Genome Wide Expression Analysis of White Blood Cells and Liver of Pre-diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) Rats Using a cDNA Microarray

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In a prior study, we reported on a significant decrease in calpain10 gene expression in white blood cells (WBC) as well as the major insulin-target tissues including liver and adipose tissue, before the onset of diabetes in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. In this study, we extended our hypothesis that some type 2 diabetes mellitus (NIDDM) susceptible genes are up/down-regulated before the onset in WBC of OLETF rats, reflecting their up/down-regulation in major insulin-target tissues, such as the liver. We tested this hypothesis using rat cDNA microarrays. The findings show that 1080 genes are up/down-regulated by more than 2-fold compared to the controls, Long-Evans Tokushima Otsuka rats, before the onset in WBC and liver under fasted or insulin administered condition. Fifty-seven of the 1080 genes were up/down-regulated in both WBC and the liver. More than half have been reported to NIDDM susceptible genes and the remainder have not been reported to be related to NIDDM. These results indicate that there some NIDDM related genes are up/down-regulated in WBC before the onset of diabetes.

Key words white blood cell; non-insulin dependent diabetes mellitus; genetic diagnosis; cDNA microarray; Otsuka Long-Evans Tokushima fatty rat

The establishment of an efficient diagnosis and safe treatment for type 2 diabetes mellitus are needed, since the number of patients who suffer from this disease is predicted to reach 333 million in 2025.[1] Diabetes mellitus can be classified into type 1 (insulin-dependent, IDDM) and type 2 (non-insulin-dependent, NIDDM), and NIDDM is the most common form of diabetes accounting for approximately 90% of all cases.[2] The mechanisms responsible for this disease have not been clarified to date, but both genetic and environmental factors are thought to be major causes of the disease.[3] Therefore, NIDDM is a typical multifactorial genetic syndrome.[4]

A genetic approach will help focus on the underlying causes of the disease and may provide new information related to diagnosis and treatment. Two common approaches for distinguishing genetic factors are 1) the candidate gene approach and 2) the genome wide scan using anonymous polymorphic markers.[5] The discovery of thousands of single nucleotide polymorphism (SNPs) and the construction of a reliable SNP linkage map would be a major factor in the discovery of new genes.[6,7] However, it may be a long time before all the susceptibility genes are found.

We approached this disease from a genomic point of view but from a completely different point of view.[8] We reported that the level of the calpain10 (CAPN10) transcript was significantly decreased in WBC before and after the onset of diabetes in Otsuka Long-Evans Tokushima Fatty (OLETF) rats compared to control rats (Long-Evans Tokushima Otsuka rats: LETO rats).[9] Significant decreases in the expression of this gene were also found in the major insulin-target tissues, liver and adipose tissue, as well as WBC before onset. We hypothesize from these results that some NIDDM susceptible genes are up/down-regulated in WBC, reflecting their expressions in the liver, one of the major insulin-target tissues.

In this study, we examined this hypothesis using a rat cDNA microarray which includes more than 15000 genes, and OLETF and LETO rats before the onset. We searched for genes that are up/down-regulated in WBC before onset under the fasted or insulin administered conditions. Only genes, which showed a significant variation in gene expression between WBC and the liver, were selected as candidate genes for predicting the onset of NIDDM.

MATERIALS AND METHODS

Animals 4-Week old male Otsuka Long-Evans Tokushima Fatty (OLETF) and Long-Evans Tokushima Otsuka (LETO) rats were generously donated by the Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan).[10] 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. In this study, each experimental group was comprised of six rats. All animal protocols were approved by the institutional animal care and research advisory committee at Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

Glucose Tolerance Test (GTT) Rats were fasted overnight and subjected to an intraperitoneal GTT (IPGTT) at 5, 8, 11, 17, 23 and 31 weeks of age.[11] A glucose solution (Otsuka Pharmaceutical, Tokushima, Japan) was injected intraperitoneally (1 g/kg BW) after a 12 h period of fasting and

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blood was then obtained from the tail vain after 1 h. The blood was incubated for 30 min on ice followed by centrifugation for 2 min, 9000 rpm at 4 °C. Serum glucose concentration was determined using a Glucose-B test kit (Wako, Osaka, Japan).

Tissue Sampling Six-week old male OLETF rats were used as a before onset model, based on IPGTT. After overnight fasting, whole blood and liver tissue were collected as fasting samples. Porcine insulin (SIGMA, ST. Louis, MO, U.S.A.) was injected into 6-week old rats intraperitoneally (5 U/kg BW) after overnight fasting, and rats were cervical dissociated 1 h after insulin administration. Whole blood and liver were collected after decapitation.[12] All experiments were carried out without 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 purification using an RNeasy Mini Kit combined with RNase-free DNase Digest Set (QIAGEN, Hilden, Germany) for the degradation of genomic DNA in total RNA samples, and 5 μg of total RNA extracted from six rats were then pooled into one sample for normalizing the individual differences, followed by gene expression analysis in both tissues by cDNA microarrays.[13] Total RNA extracts from WBC and liver were stored at −80 °C until used in the assays.

