DNA Microarray Analysis of Whole Blood Cells and Insulin-Sensitive Tissues Reveals the Usefulness of Blood RNA Profiling as a Source of Markers for Predicting Type 2 Diabetes

Yasuhiro Hayashi,[a] Kazuaki Kajimoto,[a] Shinya Iida,[b] Yuichiro Sato,[b] Shogo Mizufune,[c] Noritada Kaji,[c,d] Hiroyuki Kamiya,[b] Yoshinobu Baba,[c,d,e,f] and Hideyoshi Harashima*,[a,b]

[a] *Laboratory of Innovative Nanomedicine, Graduate School of Pharmaceutical Sciences, Hokkaido University; Sapporo, Hokkaido 060–8638, Japan:* [b] *Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University; Sapporo, Hokkaido 060–0812, Japan:* [c] *Department of Applied Chemistry, Graduate School of Engineering, Nagoya University;* [d] *MEXT Innovative Research Center for Preventive Medical Engineering, Nagoya University; Nagoya, Aichi 464–8603, Japan:* [e] *Department of Advanced Medical Science, Graduate School of Medicine, Nagoya University; Nagoya, Aichi 266–8550, Japan: and* [f] *Health Technology Research Center, National Institute of Advanced Industrial Science and Technology; Takamatsu, Kagawa 761–0395, Japan.*

Received January 22, 2010; accepted March 9, 2010; published online March 15, 2010

To determine if gene expression profiling of whole blood cells is a useful source of markers for the early diagnosis of the onset of type 2 diabetes, we examined gene expression profiling of whole blood cells and type 2 diabetes-related organs, such as liver, adipose tissue, and skeletal muscle, of Otsuka Long-Evans Tokushima Fatty (OLETF) rats. At the age of 6 weeks, RNA was isolated from tissues of fasted OLETF and control Long-Evans Tokushima Otsuka (LETO) rats. Gene expression was analyzed using the Agilent rat oligo microarray. Gene ontology analysis showed that gene expression of biologically meaningful groups of genes in liver, adipose tissue, and skeletal muscle, which are involved in the pathogenesis of type 2 diabetes, differed between OLETF and LETO rats. Three hundred genes of whole blood cells were differentially expressed. Four out of these 300 genes were related to the insulin-signaling pathway and 57 out of 300 genes were up- or down-regulated in at least one tissues in OLETF rats. These results support our hypothesis that gene expression profiling of whole blood cells might be a useful source of markers to predict the onset of type 2 diabetes.

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

It is well known that diabetes is one of the most prevalent diseases worldwide. It is estimated that 246 million people have diabetes, and the number is expected to reach 380 million by 2025 (Diabetes Atlas, third edition International Diabetes Federation 2006). Type 2 diabetes accounts for 90—95% of all cases of diabetes, so development of effective treatments is important. There are many treatment modalities such as drugs, exercise therapy, and diet therapy; however, these cures are not effective in all patients because the underlying cause of the disease remains unclear. Type 2 diabetes is caused by many factors, such as multi-genetic factors, obesity, physical inactivity, and unhealthful diet. Therefore, health maintenance and preventive care are greatly needed.

Generally, the onset of type 2 diabetes is judged by measuring the level of fasting glucose and by the results of an oral glucose tolerance test (OGTT) in combination with other clinical parameters. However, the diagnosis of risk prior to the onset of type 2 diabetes is very difficult using these methods, because the phenotypic changes characteristic of type 2 diabetes are not evident during the very early stages of the disease. Therefore, development of a new diagnostic method for predicting the onset of type 2 diabetes would require highly sensitive detection of the progress of disease along with an appraisal of the innate risks such as the genetic factors.

Advances in DNA microarray technology make it possible to monitor thousands of genes using only one assay.[1] Gene expression profiling captures daily changes caused by environmental factors, such as lifestyle, as well as permanent changes caused by structural variations in DNA. Environmental and genetic risks of some diseases can be assessed by pattern recognition of selected gene expression sets. In fact, gene expression signatures are used to predict clinical outcomes and metastatic risks in cancer.[2,3] Therefore, gene expression in disease-related tissues could be an ideal sample for the identification of signs of the onset of future disease.

To realize accurate risk assessment for the onset of type 2 diabetes, non-invasive methods that evaluate prognostic indicators in readily accessible tissues are needed. Type 2 diabetes-related tissues, such as liver, adipose tissue, and skeletal muscle, are involved in the pathogenesis of type 2 diabetes, but it is difficult to sample these tissues for the purpose of genetic diagnosis in healthy people. Thus, we focused on whole blood cells (WBC), which are easily accessible, for diagnosis. WBC are also an ideal surrogate tissue for use as a diagnostic tool[4] to predict infection, diseases, and other threats, because WBC participate in surveillance of the entire body.

We previously planned the use of gene expression profile in WBC as a diagnostic tool to predict the onset of type 2 diabetes, independent of other research groups. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a well-established animal model of spontaneous type 2 diabetes. Because the OLETF rat model is characterized by mild obesity with visceral fat accumulation and late-onset insulin resistance, it resembles human obese patients with type 2 diabetes.[5] Previously, we demonstrated differences in gene expression WBC and liver in OLETF rats prior to the onset of type 2 diabetes and control rats of the same age.[6,7] In addition, Takamura *et al.* recently showed that gene expression in blood cells re-

* To whom correspondence should be addressed. e-mail: harasima@pharm.hokudai.ac.jp

flected the pathophysiology of type 2 diabetes patients, supporting our hypothesis.[8] Thus, it is of great interest to identify genes that are differently expressed in both WBC and type 2 diabetes-related tissues, other than liver, before onset of disease.

In the present study, we carried out gene expression analysis of OLETF and Long-Evans Tokushima Otsuka (LETO) rats (control rats) to examine the gene expression profiles in WBC, adipose tissue, and skeletal muscle using oligo microarray. Moreover, we re-examined gene expression in liver because the cDNA microarray that was used in the previous study[7] was unavailable at this time. The results obtained in this study suggest that gene expression profiling of WBC might be a useful source of markers to predict the onset of type 2 diabetes.

MATERIALS AND METHODS

Animals Male OLETF rats and non-diabetic control male LETO rats (4 weeks of age) were generously provided by the Tokushima Research Institute, Otsuka Pharmaceutical, (Tokushima, Japan). 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 experiments were performed after an overnight fast. All animal procedures were carried out in accordance with the institutional animal care and research advisory committee of the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

Measurements of Plasma Glucose and Insulin When rats reached 6 weeks of age, blood was collected from the tail vein. The glucose concentration in plasma was quantified using the Glucose B Test Kit (Wako Chemical, Osaka, Japan). The insulin concentration in plasma was quantified using the Insulin-Rat T ELISA kit (Shibayagi, Gunma, Japan).

Isolation of Total RNA Total RNA was isolated from liver, adipose tissue, skeletal muscle, and WBC of 6-week old rats. Whole blood was obtained from the carotid artery, adipose tissue was obtained from epididymal fat pads, and skeletal muscle was obtained from the right hindlimb. Total RNA was isolated from WBC using the PAXgene Blood RNA kit protocol (PreAnalytiX, Hombrechtikon, Switzerland). Total RNA was isolated from liver, adipose tissue, skeletal muscle using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) and the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Genomic DNA was eliminated using the RNase-free DNase Digest Set (QIAGEN). To avoid differences between individuals, 5 μg of total RNA isolated from four rats were pooled into a single sample. The integrity of the pooled total RNA samples was evaluated using an Agilent 2100 Bioanalyzer (Agilent, Foster City, CA, U.S.A.).

