DNA Microarray Analysis of Type 2 Diabetes-Related Genes Co-regulated between White Blood Cells and Livers of Diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) Rats

Yasuhiro HAYASHI,[a] Shinya IIDA,[a] Yuichiro SATO,[a] Akihiro NAKAYA,[b] Akihiro SAWADA,[c] Noritada KAJI,[c] Hiroyuki KAMIYA,[a] Yoshinobu BABA,[c] and Hideyoshi HARASHIMA*,[a]

[a] *Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University; Sapporo, Hokkaido 060–0812, Japan:* [b] *Department of Computational Biology, University of Tokyo; Kashiwa, Chiba 277–8561, Japan: and* [c] *Department of Applied Chemistry, Graduate School of Engineering, Nagoya University; Nagoya, Aichi 464–8603, Japan.* Received December 29, 2006; accepted January 18, 2007; published online January 24, 2007

In a previous study, we hypothesized that some type 2 diabetes mellitus susceptible genes may be up/down-regulated in white blood cells (WBC) of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, reflecting their up/down-regulation in major insulin-target tissues such as the liver before the onset of diabetes. We identified 57 potential candidate genes for predicting diabetes. In this study, we examined this hypothesis further by extending the experimental conditions from before the onset (6 weeks) to after the onset (24 weeks) of diabetes that type 2 diabetes mellitus susceptible genes are co-regulated in WBC, reflecting their expression in the liver. Using rat oligo DNA microarrays, we found that 48 genes are up/down-regulated in OLETF rats compared to control Long-Evans Tokushima Otsuka (LETO) rats in WBC and liver under fasting or insulin administration conditions. Twenty nine and 33 genes were up/down-regulated in both WBC and livers, respectively, under fasting and insulin administration conditions, respectively. Eight out of 29 genes in fasting condition and 12 out of 33 genes in insulin administration conditions have been reported to be type 2 diabetes mellitus susceptible genes and the remainder have not been reported to be related to type 2 diabetes mellitus. These results support our hypothesis that the expression levels of type 2 diabetes mellitus related genes in WBC are reflective of those in the liver after the onset of diabetes.

Key words white blood cell; type 2 diabetes mellitus; gene expression; oligo DNA microarray; Otsuka Long-Evans Tokushima Fatty rat

The ultimate goal of the Human Genome Project is to elucidate and characterize the complete sequence of the 3×10^9 base pairs of the human genome. A knowledge of all the human genes and their functions may allow effective preventative measures, and have an impact on drug discovery and development processes.[1)] Under these circumstances, a genetic diagnosis would become possible, if disease-related genes could be identified and alteration of their expressions could be estimated before the onset of the disease.

In this study, we focused on type 2 diabetes mellitus, since it is considered to be a multi-factorial disorder that includes both genetic and environmental factors. Genome wide screening has been performed for genes responsible for type 2 diabetes mellitus in adipose, liver and skeletal muscle.[2—4)] The recent development of DNA microarray technology has made it possible to analyze gene expression on a genome-wide basis, to determine the gene expression profile and its relationship between organs can also be analyzed.[5)] These studies provide information on disease states at the molecular and genetic levels.

However, it is difficult to perform a genetic analysis on type 2 diabetes mellitus in a clinic using tissue samples such as liver, adipose and muscle. Therefore, we proposed an approach to the genetic diagnosis of type 2 diabetes mellitus that involves, by measuring the up/down regulation of some type 2 diabetes mellitus related genes using white blood cells (WBC), since the peripheral blood is an accessible source of cells and circulating WBC can be viewed as a surveillance of the body. The gene expression responses of circulating WBC can potentially provide an early warning of potential threats and can be used for the diagnosis of only infections but other diseases as well.[6)]

We reported that the expression of the calpain 10 gene in WBC is significantly decreased after the onset of type 2 diabetes in Otsuka-Long-Evans Tokushima Fatty (OLETF) rats, compared to Long-Evans Tokushima Otsuka (LETO) control rats. OLETF rats are used as a model of type 2 diabetes, and a decrease in gene expression was found in liver, adipose and muscle.[7)] We also performed a genome-wide gene expression analysis using OLETF and LETO rats before onset and 57 genes were found to have the same up/down gene expression pattern between WBC and the liver.[8)]

In this study, we hypothesize there are some type 2 diabetes mellitus related genes which are up/down-regulated between WBC and liver after the onset of diabetes. A genome wide gene expression analysis was performed by comparing WBC and liver using an oligo DNA microarray.

MATERIALS AND METHODS

Animals Twenty-four-week old male OLETF and LETO rats were generously donated by the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan).[9)] The rats were housed with free access to water and food at 22°C and 58% humidity with light from 7:00 to 19:00. All animal protocols were approved by the institutional animal care and research advisory committee of the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

Tissue Sampling Twenty-four-week old male OLETF rats were used as an after onset model, based on an intraperitoneal glucose tolerance test (IPGTT). After overnight fasting, whole blood and liver tissue were collected as fasting

* To whom correspondence should be addressed. e-mail: harasima@pharm.hokudai.ac.jp © 2007 Pharmaceutical Society of Japan

samples. Porcine insulin (SIGMA, St. Louis, MO, U.S.A.) was injected intraperitoneally into 24-week old rats (5 U/kg BW) after overnight fasting, and rats were cervically dissociated 1 h after insulin administration. Whole blood and liver were collected after decapitation.[10] All experiments were carried out without the use of an anesthetic, because diethyl ether affects serum glucose concentrations.

Isolation of Total RNA Whole blood was collected from the carotid artery and total RNA was extracted from WBC using a PAXgene Blood RNA kit (PreAnalytiX, Hombrechtikon, Switzerland). The liver was harvested and rapidly frozen in liquid nitrogen, and all samples were stored at −80 °C until used for assay. Total RNA from stocked liver samples were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and purified using an RNeasy Mini Kit combined with a RNase-free DNase Digest Set (QIAGEN, Hilden, Germany) for the degradation of genomic DNA in total RNA samples, and total RNA extracted from four rats (5 μg each) were then pooled into one sample for normalizing a individual differences, followed by a gene expression analysis in both tissues by oligo DNA microarrays.[11] Total RNA extracts from WBC and liver were stored at −80 °C until used in the assays.