cDNA Probe Labeling The pooled samples under the respective conditions were separately labeled using a cDNA synthesis kit (Part No. G2557A, Agilent Technologies, Palo Alto, CA, U.S.A.). In a typical run, a 30 μg aliquot of each total RNA sample was reverse-transcribed into a cDNA probe with oligo (dT) primer and labeled nucleotides. The reaction was carried out in a solution containing 50 μM dATP/dGTP/dTTP, 25 μM dCTP, 25 μM cyanine 3 (Cy3)-dCTP (for LETO samples) or cyanine 5 (Cy5)-dCTP (for OLETF samples) and 400 U MMLV reverse transcriptase at 42 °C for 1 h. The labeling reaction was terminated by incubation at 70 °C for 10 min. The RNA in each sample was then degraded by adding 0.05 μg of RNaseIA, followed by incubation at 37 °C for 30 min. Degraded RNA and unincorporated nucleotides were removed using a QIAquick PCR Purification Kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the instructions of Agilent Technologies Inc. Each Cy3-labeled probe was combined to an equal volume of the corresponding Cy5-labeled probe and hybridized to a microarray.

cDNA Microarray Procedure The total 22 μl of a hybridization mixture containing cDNA probes, the labeled orientation marker (Deposition Control SP300: Invitrogen, Carlsbad, CA, U.S.A.) and mouse Cot-1 DNA (Invitrogen, Carlsbad, CA, U.S.A.) were hybridized to a rat cDNA microarray (Part No. G4105A, Agilent Technologies, Palo Alto, CA, U.S.A.) at 65 °C for 17 h. The glass slides were then washed with 0.5×SSC containing 0.01% SDS at room temperature for 5 min, and with 0.06×SSC at room temperature for 2 min. After immediate removal of the wash buffer by centrifugation, the glass slides were scanned using Agilent G2565AA microarray scanner (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with 532 and 635-nm lasers for Cy3 and Cy5 measurements, respectively.[14]

Sixteen-bit TIFF images produced using an Axon scanner were analyzed using the Feature Extraction software (Agilent Technologies, Palo Alto, CA, U.S.A.). After obtaining Cy3 and Cy5 grayscale images, each pseudo-color image was overlaid, and all spots in the ratio image were defined by accessing the gene list file that specified the location of each gene on the microarray. The average of the signal intensity was subtracted from the median of the background intensity and output with the UniGene and GenBank descriptors to a Microsoft Excel data spreadsheet. Relative expression levels were calculated by global normalization between two samples using all detected genes, after the exclusion of a spot annotated as "Incyte Blank", "Agilent Blank" and "Buffer". Intensity-dependent normalization (LOWESS) was performed, where the ratio was reduced to residual of LOWESS fit of the intensity *vs*. ratio curve. In addition, to adjust the results from each of the samples, we performed median centering by dividing each gene's signal ratio by the median of all signal ratios for that particular gene. An ANOVA test was performed at a p value <0.05 to identify genes that are differentially expressed across conditions.

Hierarchical Clustering The expression levels of genes in WBC and liver of the rats were observed for fasting and insulin administration. The expression levels of the genes under these four conditions were screened. Each of which 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 cDNA microarray, each spot (also called as "feature") contains copies of a nucleic acid sequence (approximately 500 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 correspond to more than one spot on the DNA microarray in some cases. A set of the four expression levels of a gene is called an expression profile of the gene and we define similarity between expression patterns of two genes by 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 Condition of Concurrently Regulated Genes between WBC and Liver Genes which were up/down-regulated more than two 2-fold in OLETF rats compared to LETO rats were first selected for fasting and insulin administrated conditions. Among these genes, only those that were simultaneously up/down regulated both in WBC and liver were screened under the respective conditions.

RESULTS

Blood Glucose Level The blood glucose concentration was maintained at around 8 mmol/l from 5 to 31 week old male LETO rats. On the contrary, the blood glucose concentration of the OLETF rats increased gradually reaching a value of over 11 mmol/l at 11 weeks, which indicated glucose intolerance, since the concentration of blood glucose in excess of 11 mmol/l is borderline for the onset of NIDDM (Fig.

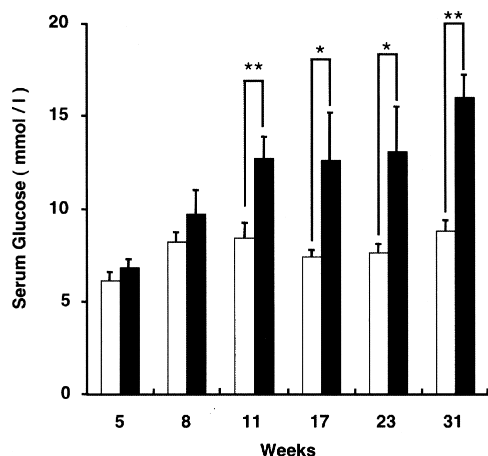

Fig. 1. Glucose Tolerance Test of OLETF Rats and Their Control Littermates, LETO Rats

Rats were tested at the ages of 5, 8, 11, 17, 23, and 31 weeks. Rats were fasted for 12 h, followed by an intraperitoneal glucose administration (1 g/kg). Blood glucose concentrations were measured 1 h after glucose administration. Open and Black columns represent the serum glucose concentrations of LETO and OLETF rats, respectively. All values are expressed as the mean±S.D. Statistical significance in values for serum glucose concentration was examined by Student's *t*-test. Bars indicate S.D. ($n=6$). ($*p<0.05$, $**p<0.01$).