DNA Microarray Experiments 500-ng aliquots of the pooled RNA samples were Cy3- or Cy5-labeled using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Product No. 5184-3568, Agilent Technologies, Palo Alto, CA, U.S.A.). 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). After the washing step, the microarray slides were analyzed using an Agilent Microarray scanner (G2565AA), using the default settings for all parameters. These experiments were carried out in duplicate using exchanged dye-labeled cRNA probes (*i.e.*, Cy3 and Cy5 dye-swapping experiments). Microarray expression data were obtained using the Agilent Feature Extraction software (Version A.6.1.1), using the default settings for all parameters.

Normalization and Data Analysis A total of 8 samples were analyzed (2 dye-swap samples per each of the 4 different tissues—WBC, liver, adipose tissue, and skeletal muscle). Microarray expression data were imported into GeneSpring GX 7.3 Expression Analysis Software (Agilent Technologies). Three normalization steps were applied as follows: data transformation, dye swap; per spot, divide by control channel; and per chip, normalization to the 50th percentile. Genes were filtered in accordance with the following three criteria; an average raw and control value >100 in all chips; a present or marginal flag value for both samples in each tissue chip; a percent difference between raw and control values within 51% in each tissue chip. Genes that met all three criteria were extracted. Data analysis focused on these genes, the expression of which changed by 1.5-fold or greater, or less than 0.67-fold, in OLETF rats compared with LETO rats.

Hierarchical Clustering Analysis Gene-tree hierarchical clustering analysis was performed to understand the differential gene expression pattern in diabetes-related tissues. Average linkage was used as the clustering algorithm. Pearson correlations were used as a measure of similarity between the gene expression patterns for each sample. Four subset clusters were identified based on the distance value, which is a measure of similarity.

Gene Ontology Analysis Gene ontology (GO) analysis was performed to assign biological meaning to the subsets of the gene clusters. Overrepresentation of genes with altered expression in OLETF rats compared with LETO rats within specific GO categories was determined using Fisher's exact probability test. GO categories that included more than 10 genes were extracted.

Quantitative Reverse Transcription Polymerase Chain Reaction (PCR) cDNA was prepared from 400-ng aliquots of pooled RNA samples from OLETF and LETO rats using the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (code No. RR019A, TaKaRa, Kusatsu, Japan). 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 the SYBR Green Realtime PCR Master Mix (Code No. QPK-201, 201T, TOYOBO, Osaka, Japan), except that the primer concentration was 0.2 μM. Gene-specific primer sequences are shown in Table 1. PCR was performed using the following sequence in an Applied Biosystems 7500 Real Time PCR System: a 10-min pre-incubation at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were run in triplicate. Data were analyzed using the comparative $\Delta\Delta$Ct method and are expressed as relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a reference.

KeyMolnet Software Analysis DNA microarray data were analyzed using KeyMolnet software, which is a new approach to mechanistic analysis, that was developed by the Institute of Medical Molecular Design, Inc. (IMMD).[9]

Table 1. Design of Primers for Quantitative RT-PCR

Gene	Accession number	Sequence of primers (5′→3′)	PCR product (bp)
Acadl	NM_012819	F: TCTACTACCTCATGCAAGAG	79
		R: AACATGAACTCACAGGCAGA	
Acbd4	NM_001012013	F: AAGTGGGATGCCTGGAACAG	80
		R: ACCAGCTTCATCTCCGTGATG	
Add2	NM_012491	F: CCCAAGAAGTGCTGGACATG	73
		R: TGAGGCCCGGCTGACTTTAT	
C2	NM_172222	F: TCTGCCCTAGGCACCTCCTT	99
		R: CGAGCGCTGGATTTGGATT	
Cfd	NM_001077642	F: GACAGCGTTGAGGACGACCTCAT	84
		R: TTGCAAGGGCAGGGGTCTCACAT	
PC-1	NM_053535	F: AGTGCCAATGTACCAGAGTT	82
		R: ATTCCTTTCTTGGGCATACC	
Fabp5	NM_145878	F: CAACAACAACCTCACCGTCAA	68
		R: CTCCCAAGGTGCAAGAAAACAC	
GAPDH	AF106860	F: GGCAAGTTCAACGGCACAGT	62
		R: ATGGGTTTCCCGTTGATGAC	
Grb2	NM_030846	F: TGACTTCAAAGCTACTGCTG	81
		R: CAGTTCTGATCACACTCTTC	
Plekhb1	NM_172033	F: CACGTATGTCCGCAGCTACT	68
		R: CCTCTCGCACGATCACATGT	
Pten	NM_031606	F: CAACCGATACTTCTCTCCAA	73
		R: TGGATTTGATGGCTCCTCTA	
SIRP	NM_013016	F: ATCACCGAAAACCATACCGT	97
		R: CGTTCCAGTTGTAGTAAGCA	
Rgs3	NM_019340	F: CTGGCTTGTGAGGATTTCAAGA	84
		R: CGCGATGAACTCAGCAAAGA	
Trf	NM_001013110	F: AGGAGCAGAGTACTTGCAAG	80
		R: TGTGGAAAGTGCAGGCTTCT	

RESULTS

Characteristics of OLETF Rats before the Onset of Type 2 Diabetes To confirm the characteristics of OLETF rats before the onset of type 2 diabetes, body weight and fasting plasma glucose and insulin concentrations were measured. The measurements of fasting plasma glucose and insulin concentrations are commonly used methods to judge the onset of type 2 diabetes. As shown in Table 2, the body weight of OLETF rats was slightly greater than that of LETO rats, but the difference was not significant ($p=0.053$). Fasting plasma glucose and insulin concentrations were not significantly different between OLETF and LETO rats ($p=0.255$, 0.185, respectively). Also, results of the glucose tolerance test (GTT) did not differ between OLETF and LETO 5- and 8-weeks-old rats, as described previously.[7] These results show that a new diagnostic method is needed to predict the onset of type 2 diabetes.

Microarray Analysis of Gene Expression Profiles in Diabetes-Related Tissues We next focused on global gene expression in diabetes-related tissues, such as liver, adipose tissue, and skeletal muscle, which are expected to express early symptoms of type 2 diabetes. We hypothesized that gene expression in diabetes-related tissues would identify signs of diabetes earlier than serum parameters, as described in the above section. Plots of cRNA signal intensity for LETO and OLETF rats showed a widely scattered pattern in each tissue (Fig. 1). In particular, the pattern in adipose tissue exhibited the widest scattering. In the liver, 503 genes had expression ratios that were at least 1.5-fold higher or lower in OLETF than in LETO rats (401 genes were up-regu-

Table 2. Characteristics of the Experimental Rats at 6 Weeks of Age

	LETO	OLETF
Body weight (g)	135.7±9.5	149.3±16.3
Fasting plasma glucose (mmol/l)	5.3±0.8	5.1±0.5
Fasting plasma insulin (ng/ml)	0.80±0.11	0.92±0.16

The value of body weight and fasting plasma glucose were expressed as means±S.D. ($n=6$) and the value of fasting plasma insulin were expressed as means±S.D. ($n=3$). Three parameters were not significant ($p<0.05$) between LETO and OLETF rats.