DNA Microarray Experiments In this study, 500 ng aliquots of pooled RNA samples under the respective conditions were labeled using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Product No. 5184-3568, Agilent Technologies, Palo Alto, CA, U.S.A.) according to the manufacture's instructions. After checking the labeling efficiency, 1 μg aliquots of Cy3-labeled LETO cRNA and Cy5-labeled OLETF cRNA were mixed and then hybridized to Agilent Rat Oligo Microarrays (G4130A) using the manufacturer's hybridization protocol (Product No. 5184-3568, Agilent Technologies, Palo Alto, CA, U.S.A.). After the washing step, the microarray slides were analyzed using an Agilent Microarray scanner (G2565AA). These experiments were carried out in duplicate using exchanged dye-labeled cRNA probes (*i.e.*, Cy3 and Cy5 dye-swapping experiments). Data were analyzed using the Agilent Feature Extraction software (Version A.6.1.1).

Hierarchical Clustering The expression levels of genes in WBC and livers were observed for both fasting and insulin administration. The expression levels of the genes under these four conditions were screened. Each sample type is represented by the logarithm (Log_{10}) of the fluorescence signal ratio (Cy5 signal to Cy3 signal), referred to hereafter as the "log ratio" for simplicity. In this rat oligo DNA microarray, each spot (also referred to as "feature") contains copies of a nucleic acid sequence (60 bases) that is complementary to some segment of the DNA sequence of the gene that the spot detects. Since a spot corresponds to one of the partial sequences of the target gene instead of the entire sequence, a single gene can, in some cases, correspond to more than one spot on the DNA microarray. A set of the four expression levels of a gene is called an expression profile of the gene and the similarity between the expression patterns of two genes is identified as the Euclidean distance between their expression profiles, in this analysis. Note that a lower distance value means that two genes share a similar expression profile.

Screening Criteria of Concurrently Regulated Genes between WBC and Liver Genes which were differentially expressed in OLETF rats compared to LETO rats were selected for fasting and insulin administrated conditions. The data were statistically analyzed by the ANOVA test and genes with p values<0.01 were selected. Dye-swapping experiment data (LETO rat RNA sample labeled with Cy5 and OLETF rat RNA samples labeled with Cy3) were also used to extract highly reproducible spots. Among these genes, only those that were simultaneously up/down regulated both in WBC and liver were screened under the respective conditions.

Quantitative Reverse Transcription PCR cDNA was prepared from 400 ng aliquots of pooled RNA samples from OLETF or LETO using TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (code No. RR019A, TaKaRa, Kusatsu, Japan) according to the manufacture's instructions. The reverse transcription reaction was performed by incubation at 55 °C for 30 min, at 99 °C for 5 min and at 4 °C for 5 min. The resulting cDNA was analyzed by quantitative PCR using SYBR Green Realtime PCR Master Mix (Code No. QPK-201, 201T, Toyobo, Osaka, Japan) according to the manufacture's instructions except that concentration of the primers used were 0.2 μM.

Each gene-specific primer set was designed using Primer Express Software Ver 2.0 (Applied Biosystems, Lincoln Centre Drive Foster City, CA, U.S.A.) and these sequences are shown in Table 1. PCR was performed using the following program; 95 °C for 15 s and 60 °C for 1 min, after 10 min pre-incubation at 95 °C in an Applied Biosystems 7500 Real Time PCR System. All samples were run in triplicate and data were calculated using the comparative Ct method and are expressed as a ratio to glyceraldehydes-3-phosphate dehydrogenase (GAPDH), as a reference.

RESULTS

Gene Expression Profiles in WBC and Liver of OLETF and LETO Rats under Fasting and Insulin Administration The gene expression profiles under fasting conditions and insulin administration in WBC and livers are shown in Fig. 1. Each row corresponds to a gene and the four columns correspond to WF, LF, WI, and LI, as described in the figure legend. One thousand ninety two genes were detected as sig-

Table 1. Sequence of PCR Primers

Gene	GenBank accession No.	Primer sequence	Size of PCR product (bp)
Acbd4	NM_001012013	Forward: 5′-aagtgggatgcctggaacag-3′ Reverse: 5′-accagcttcatctccgtgatg-3′	80
Tcn2	NM_022534	Forward: 5′-cagcttctccgagtccctgat-3′ Reverse: 5′-gccgcaattcgatggtttc-3′	85
GAPDH	AF106860	Forward: 5′-ggcaagttcaacggcacagt-3′ Reverse: 5′-atgggtttcccgttgatgac-3′	62

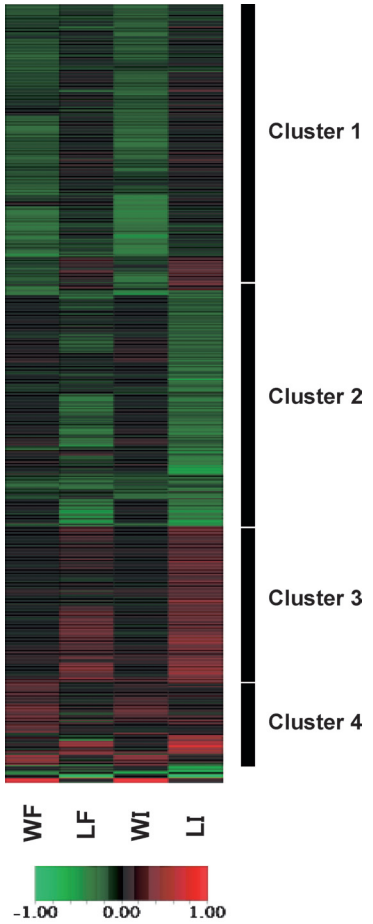

Fig. 1. Hierarchical Clustering Analysis of Genes That Are Expressed Differentially under Fasting and Insulin Administration Conditions in WBC and the Liver

Gene expression levels of OLETF rats compared to LETO rats are displayed a color-coded format. The scale bar represents the expression level of each gene of OLETF rats relative to that of LETO rats on a $\log_{10}$ scale of normalized mean signal intensities. Red and green represent an increase and decrease in expression level relative to the median value, respectively. Black represents the same degree of expression in OLETF and LETO rats. Each column represents the sampling conditions, WF: WBC under fasting, LF: liver under fasting, WI: WBC under insulin administration, LI: liver under insulin administration. Details of gene identity and expression differences are shown in the Tables 2 and 3.

