.

Gene Symbol	SD-HFDS vs. SD-ND	SHHR-ND vs. SD-ND	SHHR-HFDS vs. SD-HFDS	SHHR-HFDS vs. SHHR-ND
Up-regulated genes				
Scarb2	1.07	1.02	1.12	1.18
Dab2	1.58	2.14	3.05	2.25
Gstm5	1.22	1.79	3.36	1.54
Cd36	1.21	1.73	4.57	2.19
Down-regulated genes				
Fmo5	-1.52	-2.17	-3.09	-2.17

Table 2(c): Changed genes correlated with steatohepatitis HFDS treatment significantly increased or decreased gene expression in only SHHR. HF treatment did not lead to meaningful differences between the SD HFDS and SD ND groups. Each mean fold value is presented.

(Gstk1), disabled homolog 2, mitogen-responsive phosphoprotein (Dab2), glutathione S-transferase mu 5 (Gstm5) and flavin-containing monooxygenase 5 (Fmo5). Acp5, Tspo, Ptgis, Tnfrsf, Gsta5 (up-regulated) as well as Rab8a, Rgn and Gstk1 (down-regulated) were included in the group of genes correlated with progression of NAFLD. Scarb2, Cd36, Dab2 Gstm5 (up-regulated) and Fmo5 (down-regulated) were included in the group of genes expressed exclusively in NASH.

Acp5, Tspo, Ptgis, Tnfrsf and Gsta5 expression increased among genes correlated with the progression of NAFLD. Acp5 is also known as TRACP 5a, which is the novel marker of macrophage activation and inflammatory disease processes [21,22]. TRACP mRNA and a monomeric TRACP protein, equivalent to serum TRACP 5a, demonstrated more abundant expression in adipose-derived macrophages of obese adults in comparison with those of lean individuals [23]. Therefore, elevated Acp5 may be representative of macrophage infiltration in the liver of SS and NASH model rats.

Tspo is involved in the regulation of cholesterol transport into mitochondria in relation to bile production and steroidogenesis as well as in oxidative stress, apoptosis and inflammatory and immune responses [24-32]. Interestingly, Kugler et al. found that Tspo appears to be an active participant in the generation of Reactive oxygen Species (ROS) at mitochondrial levels and in the modulation of the mitochondrial membrane potential, thereby playing a role in the induction of the mitochondrial apoptosis cascade [33,34]. Increased Tspo may be a key factor in oxidative stress in the liver of SS and NASH model rats. The 4-1BB (also known as ILA; 4-1BB; CD137; CDw137) receptor, a recently identified molecule of the tumor necrosis factor-receptor (Tnfr) superfamily, is a type I membrane protein expressed on activated cytolytic and helper T cells [35]. The 4-1BB receptor ligand (4-1BBL) is expressed on APCs including B cells, macrophages and dendritic cells [36,37]. Ligation of 4-1BB with 4-1BBL plays an important role in sustaining T cell activation and amplifying Cytotoxic T Lymphocyte (CTL) response [38]. Enhanced Tnfr may be related to cytokine signal in the liver of SS and NASH model rats. These data suggested that Acp5, Tspo and Tnfr were already elevated in liver at the SS stage, which may initiate macrophage infiltration and oxidative stress in the liver. Prostacyclin (PGI2) inhibits platelet aggregation and vasoconstriction. PGI2 synthase (Ptgis), which is widely distributed, occurs predominantly in vascular endothelial and smooth muscle cells [39]. Ptgis is over-expressed and exhibits a strong protective effect against cytokine toxicity, which is correlated with decreased activation of the transcription factor NFkB and the inducible NO synthase promoter as well as reduced inducible NO synthase protein expression and nitrite production. Reduction in the cytokine-stimulated endoplasmic reticulum and mitochondrial stress was also observed in the Ptgis-over-expressing cells. As a result, increased Ptgis may function as a protection system in the liver of SS and NASH model

rats. The Glutathione-S-transferases (GST) comprise a major group of detoxification enzymes, which are ubiquitous in all eukaryotic species [40]. GST is responsible for catalyzing the conjugation of Glutathione (GSH) to a wide spectrum of electrophilic compounds including endogenous substances and xenobiotics [41]. Increased expression of GST in response to exposure to xenobiotics is thought to constitute an adaptive response by the exposed cells to chemical or oxidative stress [42]. Gsta5 may also play a role in the protection system in the NAFLD model liver. It is possible that Ptgis and Gsta5 are up-regulated in compensatory fashion in NAFLD liver as a protective mechanism. Rab8, Rgn and Gstk1 decreased among genes correlated with progression of NAFLD.

Rab8, which regulates endosomal cholesterol removal to apoA-I in human fibroblasts, is abundantly expressed in human atherosclerotic lesion macrophages and up-regulated on lipid loading of macrophages *in vitro*; additionally, Rab8 reduces foam cell formation by facilitating ABCA1 surface expression and stimulating endosomal cholesterol efflux to apoA-I in primary human macrophages [43]. Rab8 redistributes cholesterol from late endosomes to the cell periphery and stimulates cholesterol efflux to the ABCA1-ligand apolipoprotein A-I (apoA-I) without increasing cholesterol esterification. Depletion of Rab8 from wild-type fibroblasts results in cholesterol deposition within late endosomal compartments [44]. Diminished Rab8 expression may be related to lipid deposition in the liver of SS and NASH model rats.