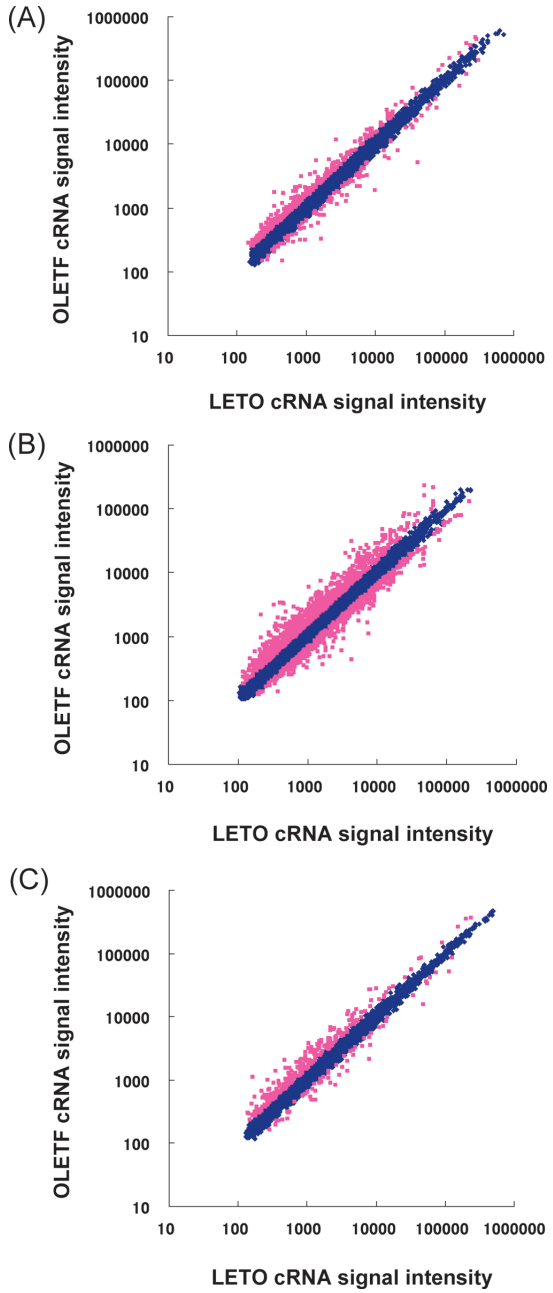

Fig. 1. Scatter Plot for LETO *vs.* OLETF in Liver (A), Adipose Tissue (B), Skeletal Muscle (C)

For each spot, the cRNA intensity signal for LETO was plotted against that for OLETF on a logarithmic scale. The value of LETO (or OLETF) cRNA intensity signal on each spot was the average value of Cy3-labeled LETO (or OLETF) cRNA intensity and Cy5-labeled LETO (or OLETF) cRNA intensity. Pink spots indicate that the gene expression ratio of OLETF to LETO was greater than 1.5 or less than 0.67.

lated and 102 genes were down-regulated). In adipose tissue, 1432 genes had significant differences in expression ratio (944 genes were up-regulated and 488 genes were down-regulated). In skeletal muscle, the expression of 314 genes was significantly altered in OLETF rats compared with LETO rats. (256 genes were up-regulated and 58 genes were down-regulated). Figure 2 shows hierarchical clustering analysis of the 2025 genes, for which the expression ratio in OLETF rats was 1.5-fold greater or less than that in LETO rats in at least one tissue. To characterize gene expression patterns among these tissues, 4 subset clusters were identified based on the distance value, which is a measure of similarity. Cluster 1 included 562 genes, which were characterized as high-expression in the liver of OLETF rats. Cluster 2 included 558 genes, which were characterized as low-expression in adipose tissue of OLETF rats. Cluster 3 included 136 genes which were characterized low expression in liver and high expression in adipose and skeletal muscle of OLETF rats. Cluster 4 included 769 genes, which were characterized as high-expression in adipose tissue of OLETF rats. To assign biological meaning to these clusters, gene ontology (GO) analysis was performed. GO can be defined by three aspects; molecular function, biological process, and cellular components. In this analysis, GO was defined by biological process. Classifications using GO terms for each cluster are shown in Table 3. Among the expression levels of the top 100 genes that are preferentially expressed in LETO and OLETF in liver, adipose tissue, and skeletal muscle, Cluster 1 included Lpin2[10—12] and Ucp3,[13,14] which are related to the progress of diabetes. In particular, Ucp3 is involved in lipid metabolism (GO:6629). Cluster 2 includes five genes Hepattic protein EIIH,[15] Nr4a3,[16] Adrb3,[17—20] Adiponectin,[21] and Rbp4.[22,23] In particular, Nr4a3 and Adrb3 genes were involved in responses to stimulus (GO:50896), and Adiponectin genes were involved in lipid metabolism (GO:6629). Cluster 3 included the IGFBP2 gene,[24,25] however, there were no statistically overrepresented genes (more than 10 genes) within a specific GO term ($p<0.01$). Cluster 4 included the Pla2g7[26] gene, which was not assigned a GO term. These specific genes are involved in the pathogenesis of type 2 diabetes, therefore, they are covered in detail in the discussion section. These results suggest that gene expression in type 2 diabetes-related tissues is sensitive to the progress of type 2 diabetes before changing serum parameters.

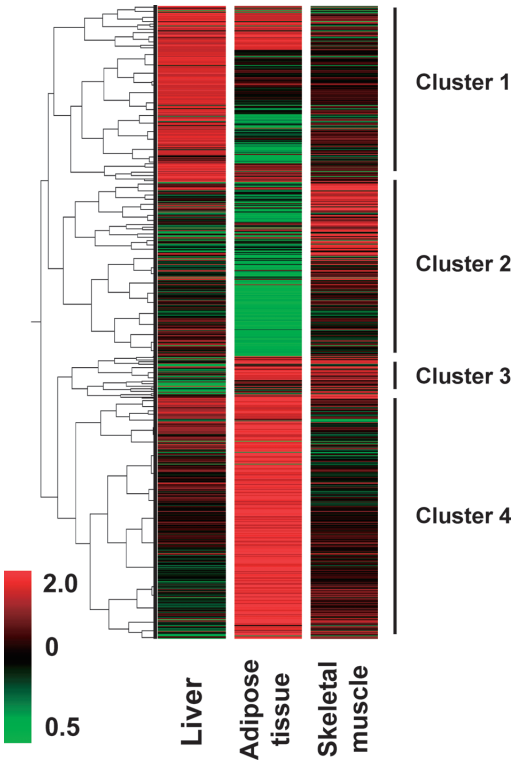

Fig. 2. Hierarchical Clustering Analysis of Differentially Expressed Genes in Diabetes-Related Tissues

2025 genes with an expression ratio of LETO to OLETF is greater than 1.5 or less than 0.67 in at least 1 tissue are shown. Red and green represent preferential expression in OLETF and LETO rats, respectively. Black represents equivalent expression in OLETF and LETO rats. In 2025 genes, 4 subset clusters were identified based on the distance value, which is a measure of similarity.