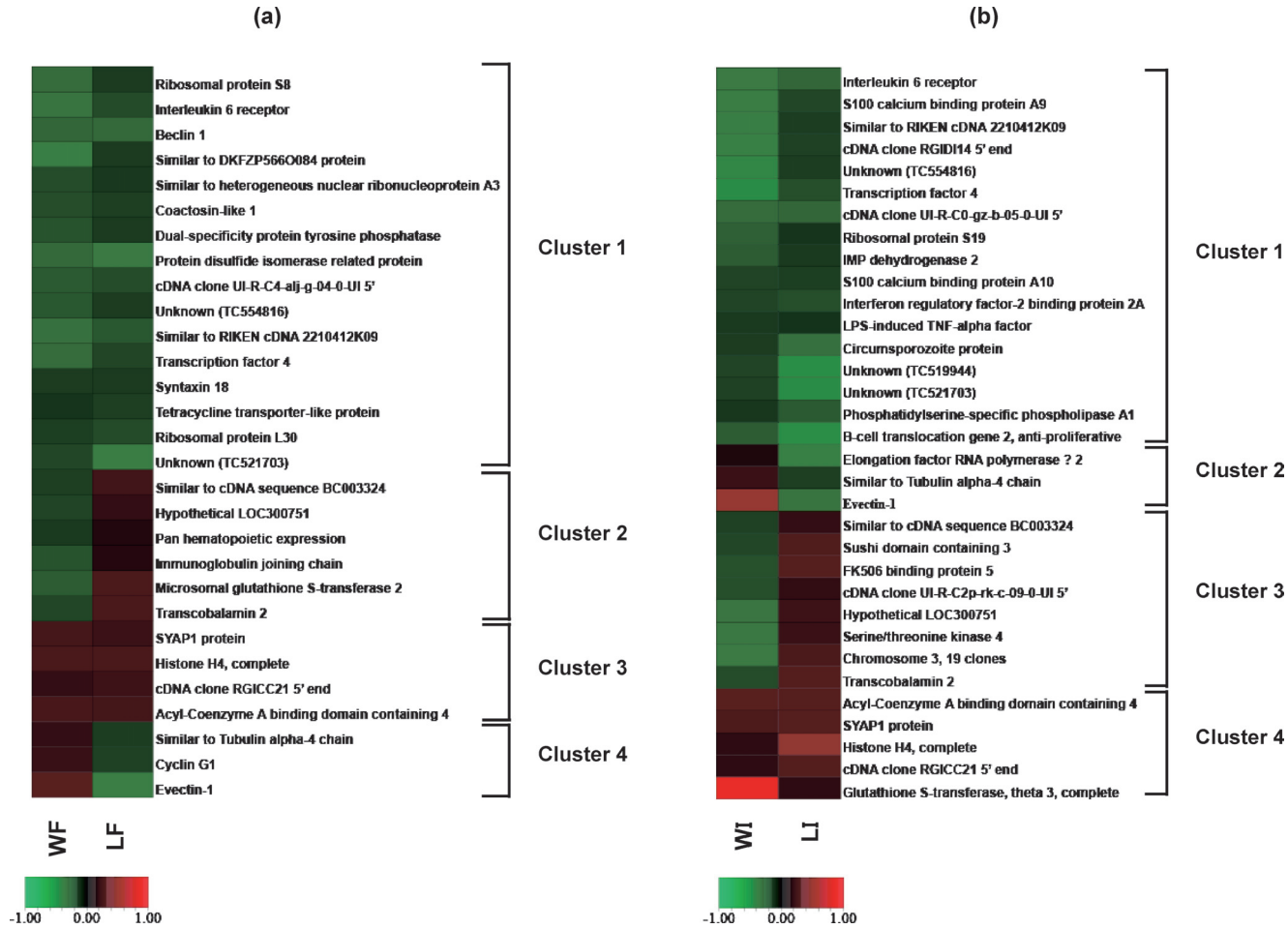

Fig. 2. Genes Whose Expressions Were Concurrently Altered in WBC and Liver Were Clustered under Conditions Described in Materials and Methods

Forty-eight genes (29 genes under fasting and 33 genes under insulin administration including 14 identical genes) were screened as candidate genes for reflecting type 2 diabetes mellitus under fasting (a) and insulin administration (b).

nificant signals at least under one condition. To extract some of the highly reproducible spots, a dye-swapping data (LETO rat RNA sample labeled with Cy5 and OLETF rat RNA samples labeled with Cy3) were also used. These genes were further analyzed using hierarchical clustering to group genes with similar expression patterns in their expression profiles (Fig. 1). These genes were subsequently divided into four main clusters. The genes in cluster 1 showed a tendency to be decreased in WBC of the OLETF rats (green color in columns WF and WI), with a relatively small alteration in the liver (black color in columns LF and LI) under both conditions. Cluster 2 contains genes that are down-regulated under both conditions in the liver (green color in columns LF and LI), with a relatively small alteration in WBC. Cluster 3 contains genes that are up-regulated under both conditions in the liver (red color in columns LF and LI), with a relatively small alteration in WBC. Cluster 4 contains genes in WBC that are up-regulated under both conditions (red color in columns WF and WI). Under both conditions for OLETF rats in the diabetic state, in WBC, the number of differentially down-regulated genes were more numerous than those of differentially up-regulated genes. On the other hand, in the liver under both conditions, a similar number of genes were up-regulated or down-regulated. These results suggest that, in the diabetic state, differences exist in the regulation of gene expression in WBC vis-à-vis the liver. In the following analysis, differences in the expression patterns depending on the different conditions in both tissues were examined. Insulin signals could be generated in both WBC and the liver by the administration of insulin (compare column WF and

Table 2. Genes That Are Differentially Expressed in WBC of OLETF Rats under Fasting and Insulin Administration

Top 20 genes that are up- or down-regulated in OLETF under fasting.