Rgn (also known as Senescence Marker Protein-30 (SMP30)) acts as an antioxidant and anti-apoptotic protein. Hepatic SMP30 is closely associated with the pathogenesis of NAFLD [45]; over-expression of SMP30/GNL in Hep G2 cells contributes to decreased ROS formation accompanied by declines in lipid peroxidation, SOD activity and GSH levels [46]. SMP30, which is a potential biomarker for the diagnosis and prognosis of acute liver failure, also plays a very important role in a self-protective mechanism in survival and participates in the pathophysiological processes of acute liver failure [47]. Therefore, the down-regulation of Rgn may be representative of increased oxidative stress in the liver of SS and NASH model rats.

The kappa class GST, which occurs specifically within mitochondria and peroxisomes [48], conjugates glutathione to the classic xenobiotic substrate 1-chloro-2,4-dinitrobenzene. Detection of mGstk1 in the mitochondria of hepatic and renal tissue suggests that this kappa class transferase likely functions as an antioxidant in this organelle [49]. These data suggested that the anti-lipid deposition and antioxidant systems are diminished in the liver of SS and NASH model rats. Scarb2, Cd36, Dab2 and Gstm5 increased among genes expressed exclusively in NASH. Scarb2 is also known as AMRF, EPM4, LGP85, CD36L2, HLGP85, LIMP-2, LIMPII and Scavenger receptors type II (SR-BII). Ishikawa et al. [50] previously reported that SR-B type I and II are expressed in macrophages. Foam cell formation of macrophages is mediated by the uncontrolled uptake of modified and oxidized Low-Density Lipoprotein (LDL) via scavenger receptors, which produces excessive lipoprotein-derived Cholesteryl Ester (CE) accumulation [51]. Elevated scarb2 may be indicative of macrophage infiltration in the liver of NASH model animals.

Cd36 was described as a transporter governing the rate-limiting steps of fatty acid uptake on the plasma membrane of hepatocytes [52]. Involvement of the Cd36 antigen has been demonstrated in phagocytosis of apoptotic cells [53] as well as in the endocytosis of long-chain fatty acids, anionic phospholipids and oxidized lipoproteins [54]; moreover, Cd36 is a class B scavenger receptor. High LDL levels have been shown to become atherogenic when oxidized to modify LDL (Ox-LDL) by inducing foam cell formation via enhanced Cd36 expression on

macrophages. In addition to Ox-LDL, elevated levels of glucose, insulin resistance, low HDL cholesterol and increased levels of free fatty acid (FFA) all result in enhanced expression of CD36, thereby contributing to type 2 diabetes mellitus (T2DM) and related atherosclerosis. Therefore, up-regulation of Cd36 may lead to progression of lipid deposition in the liver of SS and NASH model rats. The cytokine TGF-beta acts as a tumor suppressor in normal epithelial cells during the early stages of tumorigenesis. It was suggested that down-regulation of Dab2 blocks TGF-beta-mediated inhibition of cell proliferation and migration and enables TGF-beta promotion of cell motility, anchorage-independent growth and tumor growth *in vivo* [55]. Up-regulation of Dab2 may be related to cytokine signaling in the liver of SS and NASH model rats.

Members of the GST isoenzyme families, alpha, mu and pi, are elevated in response to chemical and oxidative stress [56]. An increasing volume of data suggests that African-Americans with NAFLD tend to display less progressive liver disease. In comparison to Caucasian NASH patients, African-American NASH patients exhibit over-expression of GSTM 2, GSTM4, GSTM5, FH and ASCL4 [57]. Over-expression of glutathione S-transferase mu transcripts (GSTM1, GSTM3, GSTM4 and GSTM5) may contribute to a decrease in oxidative stress [58]. These findings suggested that increased Scarb2, Cd36 and Dab2 may be related to pathogenesis of NASH and that GSTm5 may be enhanced in compensatory fashion.

Fmo5, which belongs to a family of enzymes that catalyzes the oxygenation of nucleophilic N- and S-containing compounds, decreased among genes expressed exclusively in NASH. The FMO enzyme family consists of five forms (FMOs1-5) that share approximately 50-60% sequence identity with one another [59]. Fmo1, Fmo3 and Fmo5 mRNAs were also found to be down-regulated in LPS models of inflammation [60]. Toll-Like Receptor (TLR) 4 is responsible for LPS signaling in association with several proteins. The down-regulation of Fmo3 and Fmo5 in this model is TLR4-dependent [61]. These data suggested that decreased Fmo5 may be related to inflammation of NASH liver.

Conclusion

Hepatic gene expressions in NAFLD and NASH model rats were elucidated. We hypothesized that scavenger receptor class B and Glutathione S-transferase (GST) play important roles in the progression from simple NAFLD to NASH. The current data afford beneficial information regarding therapeutic targets of NAFLD/NASH progression. Additional studies are necessary in order to confirm the role of these genes.

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