Three-Hundred Genes Were Differentially Expressed in WBC As revealed by GO analysis and literature research, it might be possible to detect pre-clinical signs of type 2 diabetes by use of groups of gene expression in diabetes-related tissues. However, it is unethical to obtain tissue

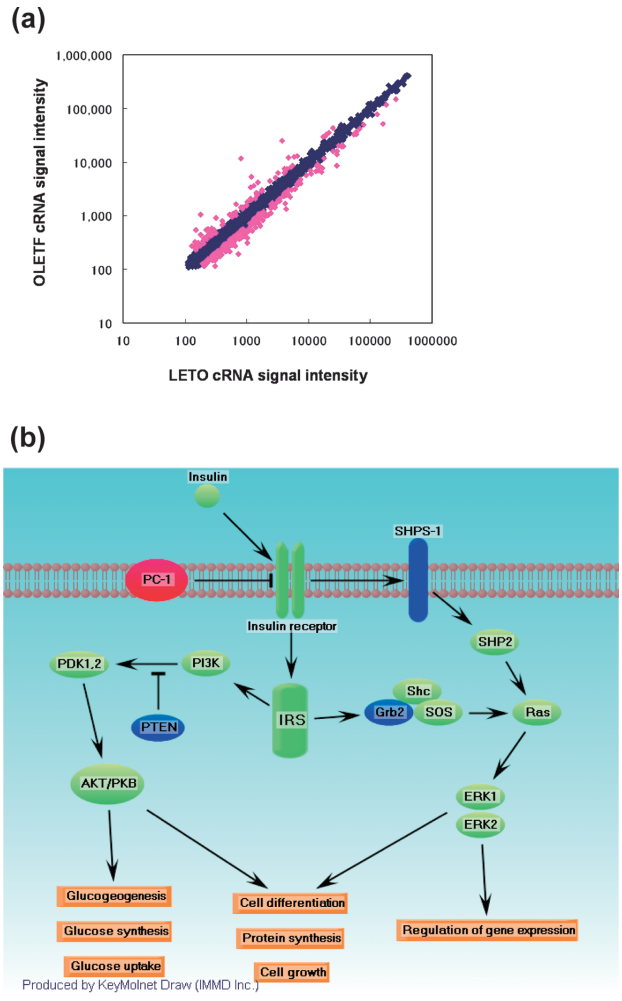

Fig. 3. Scatter Plot for LETO *vs.* OLETF in WBC (a) and Four Genes Related to the Insulin-Signaling Pathway (b)

The condition of Scatter plot for WBC is the same as Fig. 1. KeyMolnet software extracted 4 genes related to the insulin-signaling pathway from 300 genes with expression ratios of OLETF to LETO greater than 1.5 or less than 0.67 in whole blood cells. Red indicates up-regulated genes, whereas blue indicates down-regulated genes. PC-1, ectonucleotide pyrophosphatase/phosphodiesterase 1; SIRP, signal-regulatory protein alpha; Grb2, growth factor receptor bound protein 2; Pten, phosphatase and tensin homolog.

Table 3. Statistically Overrepresented GO Terms (Biological Process) in Each Cluster

Cluster 1 (562 genes distance=0.645) $p<0.01$

Category	Genes in category	% of genes in category	Genes in list in category	% of genes in list in category	*p*-Value
GO:42221: response to chemical stimulus	598	6.5	30	11.2	0.00217
GO:6629: lipid metabolism	546	5.9	27	10.1	0.00437
GO:44255: cellular lipid metabolism	454	4.9	23	8.6	0.00628
GO:19752: carboxylic acid metabolism	481	5.2	24	9.0	0.00636
GO:6082: organic acid metabolism	482	5.2	24	9.0	0.00652
GO:8610: lipid biosynthesis	215	2.3	13	4.9	0.00959

Cluster 2 (558 genes distance=0.345) $p<0.000001$

Category	Genes in category	% of genes in category	Genes in list in category	% of genes in list in category	*p*-Value
GO:9613: response to pest, pathogen or parasite	361	3.9	52	16.4	3.10E-19
GO:43207: response to external biotic stimulus	381	4.1	52	16.4	3.50E-18
GO:6955: immune response	575	6.2	64	20.1	1.19E-17
GO:9605: response to external stimulus	707	7.6	71	22.3	4.01E-17
GO:6952: defense response	615	6.6	65	20.4	8.94E-17
GO:9607: response to biotic stimulus	643	6.9	66	20.8	2.20E-16
GO:50874: organismal physiological process	1600	17.3	113	35.5	1.26E-15
GO:9611: response to wounding	426	4.6	47	14.8	6.37E-13
GO:6950: response to stress	1044	11.3	80	25.2	1.35E-12
GO:50896: response to stimulus	1802	19.5	112	35.2	1.39E-11
GO:6954: inflammatory response	212	2.3	29	9.1	1.48E-10
GO:6953: acute-phase response	25	0.3	11	3.5	1.91E-10
GO:6956: complement activation	32	0.3	11	3.5	4.45E-09
GO:8015: circulation	141	1.5	20	6.3	6.32E-08
GO:42221: response to chemical stimulus	598	6.5	45	14.2	4.27E-07
GO:6629: lipid metabolism	546	5.9	42	13.2	6.06E-07
GO:6091: generation of precursor metabolites and energy	515	5.6	40	12.6	9.03E-07

Cluster 4 (769 genes distance=0.158) $p=0.001$

Category	Genes in category	% of genes in category	Genes in list in category	% of genes in list in category	*p*-Value
GO:19752: carboxylic acid metabolism	481	5.2	43	10.9	2.60E-06
GO:6082: organic acid metabolism	482	5.2	43	10.9	2.75E-06
GO:7283: spermatogenesis	160	1.7	21	5.3	3.87E-06
GO:48232: male gamete generation	160	1.7	21	5.3	3.87E-06
GO:19953: sexual reproduction	247	2.7	26	6.6	1.74E-05
GO:3: reproduction	346	3.7	32	8.1	2.72E-05
GO:226: microtubule cytoskeleton organization and biogenesis	73	0.8	12	3.0	5.18E-05
GO:7276: gametogenesis	219	2.4	22	5.6	0.000154
GO:7010: cytoskeleton organization and biogenesis	429	4.6	34	8.6	0.000325
GO:6520: amino acid metabolism	242	2.6	22	5.6	0.000626
GO:7017: microtubule-based process	166	1.8	17	4.3	0.000683
GO:16053: organic acid biosynthesis	70	0.8	10	2.5	0.000698
GO:46394: carboxylic acid biosynthesis	70	0.8	10	2.5	0.000698

Genes in list in category were the number of genes which were classified with statistically overrepresented GO term. GO terms with biological process were shown. GO, Gene ontology.

biopsies for the purpose of early diagnosis in healthy people. To realize early diagnosis of type 2 diabetes, easily accessible tissues are needed. Then we focused on the gene expression profile of WBC. In WBC, expression of 300 genes in OLETF rats was at least 1.5-fold greater or less than expression in LETO rats (Fig. 3a). This result suggests that gene expression in WBC could be used to diagnose type 2 diabetes prior to the onset of the disease, as fasting glucose and insulin concentrations and GTT are not significantly changed early in the pathogenesis of diabetes. However, the functional associations of the 300 genes were unknown. Therefore, KeyMolnet software was used to determine if some genes were related by function or pathway. As shown in Fig. 3b, 4 genes are involved in the insulin-signaling pathway. Quantitative RT PCR analysis of the 4 genes showed similar expression profiles, which was confirmed by the microarray data (Fig. 4). These results suggest an active insulin signal pathway in whole blood cells, and that insulin resistance begins at the transcriptional level.