GenBank accession No.	Annotation	Log_{10}(OLETF/LETO) ratio
NM_130740	Protein kinase C and casein kinase substrate in neurons 2 (Pacsin2)	−0.45
NM_022510	Ribosomal protein L4 (Rpl4)	−0.45
NM_173329	Defensin, alpha 5, Paneth cell-specific (Defa)	−0.42
NM_001007653	Mitochondrial ribosomal protein S15 (Mrps15)	−0.41
NM_017317	Low Mr GTP-binding protein (Rab27a)	−0.38
XM_213656	Similar to mitochondrial ribosomal protein S6	−0.36
XM_344706	Mitochondrial ribosomal protein S21 (Mrps21_predicted)	−0.32
NM_053660	Guanine nucleotide binding protein gamma 10 (Gng10)	−0.32
NM_001006969	Interferon regulatory factor 3 (Irf3)	−0.32
NM_017020	Interleukin 6 receptor (Il6r)	−0.32
NM_172065	Proline-rich proteoglycan 2 (Prpg2)	1.58
NM_052808	Parotid secretory protein (Psp)	1.18
NM_012536	Chymotrypsinogen B (Ctrb)	0.91
NM_013097	Deoxyribonuclease I (Dnase1)	0.74
TC535594	Glutathione *S*-transferase, theta 3, complete	0.72
NM_207605	SH2 domain protein 2A (Sh2d2a)	0.52
XM_218343	Exosome component 5 (Exosc5_predicted)	0.43
XM_233798	Cysteine-rich motor neuron 1 (Crim1_predicted)	0.37
XM_237521	Similar to Lipin 2	0.35
NM_172033	Evectin-1 (Plekhb1)	0.32

Top 20 genes that are up- or down-regulated in OLETF under insulin administration.

GenBank accession No.	Annotation	Log_{10}(OLETF/LETO) ratio
NM_173329	Defensin, alpha 5, Paneth cell-specific (Defa)	−0.74
NM_130740	Protein kinase C and casein kinase substrate in neurons 2 (Pacsin2)	−0.65
NM_053369	Transcription factor 4 (Tcf4)	−0.47
XM_213346	Ribosomal protein L26 (Rpl26_predicted)	−0.46
NM_053982	Ribosomal protein S15a (Rps15a)	−0.46
NM_017317	Low Mr GTP-binding protein (Rab27a)	−0.43
NM_021576	5 Nucleotidase (Nt5)	−0.41
NM_012491	Adducin 2 (beta) (Add2)	−0.41
NM_022514	Ribosomal protein L27 (Rpl27)	−0.40
XM_344706	Mitochondrial ribosomal protein S21 (Mrps21_predicted)	−0.40
NM_172065	Proline-rich proteoglycan 2 (Prpg2)	1.39
NM_052808	Parotid secretory protein (Psp)	1.07
NM_012536	Chymotrypsinogen B (Ctrb)	1.06
NM_013097	Deoxyribonuclease I (Dnase1)	0.92
TC535594	Glutathione *S*-transferase, theta 3, complete	0.84
NM_172033	Evectin-1 (Plekhb1)	0.47
XM_218343	Exosome component 5 (Exosc5_predicted)	0.46
XM_341353	Exportin 7 (Xpo7_predicted)	0.44
NM_053535	Ectonucleotide pyrophosphatase/phosphodiesterase 1 (Enpp1)	0.38
NM_207605	SH2 domain protein 2A (Sh2d2a)	0.37

Twenty genes (top 10 down- and up-regulated genes) in WBC are shown. The scale of genes alteration represent as log_{10}(OLETF/LETO) ratio value. These genes were selected as described in Materials and Methods.

LF to column WI and LI, respectively). The top 20 genes for which their expressions were highly altered are listed in Tables 2 and 3. The upper and lower lists show genes for which the expression is remarkably altered under fasting and insulin administration in WBC, respectively (Table 2). Under fasting conditions, in the case of WBC, 339 genes were extracted, with 254 being down-regulated and 85 genes being up-regulated (Table 2, upper list). Under insulin administration in WBC, 354 genes were extracted, with 302 genes being down-regulated and 52 genes being up-regulated (Table 2, lower list). In the liver, under fasting conditions, 410 genes were extracted, 217 genes were down-regulated and 193 genes were up-regulated (Table 3, upper list). Under insulin administration in the liver, 665 genes were extracted, of which 368 were down-regulated and 297 genes were up-regulated (Table 3, lower list).

Screening of Concurrently Regulated Genes in WBC and Liver of OLETF Rats In the following analysis, genes that were concurrently altered in WBC and the liver of OLETF were screened. Twenty-nine genes under fasting and 33 genes under insulin administration were detected (Fig. 2, Table 4). Since 14 genes were detected in both stimulations, forty-eight were screened in both conditions. Under fasting conditions, in cluster 1, sixteen genes were down-regulated in both WBC and the liver (colored green in column WF, LF, Fig. 2a). In cluster 2, six genes were down- and up-regulated in WBC and the liver, respectively (colored green and red in columns WF and LF). In cluster 3, four genes were up-regu-

Table 3. Genes That Are Differentially Expressed in the Liver of OLETF Rats under Fasting and Insulin Administration

Top 20 genes that are up- or down-regulated in OLETF under fasting.