Fifty-Seven Genes in WBC Were Concurrently Expressed in Diabetes-Related Tissues In the above section, 4 genes in whole blood cells are involved in the insulin-signaling pathway. The PC-1 (ectonucleotide pyrophosphatase/phosphodiesterase 1) gene was among those that also were

upregulated in adipose tissue (Table 4). The PC-1 gene is associated with type 2 diabetes,[27—29] therefore, we hypothesized that some of the genes exhibited an altered expression in diabetes-related tissues and could be detected in WBC. A total of 57 genes were detected in WBCs: 25 genes (14 genes were up-regulated, 7 genes were down-regulated) in liver, 41 genes (15 genes were up-regulated, 25 genes were down-regulated) in adipose tissue, and 14 genes (11 genes were up-regulated, 3 genes were down-regulated) in skeletal muscle (Table 4). To validate the microarray results, quantitative RT PCR analyses were performed. As shown in Fig. 5, gene expression of the 10 selected genes showed a similar expression profile, which was consistent with the microarray data. Among the 57 genes, FABP5,[30,31] Cfd,[32,33] PC-1,[27—29] and Ucp3[13,14] were reported to be associated with the progress of type 2 diabetes. Possible interpretations of these results are discussed in the last section.

DISCUSSION

The results of the present study demonstrate that the gene expression profile of WBC might be useful for detection of pre-clinical symptoms of type 2 diabetes in OLETF rats. To the best of our knowledge, this is the first report of screening WBC candidate genes, which are also up- or down-regulated in adipose tissue or skeletal muscle.

Fasting plasma glucose and insulin concentrations and, the glucose tolerance test (GTT) were not significantly different between OLETF and LETO rats (Table 2, ref. 7). These results suggest that another diagnostic method is needed to detect type 2 diabetes during the very early stage. In contrast, dramatic alternations in gene expression were seen in liver, adipose tissue and skeletal muscle prior to the onset of type 2 diabetes (Figs. 1, 2). For example, the IGFBP2 (insulin-like growth factor binding protein 2) gene in liver was down-regulated (0.53 fold) in OLETF rats. I have previously mentioned that IGFBP2 gene expression is much lower (about 10 times) in the liver of OLETF rats.[25] Heald *et al.* showed that a lower concentration of IGFBP2 in serum is a marker for elevating serum fasting glucose in patients with type 2 diabetes.[24] Therefore, these results suggest that lower IGFBP2 in serum might be influenced by lower IGFBP2 gene expression in the liver.

In adipose tissue, the Pla2g7 (phospholipase A2, group 7) gene was up-regulated (3.3 fold) in OLETF rats, which was also up-regulated in obese Pima Indians in microarray and Quantitative RT-PCR analysis.[26] Pima Indians are believed to be a genetic high-risk group for developing obesity, insulin resistance, and type 2 diabetes. Therefore, there is some possibility that the progress of these symptoms begins in adipose tissue at an early stage.

In skeletal muscle, Adiponectin and Rbp4 genes were both up-regulated (2.5 fold) in OLETF rats. Adiponectin is expressed at the mRNA and protein levels in skeletal muscle of ob/ob mice.[21] This is viewed as a result of lipotoxicity and related oxidative stress. On the other hand, Rbp4 (retinol-binding protein 4) expression in skeletal muscle correlates highly with both insulin resistance (Graham *et al.* the 67th annual meeting of the American Diabetes Association Scientific Sessions 2007) and an increase in body fat percentage, as does adiponectin mRNA expression.[22] Therefore, it is suggested that insulin resistance and lipotoxicity begins in skeletal muscle.

These changes of gene expression in three tissues suggests that type 2 diabetes is progresses gradually in liver, adipose

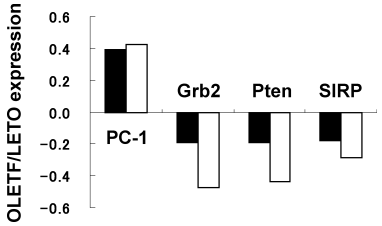

Fig. 4. Confirmation of the Changes in the Transcript Levels of 4 Genes in WBC That Were Extracted Using KeyMolnet Software Analysis

Black and open bars represents each gene expression ratios of OLETF to LETO on a log10 scale in the microarray and quantitative RT-PCR experiments, respectively. cDNA was prepared from 400-ng aliquots of each total WBC RNA sample obtained from LETO and OLETF rats. The levels of each transcript were measured using an SYBR green real time PCR system. The GAPDH transcript was used to adjust the amount of transcript in LETO and OLETF. PC-1, ectonucleotide pyrophosphatase/phosphodiesterase 1; SIRP, signal-regulatory protein alpha; Grb2, growth factor receptor bound protein 2; Pten, phosphatase and tensin homolog.

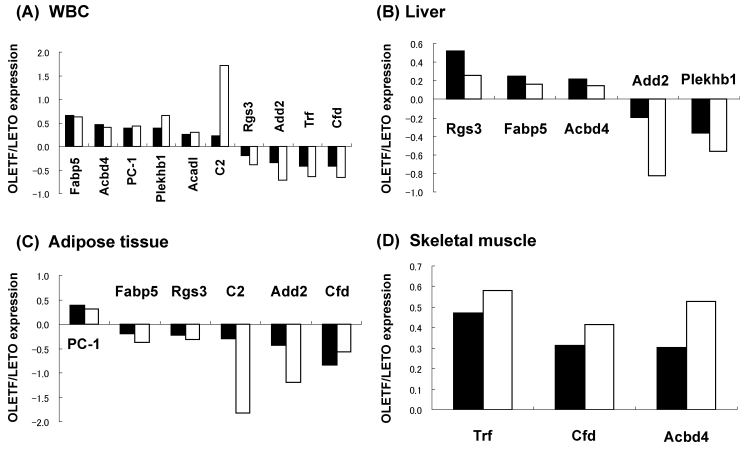

Fig. 5. Validation of Microarray Results Using Quantitative RT PCR in WBC (A), Liver (B), Adipose Tissue (C), Skeletal Muscle (D)

Black and open bars represents each gene expression ratio of OLETF to LETO on a log10 scale in the microarray and quantitative RT-PCR experiments, respectively. The experimental conditions were the same as in Fig 4. Fabp5, fatty acid binding protein 5; Acbd4, acyl-coenzyme A binding domain containing 4; PC-1, ectonucleotide pyrophosphatase/phosphodiesterase 1; Plekhb1, pleckstrin homology domain containing, family B (evectins) member 1; Acadl, acetyl-coenzyme A dehydrogenase, long chain; C2, complement component 2; Rgs3, regulator of G-protein signaling 3; Add2, adducin 2 (beta); Trf, transferrin; Cfd, complement factor D (adipsin).

Table 4. Concurrently Expressed Genes in WBC, Liver, Adipose Tissue, and Skeletal Muscle of OLETF Rats

Probe ID	Description	Genbank	UniGene	WBC			Liver			Adipose tissue			Skeletal muscle		
				Expression level		Ratio	Expression level		Ratio	Expression level		Ratio	Expression Level		Ratio
				LETO	OLETF		LETO	OLETF		LETO	OLETF		LETO	OLETF	
WBC-Liver-Adipose tissue-Skeletal muscle															
A_42_P650592	Glutathione *S*-transferase, theta 3 (Gstt3)	NM_001137643	Rn.92581	178	1034	5.73	15403	29524	1.92	2176	6296	2.89	476	860	1.83
A_42_P762871	Putative ISG12(b) protein (isg12(b))	NM_206846	Rn.22509	1477	836	0.57	82967	143797	1.74	9204	14076	1.53	2105	4613	2.19
A_43_P14036	Putative ISG12(b) protein (isg12(b))	NM_206846	Rn.22509	1547	851	0.56	96402	172279	1.80	8977	13971	1.56	1619	3897	2.37
A_43_P14573	Transcribed locus	AA944679	Rn.7774	899	416	0.47	3927	2344	0.60	5983	3551	0.59	2669	1740	0.66
WBC-Liver-Adipose tissue															
A_42_P766084	Fatty acid binding protein 5, epidermal	NM_145878	Rn.98269	1178	5260	4.47	8801	15838	1.79	74565	47379	0.63	9310	10541	1.13
A_42_P843603	Thymidine kinase 1, soluble (TK1)	NM_052800	Rn.217952	664	1152	1.72	511	1023	2.05	538	1083	2.00	328	451	1.38
A_42_P665879	Activating transcription factor 5	NM_172336	Rn.195729	7471	4819	0.63	210462	125506	0.59	73093	42809	0.59	14666	14983	1.02
A_42_P738549	Napsin A aspartic peptidase	NM_031670	Rn.17097	1643	928	0.56	2219	1479	0.66	949	599	0.63	545	521	0.94
A_42_P599390	Transcribed locus	BQ781740	Rn.168428	528	215	0.42	6262	3098	0.50	6845	3982	0.58	4232	2979	0.70
A_42_P762691	Transcribed locus	AW916109	Rn.17129	4155	1700	0.41	2990	1781	0.60	694	417	0.60	336	348	1.08
WBC-Liver-Skeletal muscle															
A_42_P482542	Acyl-Coenzyme A binding domain containing 4	NM_001012013	Rn.18284	1153	3437	2.89	4844	7999	1.65	4710	6380	1.36	2204	4427	2.01
WBC-Adipose tissue-Skeletal muscle															
A_42_P754654	Histone H4 variant H4-v.1 (RGD1562378_predicted)	XM_001072344	—	241	513	2.10	374	469	1.26	611	1711	2.81	1519	3114	2.06
A_42_P639047	Similar to transthyretin (4L369) (RGD1309350)	NM_001134507	Rn.198530	21413	35708	1.66	64180	60560	0.94	740	1928	2.60	602	963	1.61
A_43_P11165	Unkonwn	—	—	615	315	0.52	4608	3947	0.86	19855	12163	0.61	14602	9696	0.66
WBC-Liver															
A_42_P703688	Pleckstrin homology domain containing, family B member 1	NM_172033	Rn.8415	4784	11955	2.46	7496	3242	0.43	774	754	0.98	1655	1851	1.12
A_43_P17704	Solute carrier family 17 , member 5	NM_001009713	Rn.74591	533	1045	2.00	880	1337	1.54	1912	2064	1.08	698	756	1.10
A_42_P603488	Proteasome maturation protein (Pomp)	NM_001100942	Rn.28242	1488	2475	1.65	16779	29919	1.79	9667	14007	1.45	19617	24128	1.20
A_42_P526910	Unknown	—	—	227	154	0.67	1577	2410	1.53	5463	4310	0.79	2315	2863	1.24
A_42_P568943	Hypothetical LOC300751 (RGD1311874)	NM_001106825	Rn.163007	17626	11566	0.64	343	626	1.83	313	266	0.86	236	255	1.10
A_42_P465144	DENN/MADD domain containing 2D (Dennd2d)	NM_001107714	Rn.105855	511	314	0.62	498	781	1.57	1528	1367	0.90	160	190	1.19
A_43_P10606	Breast cancer metastasis-suppressor 1 (Brms1)	NM_001009605	Rn.2839	810	459	0.57	3106	4738	1.51	1418	1472	1.04	1023	1138	1.11
A_43_P11918	Guanine nucleotide binding protein, alpha o	NM_017327	Rn.90161	322	170	0.53	654	1117	1.72	1130	1009	0.89	658	595	0.89
A_42_P616370	Chromatin modifying protein 5	NM_001025410	Rn.9339	364	177	0.48	6182	3879	0.63	5805	4219	0.73	3011	2013	0.67
A_43_P14324	Kruppel-like factor 6	NM_031642	Rn.163355	1546	724	0.47	4649	7763	1.70	17997	14112	0.78	7597	9410	1.24
WBC-Adipose tissue															
A_43_P12070	Ly6-C antigen	NM_020103	Rn.40119	151	550	3.56	494	472	0.98	925	484	0.53	414	405	0.98
A_43_P12841	Ectonucleotide pyrophosphatase/phosphodiesterase 1	NM_053535	Rn.1199	179	449	2.49	1250	1562	1.25	2754	6781	2.46	371	378	1.03
A_43_P19799	Similar to RIKEN cDNA A930018P22 (RGD1563222)	NM_001108585	Rn.57377	233	565	2.47	442	430	1.01	434	689	1.58	438	422	0.96
A_42_P814597	cDNA clone UI-R-C1-jy-h-08-0-UI 3′	AI045743	—	277	591	2.15	1073	1475	1.39	2893	1756	0.61	2351	2434	1.02
A_42_P563285	Enoyl coenzyme A hydratase 1, peroxisomal	NM_022594	Rn.6148	272	484	1.80	70600	61224	0.87	16668	9946	0.60	21533	16058	0.75
A_43_P16609	Similar to Mkrn1 protein	NM_001004233	Rn.101798	17247	29979	1.71	1300	1281	0.99	1416	2847	2.02	543	597	1.11
A_42_P484186	Complement component 2	NM_172222	Rn.98333	573	961	1.68	9541	7735	0.83	7531	1099	0.15	522	482	0.94
A_43_P10746	Tubulin, alpha 4	NM_001007004	Rn.92961	18739	30300	1.62	8807	6199	0.71	2320	4175	1.80	81585	74487	0.91
A_43_P12729	Glycerophosphodiester phosphodiesterase 1 (Gde1)	NM_032615	Rn.170542	1195	1919	1.59	8975	8895	0.99	7709	5100	0.66	2520	2804	1.11
A_42_P651285	Catalase	NM_012520	Rn.3001	5193	8063	1.52	48446	52550	1.20	8752	5355	0.62	3420	4318	1.26
A_42_P723250	Transcribed locus	BF552994	Rn.155302	4104	2700	0.65	151	110	0.65	611	1198	1.98	147	132	0.89
A_43_P 12028	Regulator of G-protein signalling 3	NM_019340	Rn.53900	400	259	0.65	1951	5921	3.30	17334	10373	0.60	1227	1359	1.10

Table 4. Concurrently Expressed Genes in WBC, Liver, Adipose Tissue, and Skeletal Muscle of OLETF Rats (Continued)