GenBank accession No.	Annotation	Log_{10}(OLETF/LETO) ratio
NM_024352	Macrophage stimulating 1 (hepatocyte growth factor-like) (Mst1)	−0.98
NM_013122	Insulin-like growth factor binding protein 2 (Igfbp2)	−0.94
XM_343823	Serine (or cysteine) peptidase inhibitor, clade A member 7 (Serpina7)	−0.85
NM_001007627	STAR-related lipid transfer (START) domain containing protein 6 (Stard6)	−0.85
NM_030832	Fatty acid binding protein 7, brain (Fabp7)	−0.82
NM_130422	Caspase 12 (Casp12)	−0.61
NM_134379	Integral membrane transport protein UST4r	−0.60
NM_017270	Alcohol dehydrogenase 4 (class II) (Adh4)	−0.58
NM_053380	Solute carrier family 34, member 2 (Slc34a2)	−0.57
NM_031605	Cytochrome P450, 4a12 (Cyp4a12)	−0.56
NM_031741	Solute carrier family 2, member 5 (Slc2a5)	0.97
TC540475	Cytochrome b (Fragment), partial (12%)	0.52
XM_227134	Similar to carbon catabolite repression 4 protein homolog	0.50
NM_144755	Tribbles homolog 3 (Drosophila) (Trib3)	0.50
NM_017360	Coilin (Coil)	0.47
XM_237039	Zinc finger protein 451 (Zfp451_predicted)	0.47
NM_012703	Thyroid hormone responsive protein (Thrsp)	0.45
XM_214618	Abhydrolase domain containing 3 (Abhd3_predicted)	0.44
XM_213883	Transmembrane protein 9 (Tmem9_predicted)	0.42
NM_053703	Mitogen-activated protein kinase kinase 6 (Map2k6)	0.40

Top 20 genes that are up- or down-regulated in OLETF under insulin administration.

GenBank accession No.	Annotation	Log_{10}(OLETF/LETO) ratio
NM_013122	Insulin-like growth factor binding protein 2 (Igfbp2)	−1.12
NM_001007627	STAR-related lipid transfer (START) domain containing protein 6 (Stard6)	−1.07
NM_024352	Macrophage stimulating 1 (hepatocyte growth factor-like) (Mst1)	−1.06
NM_031628	Nuclear receptor subfamily 4, group A, member 3 (Nr4a3), transcript variant 1	−0.96
XM_343823	Serine (or cysteine) peptidase inhibitor, clade A member 7 (Serpina7)	−0.91
NM_024388	Nuclear receptor subfamily 4, group A, member 1 (Nr4a1)	−0.66
NM_021836	Jun-B oncogene (Junb)	−0.66
NM_053698	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (Cited2)	−0.60
NM_001009642	Processing of precursor 4, ribonuclease P/MRP family, (Pop4_predicted)	−0.60
NM_001008321	Growth arrest and DNA-damage-inducible 45 beta (Gadd45b_predicted)	−0.59
TC540475	Cytochrome b (Fragment), partial (12%)	0.88
NM_031741	Solute carrier family 2, member 5 (Slc2a5)	0.80
NM_138504	Pregnancy-induced growth inhibitor (Okl38)	0.78
NM_012565	Glucokinase (Gck)	0.68
NM_052798	Zinc finger protein 354A (Znf354a)	0.68
NM_017360	Coilin (Coil)	0.57
NM_013039	ATP-binding cassette, sub-family C (CFTR/MRP), member 8 (Abcc8)	0.56
NM_031815	Inhibin beta E (Inhbe)	0.54
NM_057133	Nuclear receptor subfamily 0, group B, member 2 (Nr0b2)	0.53
XM_237039	Zinc finger protein 451 (Zfp451_predicted)	0.53

Twenty genes (top 10 down- and up-regulated genes) in liver are shown. The scale of genes alteration represent as log_{10}(OLETF/LETO) ratio value. These genes were selected as described in Material and Methods.

Table 4. Concurrently Expressed Genes in WBC and Livers of OLETF Rats under Fasting and Insulin Administration

Fasting

GenBank accession No.	Annotation	Log_{10}(OLETF/LETO) ratio		Type 2 diabetes mellitus related function
		WBC	Liver	
TC555933	Histone H4, complete	0.26	0.26	Insulin biosynthesis
NM_001012013	Acyl-Coenzyme A binding domain containing 4 (Acbd4_predicted)	0.25	0.24	Lipid metabolism
NM_001004253	SYAP1 protein (Syap1)	0.24	0.21	Not reported
AW143886	cDNA clone RGICC21 5′ end	0.19	0.22	Not reported
NM_172033	Evectin-1 (Plekhb1)	0.32	−0.37	Not reported
NM_012923	Cyclin G1 (Ccng1)	0.20	−0.19	Not reported
NM_001007004	Similar to Tubulin alpha-4 chain (Alpha-tubulin 4)	0.19	−0.17	Type 1 diabetes immune response
XM_215562	Microsomal glutathione *S*-transferase 2 (Mgst2_predicted)	−0.28	0.27	Not reported
XM_341195	Immunoglobulin joining chain (Igj_predicted)	−0.25	0.16	Not reported
NM_022534	Transcobalamin 2 (Tcn2)	−0.21	0.26	Diabetic neuropathy
XM_217167	Hypothetical LOC300751 (predicted)	−0.20	0.19	Not reported
BC088266	Pan hematopoietic expression (predicted)	−0.20	0.15	Not reported
NM_001004205	Similar to cDNA sequence BC003324	−0.18	0.22	Not reported
NM_001008507	Similar to DKFZP566O084 protein (RGD1311243_predicted)	−0.37	−0.17	Not reported
NM_017020	Interleukin 6 receptor (Il6r)	−0.32	−0.22	Type 2 diabetic immune response
XM_216371	Similar to RIKEN cDNA 2210412K09 (RGD1305968_predicted)	−0.31	−0.27	Not reported
NM_053369	Transcription factor 4 (Tcf4)	−0.30	−0.20	Glucose homeostasis
NM_031706	Ribosomal protein S8 (Rps8)	−0.30	−0.17	Not reported
NM_053849	Protein disulfide isomerase related protein (Erp70)	−0.30	−0.34	Not reported
NM_053739	Beclin 1 (Becn1)	−0.29	−0.30	Not reported
BF564210	cDNA clone UI-R-C4-alj-g-04-0-UI 5′	−0.27	−0.23	Not reported
TC554816	Unknown	−0.27	−0.17	Not reported
U42627	Dual-specificity protein tyrosine phosphatase (rVH6)	−0.25	−0.16	Not reported
XM_341700	Coactosin-like 1 (Cotl1_predicted)	−0.23	−0.18	Diabetic neuropathy
XM_237842	Similar to heterogeneous nuclear ribonucleoprotein A3	−0.23	−0.16	Insulin Biosynthesis
TC521703	Unknown	−0.21	−0.36	Not reported
NM_022699	Ribosomal protein L30 (Rpl30)	−0.17	−0.22	Not reported
NM_001012151	Syntaxin 18 (Stx18_predicted)	−0.17	−0.16	Insulin resistance
TC558575	Tetracycline transporter-like protein, partial (24%)	−0.15	−0.18	Not reported