Probe ID	Description	Genbank	UniGene	WBC			Liver			Adipose tissue			Skeletal muscle		
				Expression level		Ratio	Expression level		Ratio	Expression level		Ratio	Expression Level		Ratio
				LETO	OLETF		LETO	OLETF		LETO	OLETF		LETO	OLETF	
A_43_P14228	Coactosin-like 1 (Dictyostelium) (Cotl1)	NM_001108452	Rn.98506	6930	4480	0.64	4974	4934	1.00	17952	29319	1.64	2558	2849	1.12
A_42_P641528	Linker for activation of T cells	NM_030853	Rn.9773	448	295	0.64	496	453	0.91	371	643	1.73	208	183	0.89
A_42_P740209	*N*-Methylpurine-DNA glycosylase	NM_012601	Rn.11241	3903	2499	0.64	2231	2018	0.91	3093	5814	1.88	789	879	1.10
A_42_P646290	Guanine nucleotide binding protein, alpha z subunit	NM_013189	Rn.10943	565	353	0.62	159	214	1.45	254	379	1.51	277	281	1.05
A_43_P16707	Chemokine (C–C motif) ligand 6	NM_001004202	Rn.7857	1421	854	0.60	13984	13858	0.99	8333	3957	0.48	1584	1698	1.07
A_42_P511328	Serum deprivation response protein	NM_001007712	Rn.203035	227	134	0.60	1190	1148	0.97	9380	4277	0.46	2703	1992	0.74
A_43_P11884	B-Cell translocation gene 2, anti-proliferative	NM_017259	Rn.27923	603	347	0.58	1531	2047	1.33	8775	4637	0.53	4844	3854	0.80
A_42_P826191	Platelet/endothelial cell adhesion molecule 1 (Pecam1)	NM_031591	Rn.1878	461	275	0.58	2911	3121	1.08	8292	5396	0.65	5971	5972	1.01
A_42_P500038	Transcribed locus	AA848554	Rn.164402	437	244	0.57	718	746	1.04	5807	3834	0.66	2561	2735	1.07
A_42_P595923	Unknown	—	—	718	387	0.54	591	604	1.03	2158	1361	0.63	924	847	0.92
A_42_P717482	Retinoid X receptor alpha (Rxra)	NM_012805	Rn.108206	520	269	0.52	61198	62998	1.04	22857	14051	0.61	21855	24373	1.11
A_43_P13994	cDNA clone UI-R-A0-az-c-02-0-UI 3′	AA818696	—	13048	6687	0.51	2220	2549	1.16	1434	853	0.59	1798	1587	0.88
A_43_P15399	Adducin 2 (beta)	NM_012491	Rn.11353	16641	7632	0.46	130	83	0.63	380	138	0.37	145	122	0.84
A_43_P14911	Interleukin 1 beta	NM_031512	Rn.9869	1026	390	0.39	1251	871	0.70	557	340	0.61	175	160	0.91
A_42_P620915	S100 Calcium binding protein A9 (calgranulin B)	NM_053587	Rn.6703	28615	8514	0.29	1509	1090	0.72	642	342	0.53	1186	914	0.77
WBC-Skeletal muscle															
A_43_P11743	Uncoupling protein 3 (mitochondrial, proton carrier)	NM_013167	Rn.9902	2404	4518	1.84	215	187	0.88	406	369	0.91	2033	1057	0.52
A_42_P822638	TSC22 domain family 3	NM_031345	Rn.21970	1588	949	0.59	6189	8509	1.60	17550	21276	1.22	14298	24216	1.69
A_42_P727231	Meteorin, glial cell differentiation regulator-like	NM_001014104	Rn.64557	226	132	0.58	538	546	1.04	3158	3558	1.13	815	1362	1.67
A_42_P774977	Fibronectin 1	NM_019143	Rn.1604	832	469	0.56	125996	100863	0.80	49883	42536	0.86	17792	29696	1.67
A_43_P15750	Complement factor D (adipsin) (Cfd)	NM_001077642	Rn.16172	310	114	0.38	3295	3456	1.07	255115	129450	0.50	2918	6044	2.06
A_43_P11796	Transferrin	NM_001013110	Rn.91296	1663	637	0.38	516744	657294	1.30	15651	15489	0.99	3992	11923	2.95

The value of expression level in LETO and OLETF is the arithmetical average of LETO (or OLETF) cRNA intensity in two chips, and the ratio is the geometric average of two chips.

tissue, and skeletal muscle before changing serum parameters. However, it is unethical to obtain tissue biopsies for the purpose of early diagnosis in healthy people. Therefore, we focused on gene expression in WBC, which contact all tissues, including liver, adipose tissue, and skeletal muscle. WBC directly reflect the conditions of the liver, adipose tissue, and skeletal muscle so that the continuous interaction among WBC and these tissues gives rise to the possibility that gene expression in WBC might be a sensitive diagnostic indicator to catch the changes of these tissues. The results shows that 300 genes were up- or down-regulated in OLETF rats (Fig. 3a), and 57 genes were concurrently expressed in WBCs, liver, adipose tissue, and skeletal muscle in OLETF rats (Table 4). Borovecki *et al.* showed the utility of the some genes whose expression is altered at the transcriptional level in common in WBC and the brain as biomarkers for Huntington's disease.[27] Therefore, concurrently expressed genes in WBC and diabetes-related tissues could be a diagnostic marker for type 2 diabetes.

For example, PC-1 gene was up-regulated in WBC and Adipose tissue (Fig. 5). PC-1 is an inhibitor of insulin receptor tyrosine kinase activity, and is involved in the pathogenesis of insulin resistance in type 2 diabetes.[28—30] The activity of PC-1 in lymphocytes, which are a type of WBC, was increased in type 2 diabetes patients.[28] On the other hand, increased PC-1 protein in adipose tissue was associated with whole-body insulin resistance in healthy non-obese subjects.[29] These results suggest that monitoring mRNA expression of the PC-1 gene in WBC and adipose tissue might help predict the onset of type 2 diabetes and its mRNA expression of adipose tissue. The FABP5 (fatty acid binding protein 5) gene was up-regulated in WBCs and liver, and down-regulated in adipose tissue (Fig. 5) and is involved in the pathogenesis of obesity-related insulin resistance and type 2 diabetes.[31] In macrophage, depletion of FABP4 and FABP5 led to enhanced insulin signaling and glucose uptake in adipocytes.[32] This phenomenon is very interesting because gene expression in macrophage, as a part of WBC, affects the function of adipocyte, and is a powerful supporter of the usefulness of gene expression in WBCs.

There are some reports indicating the utility of blood gene expression as a potential diagnostic tool. Hayashi *et al.* showed that several type 2 diabetes-related genes were up- or down-regulated in both WBC and liver after the onset of type 2 diabetes.[25] Takamura *et al.* showed that c-Jun terminal kinase (JNK) and mitochondrial oxidative phosphorylation (OXPHOS) pathways of peripheral blood mononuclear cells (PBMCs) could be used as surrogate transcriptional markers in type 2 diabetes patients.[8] Liew *et al.* showed that the peripheral blood transcriptome was a potential diagnostic tool and approximately 80% of genes expressed in any of nine human tissues were also found expressed in blood cells.[4] These results strongly suggest that blood gene expression provides useful information for whole-body analysis. However, the influence of gene expression in WBCs on the onset of type 2 diabetes is unclear at this time. Therefore, the usefulness should become clear as the relationship between gene expression in WBCs and disease is better understood.