Insulin injection

GenBank accession No.	Annotation	Log_{10}(OLETF/LETO) ratio		Type 2 diabetes mellitus related function
		WBC	Liver	
TC535594	Glutathione *S*-transferase, theta 3, complete	0.84	0.18	Lipid metabolism
NM_001012013	Acyl-Coenzyme A binding domain containing 4 (Acbd4_predicted)	0.32	0.31	Lipid metabolism
NM_001004253	SYAP1 protein (Syap1)	0.28	0.31	Not reported
AW143886	cDNA clone RGICC21 5′ end	0.18	0.31	Not reported
TC555933	Histone H4, complete	0.18	0.44	Insulin biosynthesis
NM_172033	Evectin-1 (Plekhb1)	0.47	−0.32	Not reported
NM_001007004	Similar to Tubulin alpha-4 chain (Alpha-tubulin 4)	0.20	−0.18	Type 1 diabetes immune response
XM_226624	Elongation factor RNA polymerase II 2 (Ell2_predicted)	0.15	−0.39	Not reported
AABR03024169	Chromosome 3, 19 clones	−0.35	0.27	Not reported
XM_230833	Serine/threonine kinase 4 (Stk4_predicted)	−0.34	0.20	Not reported
XM_217167	Hypothetical LOC300751 (predicted)	−0.32	0.22	Not reported
NM_001012174	FK506 binding protein 5 (Fkbp5_predicted)	−0.25	0.31	Glucocorticoid marker
BF547336	cDNA clone UI-R-C2p-rk-c-09-0-UI 5′	−0.24	0.18	Not reported
NM_022534	Transcobalamin 2 (Tcn2)	−0.23	0.31	Diabetic neuropathy
XM_225203	Sushi domain containing 3 (Susd3_predicted)	−0.21	0.29	Not reported
NM_001004205	Similar to cDNA sequence BC003324	−0.19	0.19	Not reported
NM_053369	Transcription factor 4 (Tcf4)	−0.47	−0.23	Glucose homeostasis
TC554816	Unknown	−0.43	−0.17	Not reported
AW916109	cDNA clone RGIDI14 5′ end	−0.39	−0.19	Not reported
XM_216371	Similar to RIKEN cDNA 2210412K09 (RGD1305968_predicted)	−0.37	−0.17	Not reported
NM_053587	S100 calcium binding protein A9 (S100a9)	−0.36	−0.21	Atherosclerosis
NM_017020	Interleukin 6 receptor (Il6r)	−0.35	−0.30	Type 2 diabetes immune response
BF556693	cDNA clone UI-R-C0-gz-b-05-0-UI 5′	−0.30	−0.29	Not reported
XM_574391	Ribosomal protein S19 (Rps19)	−0.29	−0.15	Not reported
NM_017259	B-cell translocation gene 2, anti-proliferative (Btg2)	−0.28	−0.47	Not reported
NM_199099	IMP dehydrogenase 2 (Impdh2)	−0.27	−0.16	Not reported
NM_031114	S100 calcium binding protein A10 (S100a10)	−0.20	−0.17	GLUT translocation
TC519944	Unknown	−0.20	−0.48	Not reported
TC538123	Interferon regulatory factor-2 binding protein 2A, partial (64%)	−0.20	−0.24	Not reported
TC521703	Unknown	−0.19	−0.47	Not reported
TC537997	Circumsporozoite protein, partial (26%)	−0.17	−0.32	Not reported
XM_343856	LPS-induced TNF-alpha factor (Litaf)	−0.16	−0.14	Insulin resistance
NM_138882	Phosphatidylserine-specific phospholipase A1 (Pspla1)	−0.15	−0.27	Lipid metabolism

Genes shown in Fig. 2 are listed. Accession No., annotation, scale of alteration, and function of genes are described. These genes were selected as described in Materials and Methods.

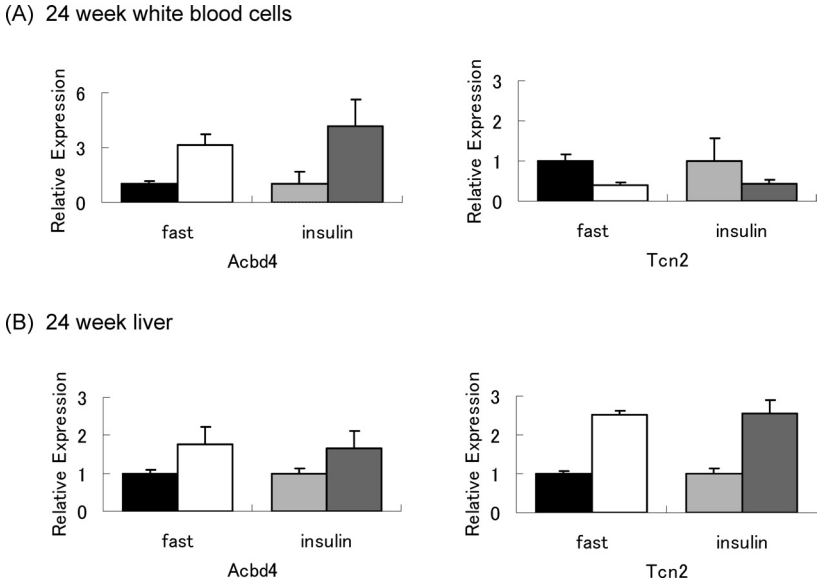

Fig. 3. mRNA Expression in the White Blood Cells or Liver of LETO and OLETF Rats Quantitated by RT PCR

Total RNA was isolated from white blood cells or livers of LETO and OLETF rats at 24 weeks of age under fasting and insulin administration. The gene expression of Acbd4 (acyl-Coenzyme A binding domain containing 4) and Tcn2 (transcobalamin 2) were measured by RT PCR. The levels of each transcript are normalized to the levels of GAPDH. The level of mRNA is the relative expression to the corresponding value of LETO rats at 24 weeks. Black columns, LETO rats under fasting; open columns, OLETF rats under fasting; hatched columns, LETO rats under insulin administration; gray columns, OLETF rats under insulin administration. Bars represent the means±S.E. (n=3).

lated in both tissues (colored red in column WF, LF). In cluster 4, three genes were up- and down-regulated in WBC and the liver, respectively (colored red and green in columns WF and LF). Under insulin administration, in cluster 1, seventeen genes were down-regulated in both WBC and the liver (colored green in column WI, LI , Fig. 2b). In cluster 2, three genes were up- and down-regulated in WBC and the liver, respectively (colored red and green in columns WI and LI). In cluster 3, eight genes were down- and up-regulated in WBC and the liver (colored green and red in columns WI, LI). In cluster 4, five genes were up-regulated in both tissues (colored red in columns WI and LI). The expression of a total of 48 out of 1092 genes were simultaneously altered in WBC and the liver. The selected genes in Fig. 2 with expression ratios on a $\log_{10}$ scale and the putative biological functions of their encoded proteins are also summarized (Table 4). Fourteen out of 48 genes reported to be type 2 diabetes mellitus related genes were screened, while no information related to type 2 diabetes mellitus for the remaining 34 genes.

Quantitative Reverse Transcription PCR Quantitative reverse transcription PCR was performed to confirm the microarray data. We focused on genes that were screened in both conditions. In these genes, we selected two genes, acyl-Coenzyme A binding domain containing 4 (Acbd4) and transcobalamin 2 (Tcn2). As shown in Fig. 3, the relative expression of Acbd4 was increased in WBC and the liver of 24-week old OLETF rats under fasting and insulin administration conditions, compared with LETO rats of the same age, which is consistent with the microarray data. The relative expression of Tcn2 was decreased in WBC and increased in the liver of 24-week old OLETF rats under both conditions compared with LETO rats of the same age, which is consistent with the microarray data.

DISCUSSION

WBC are easily obtained and thus useful as specimens for diagnosis. In addition, WBC can be used to determine individual variability and certain pathological conditions. For example, variations in gene expression in WBC from healthy individuals were analyzed and genes whose expression varies depending on genetic factors, sex, and age were identified.[12,13] A genome-wide expression analysis was applied for patients and healthy persons and the possible use of WBC for clinical diagnosis was examined.[14] Personalized medicine based on gene expression profiles of WBC is expected to be carried out in the near future.

Preliminary clinical applications of the global gene expression analysis of WBC to the diagnosis of neurological diseases in humans have been reported.[15] In fact, the expression of marker genes was reported to be significantly up-regulated in WBC as well as caudate nuclei in Huntington's disease patients.[16] These reports demonstrate the presence of genes whose expression is altered at the transcriptional level in common in WBC and the brain although the number of genes was not given. Thus, it is possible to predict and understand pathological conditions using blood mRNAs.

We examined this possibility independently for type 2 diabetes mellitus. First, we demonstrated that the expression of the calpain 10 gene, a diabetes-related gene, was significantly down-regulated in WBC and the liver of precritical OLETF rats, indicating the possibility of predicting gene expression patterns in other organs by that in WBC.[7] We then performed a genome-wide expression analysis of WBC and the liver of OLETF rats before onset to identify novel candidate genes for predicting the onset of type 2 diabetes. Fifty seven genes were identified as genes whose expression was altered both in WBC and the liver, suggesting the possibility of predicting and understanding pathological conditions by a genome-wide analysis of WBC mRNAs.[8]

In this study, we created gene expression profiles for WBC and the liver of OLETF rats after onset. 339 and 410, and 354 and 665 genes were detected as significant, reproducible spots, in WBC and the liver under fasting, and in WBC and the liver after insulin administration, respectively. The number of detected genes was similar in WBC under the two conditions, while the number increased by 1.6-fold in the liver upon insulin administration. However, a dynamic alteration in expression pattern (*i.e.* increase or decrease) was not observed in the liver (Fig. 1, LF and LI columns), possibly due to the acquisition of insulin-resistance after the onset of type 2 diabetes.

We found that 29 and 33 genes (total of 62 genes including 14 identical genes) were expressed differently in both WBC and the liver of OLETF and LETO rat strains under fasting and after insulin administration, respectively (Table 4). Before onset, fifty seven genes were found to be expressed differently in both WBC and the liver under the same conditions.[8] This suggests that altered expressions in WBC may reflect those in liver for these genes.

These 48 genes include 14 which are known to be related to type 2 diabetes (Table 4). Among the 20 genes, 8 and 12 were detected under fasting and after insulin administration, respectively. Genes related to an immune response and lipid metabolism were also included in the 48 genes.

The interleukin 6 receptor gene was detected as a gene related to an immune response. The interleukin 6 receptor may affect pathological conditions of type 2 diabetes by complex formation with interleukin 6, the expression of which is elevated by hyperglycemia.[17] The LPS-induced TNF α gene is activated by a lipopolysaccharide in a murine cell line derived from macrophages.[18] TNF α is an important factor involved in the onset of diabetes and TNF α production in subjects with type 2 diabetes may contribute to insulin resistance.[19] Although the expression of the TNF α gene decreased in WBC and the liver (Table 4), the elucidation of the regulation mechanism for the gene may link TNF α production with diabetes. The expression of the glutathione *S*-transferase theta 3 gene was altered in WBC and the liver. This gene may be involved in the pathogenesis since an association between a variant of glutathione *S*-transferase theta 1, and markers of inflammation and lipid peroxidation has been reported.[20]

The function of the acyl-coenzyme A binding domain containing 4 is uncertain at this time. However, it may be related to lipid metabolism since acyl-coenzyme A is a key in lipid metabolism.[21] It would be of interest to examine the role of the protein encoded by this gene.