In summary, blood gene expression profiling is useful for markers predicting type 2 diabetes at an very early stage. The results of the present study will advance our progress towards diagnosis of type 2 diabetes.

Acknowledgements This work was supported by Grants-in-Aid for Scientific Research (A) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and 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. Jim McDonald for his helpful advice in preparing the English manuscript.

REFERENCES

1) Golub T. R., Slonim D. K., Tamayo P., Huard C., Gaasenbek M., Mesirov J. P., Coller H., Loh M. L., Downing J. R., Caligiuri M. A., Bloomfield C. D., Lander E. S., *Science*, **286**, 531—537 (1999).
2) van de Vijver M. J., He Y. D., van't Veer L. J., Dai H., Hart A. A., Voskuil D. W., Schreiber G. J., Peterse J. L., Roberts C., Marton M J., Parrish M., Atsma D., Witteveen A., Glas A., Delahaye L., van der Velde T., Bartelink H., Rodenhuis S., Rutgers E. T., Friend S. H., Bernards R., *N. Engl. J. Med.*, **347**, 1999—2009 (2002).
3) Glas A. M., Floore A., Delahaye L. J., Witteveen A. T., Pover R. C., Bakx N., Lahti-Domenici J. S., Bruinsma T. J., Warmoes M. O., Bernards R., Wessels L. F., Van't Veer L. J., *BMC Genomics*, **7**, 278 (2006).
4) Liew C. C., Ma J., Tang H. C., Zheng R., Dempsey A. A., *J. Lab. Clin. Med.*, **147**, 126—132 (2006).
5) Kawano K., Hirashima T., Mori S., Saitoh Y., Kurosumi M., Natori T., *Diabetes*, **41**, 1422—1428 (1992).
6) Sato Y., Kuwajima M., Kamiya H., Harashima H., *Biol. Pharm. Bull.*, **26**, 1765—1768 (2003).
7) 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).
8) Takamura T., Honda M., Sakai Y., Ando H., Shimizu A., Ota T., Sakurai M., Misu H., Kurita S., Matsuzawa-Nagata N., Uchikawa M., Nakamura S., Matoba R., Tanino M., Matsubara K., Kaneko S., *Biochem. Biophys. Res. Commun.*, **361**, 379—384 (2007).
9) Sato H., Ishida S., Toda K., Matsuda R., Hayashi Y., Shigetaka M., Fukuda M., Wakamatsu Y., Itai A., *Curr. Drug. Discov. Technol.*, **2**, 89—98 (2005).
10) Donkor J., Sariahmetoglu M., Dewald J., Brindley D. N., Reue K., *J. Biol. Chem.*, **282**, 3450—3457 (2007).
11) Reue K., Donkor J., *Diabetes*, **56**, 2842—2843 (2007).
12) Aulchenko Y. S., Pullen J., Kloosterman W. P., Yazdanpanah M., Hofman A., Vaessen N., Snijders P. J., Zubakov D., Mackay I., Olavesen M., Sidhu B., Smith V. E., Carey A., Berezikov E., Uitterlinden A. G., Plasterk R. H., Oostra B. A., van Duijn C. M., *Diabetes*, **56**, 3020—3026 (2007).
13) Krook A., Digby J., O'Rahilly S., Zierath J. R., Wallberg-Henriksson H., *Diabetes*, **47**, 1528—1531 (1998).
14) Schrauwen P., Hesselink M. K., Blaak E. E., Borghouts L. B., Schaart G., Saris W. H., Keizer H. A., *Diabetes*, **50**, 2870—2873 (2001).
15) Coffy S., Decaux J. F., Girard J., de Keyzer Y., Asfari M., *Biochem. J.*, **385**, 165—171 (2005).
16) Fu Y., Luo L., Luo N., Zhu X., Garvey W. T., *J. Biol. Chem.*, **282**, 31525—31533 (2007).
17) Muzzin P., Revelli J. P., Kuhne F., Gocayne J. D., McCombie W. R., Venter J. C., Giacobino J. P., Fraser C. M., *J. Biol. Chem.*, **266**, 24053—24058 (1991).
18) Collins S., Daniel K. W., Rohlfs E. M., Ramkumar V., Taylor I. L., Gettys T. W., *Mol. Endocrinol.*, **8**, 518—527 (1994).
19) Walston J., Silver K., Bogardus C., Knowler W. C., Celi F. S., Austin S., Manning B., Strosberg A. D., Stern M. P., Raben N. *et al.*, *N. Engl. J. Med.*, **333**, 343—347 (1995).
20) Clement K., Vaisse C., Manning B. S., Basdevant A., Guy-Grand B., Ruiz J., Silver K. D., Shuldiner A. R., Froguel P., Strosberg A. D., *N. Engl. J. Med.*, **333**, 352—354 (1995).
21) Delaigle A. M., Senou M., Guiot Y., Many M. C., Brichard S. M., *Diabetologia*, **49**, 1311—1323 (2006).
22) Ribel-Madsen R., Friedrichsen M., Vaag A., Poulsen P., *Diabetes*, **58**, 54—60 (2009).

23) Chaves A. O., Coletta D. K., Kamath S., Cromack D. T., Monroy A., Folli F., DeFronzo R. A., Tripathy D., *Am. J. Physiol. Endocrinol. Metab.*, **296**, E758—E764 (2009).
24) Heald A. H., Kaushal K., Siddals K. W., Rudenski A. S., Anderson S. G., Gibson J. M., *Exp. Clin. Endocrinol. Diabetes*, **114**, 371—376 (2006).
25) Hayashi Y., Iida S., Sato Y., Nakaya A., Sawada A., Kaji N., Kamiya H., Baba Y., Harashima H., *Biol. Pharm. Bull.*, **30**, 763—771 (2007).
26) Nair S., Lee Y. H., Rousseau E., Cam M., Tataranni P. A., Baier L. J., Bogardus C., Permana P. A., *Diabetologia*, **48**, 1784—1788 (2005).
27) Borovecki F., Lovrecic L., Zhou J., Jeong H., Rosas H. D., Hersch S. M., Hogarth P., Bouzou B., Jensen R. V., Krainc D., *Proc. Natl. Acad. Sci. U.S.A.*, **102**, 11023—11028 (2005).
28) Stefanovic V., Antic S., Mitic-Zlatkovic M., Vlahovic P., *Diabetes Metab. Res. Rev.*, **15**, 400—404 (1999).
29) Frittitta L., Youngren J. F., Sbraccia P., D'Adamo M., Buongiorno A., Vigneri R., Goldfine I. D., Trischitta V., *Diabetologia*, **40**, 282—289 (1997).
30) Goldfine I. D., Maddux B. A., Youngren J. F., Reaven G., Accili D., Trischitta V., Trischitta V., Vigneri R., Frittitta L., *Endocr. Rev.*, **29**, 62—75 (2008).
31) Westerbacka J., Kolak M., Kiviluoto T., Arkkila P., Siren J., Hamsten A., Fisher R. M., Yki-Jarvinen H., *Diabetes*, **56**, 2759—2765 (2007).
32) Furuhashi M., Fucho R., Gorgun C. Z., Tuncman G., Cao H., Hotamisligil G. S., *J. Clin. Invest.*, **118**, 2640—2650 (2008).