Expression of the gene for the FK506-binding protein 5 was altered. It has been reported that the induction of 51-kDa FK506-binding protein mRNA by glucocorticoids may be a suitable marker for assessing individual glucocorticoid sensitivity.[22] The transcobalamin 2 protein binds vitamin B12 and diabetic patients have a higher total B12 binding capacity and higher serum B12 levels.[23] Thus, these genes are potential markers for judging the onset and progression of diabetes.

We used an oligo DNA microarray for a comprehensive analysis of gene expression. The altered expression of the 48 genes selected in this study were checked in a dye-swapping experiment to ensure the reproducibility of the data.[24] Indeed, very similar tendencies in expression were observed with quantitative RT PCR and with the microarray analysis (Fig. 3), indicating the reliability of the data.

In this study, we found that 48 genes are up/down-regulated in OLETF rats compared to control LETO rats in WBC and the liver under fasting or insulin administered conditions. It will be necessary to analyze functions of these genes *in vivo* and studies concerning this are currently in progress with siRNAs. Based on the results obtained with the knockdown experiments, the diagnosis of type 2 diabetes by gene expression will pave the way to clinical applications.

Acknowledgements This work was supported by a Grant-in-Aid for Exploratory Research from the Japan Society for the Promotion of Sciences (JSPS). The authors also wish to thank Dr. M. S. Feather for his helpful advice in preparing the English manuscript.

REFERENCES

1) Emilien G., Ponchon M., Caldas C., Isacson O., Maloteaux J. M., *QJM*, **93**, 391—423 (2000).
2) Lee Y. H., Nair S., Rousseau E., Allison D. B., Page G. P., Tataranii P. A., Bogardus C., *Diabetologia*, **48**, 1776—1783 (2005).
3) Heishi M., Ichihara J., Teramoto R., Itakura Y., Hayashi K., Gomi H., Sakai J., Kanaoka M., Taiji M., Kimura T., *Diabetologia*, **49**, 1647—1655 (2006).
4) Nguyen L. L., Kriketos A. D., Hancock D. P., Caterson I. D., Denyer G. S., *J. Biochem. Mol. Biol.*, **39**, 457—463 (2006).
5) Suh Y. H., Kim Y., Bang J. H., Choi K. S., Lee J. W., Kim W. H., Oh T. J., An S., Jung M. H., *J. Mol. Endocrinol.*, **34**, 299—315 (2005).
6) Whitney A. R., Diehn M., Popper S. J., Alizaden A. A., Boldrick J. C., Relman D. A., Brown P. O., *Proc. Natl. Acad. Sci. U.S.A.*, **100**, 1896—1901 (2003).
7) Sato Y., Kuwajima M., Kamiya H., Harashima H., *Biol. Pharm. Bull.*, **26**, 1765—1768 (2003).
8) Iida S., Sato Y., Nakaya A., Shinohara Y., Hayashi Y., Sawada A., Nagata H., Kaji N., Kamiya H., Baba Y., Harashima H., *Biol. Pharm. Bull.*, **29**, 2451—2459 (2006).
9) Kawano K., Hirashima T., Mori S., Saitoh Y., Kurosumi M., Natori T., *Diabetes*, **41**, 1422—1428 (1992).
10) Cefalu W. T., Wang Z. Q., Zhang X. H., Baldor L. C., Russell J. C., *J. Nutr.*, **132**, 1107—1114 (2002).
11) Yang Y. H., Dudoit S., Luu P., Lin D. M., Peng V., Ngai J., Speed T. P., *Nucleic Acids Res.*, **30**, e15 (2002).
12) Radich J. P., Mao M., Stepaniants S., Biery M., Castle J., Ward T., Schimmack G., Kobayashi S., Carleton M., Lampe J., Linsley P. S., *Genomics*, **83**, 980—988 (2004).
13) Eady J. J., Wortley G. M., Wormstone Y. M., Hughes J. C., Astley S. B., Foxall R. J., Doleman J. F., Elliott R. M., *Physiol. Genomics*, **22**, 402—411 (2005).
14) Cobb J. P., Mindrios M. N., Graziano C. M., Calvano S. E., Baker H. V., Xiao W., Laudanski K., Brownstein B. H., Elson C. M., Hayden D. L., Herndon D. N., Lowry S. F., Maier R. V., Schoenfeld D. A., Moldawer L. L., Davis R. W., Tompkins R. G., *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 4801—4806 (2005).
15) Sharp F. R., Xu H., Lit L., Walker W., Apperson M., Gilbert D. L., Glauser T. A., Wonq B., Hershey A., Liu D. Z., Pinter J., Zhan X., Liu X., Ran R., *Arch. Neurol.*, **63**, 1529—1536 (2006).
16) Borovecki F., Lovrecic L., Zhou J., Jeonq H., Then F., Rosas H. D., Hersch S. M., Hoqarth P., Bouzou B., Jensen R. V., Krainc D., *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 11023—11028 (2005).
17) Kado S., Nagase T., Nagata N., *Acta Diabetol.*, **36**, 67—72 (1999).
18) Means T. K., Pavlovich R. P., Roca D., Vermeulen M. W., Fenton M. J., *J. Leukocyte Biology*, **67**, 885—893 (2000).
19) Enqebretson S., Chertoq R., Nichols A., Hey-Hadavi J., Celenti R., Grbic J., *J. Clin. Periodontol.*, **34**, 18—24 (2007).
20) Hayek T., Stephens J. W., Hubbart C. S., Acharya J., Caslake M. J., Hawe E., Miller G. J., Hurel S. J., Humphries S. E., *Atherosclerosis*,

184, 404—412 (2006).
21) Murase T., Aoki M., Tokimitsu I., *Biochim. Biophys. Acta*, **1733**, 224—231 (2005).
22) Vermeer H., Hendriks-Stegeman B. I., Burg B., Sylvia C., Offers B., Jansen M., *J. Clin Endocrinol. Metab.*, **88**, 277—284 (2003).
23) Takahashi Y., Takayama S., Itou T., Owada K., Omori Y., *Diabetes Res. Clin. Pract.*, **25**, 13—17 (1994).
24) Suzuki Y., Nakayama M., *DNA Res.*, **10**, 263—275 (2003).