1).[11] Therefore, 6-week old OLETF rats were considered to being pre-diabetic condition and were used in the following study.

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 of OLETF are shown (Fig. 2). 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 eighty genes, in which gene expression was altered by more than 2-fold at least in one condition were screened. These genes were further analyzed using hierarchical clustering to group genes with similar changes in their expression profiles. These genes were subsequently divided into four main clusters. Nearly 50% of the genes belong to cluster 1. Most of the genes in cluster 1 showed an increased expression in WBC for the OLETF rats (red color in columns WF and WI) and a relatively small alteration in liver (black color in columns LF and LI) under both conditions. Cluster 2 contains genes in WBC that are up-regulated under both conditions and in liver under insulin administration (colored red in columns WF, WI, and LI). Cluster 3 contains genes that are predominantly down-regulated in liver under insulin administration (colored green in column LI). Cluster 4 contains genes in WBC that are down-regulated under both conditions (green color in columns WF and WI). Unexpectedly, alterations in gene expression were more prominent in WBC than liver under both conditions for OLETF rats in the pre-diabetic state (compare column WF to LF and WI to LI). Many genes were altered in WBC even under fasting. These results suggest that WBC are sensitive to the altered homeostasis that accompanies the pathology of NIDDM in pre-diabetic OLETF rats. In the following analysis, different expression patterns depending on the different conditions in both tissues were examined. Insulin signals could be generated in both WBC and liver by the administration of insulin (compare column WF and LF to column WI and LI, respectively). The major genes for which their expressions were greatly altered are listed (Tables 1, 2). The top 10 genes are represented in the lists. The upper and lower lists show genes in which the expression is remarkably altered under fasting and insulin administration in WBC, respectively (Table 1). Under fasting conditions, in the case of WBC, 400 genes in which the expressions were altered by more than 2-fold were selected, with 89 genes being down-regulated and 311 genes being up-regulated (Table 1, upper list). In the case of WBC, fasting conditions induced the down-regulation of the mouse pancreatic amylase B-1 gene,[15] the rat carboxypeptidase B gene[16] and the rat cathepsin B gene,[17] which have been reported to be NIDDM-related genes and also induced on up-regulation of the mouse complement component C2 gene which has been reported to be a blood marker of type 1 diabetes.[18] Under insulin administration in WBC, 652 genes, in which their expressions were altered by more than 2-fold in OLETF were screened, and 127 were down-regulated and 525 were up-regulated (Table 1, lower list). Insulin administration induced the down-regulation of the rat carboxypeptidase B gene[16] and the rat tyrosine aminotransferase gene[19] and also induced the up-regulation of the myelin basic protein gene.[20] These three genes have been reported to be NIDDM-related genes. The upper and lower lists show genes that are expressed remarkably under fasting and insulin administration in the liver, respec-

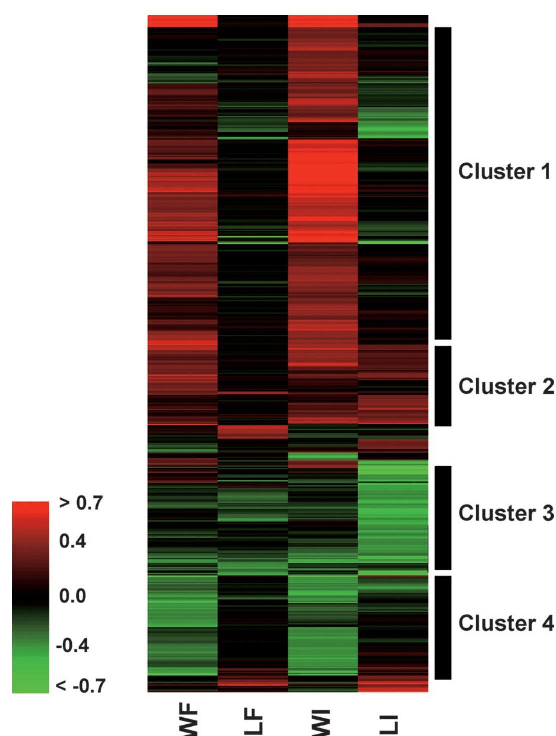

Fig. 2. Hierarchical Clustering Analysis of Genes That Are Expressed Differentially under Fasting and Insulin Administration in WBC and Liver

Gene expression levels of OLETF rats compared to LETO rats are displayed colorimetrically. In 1080 genes, their expressions were altered by more than 2-fold. The scale bar represents the expression level of each gene of OLETF rats relative to that of LETO rats by $\log_{10}$ scale of normalized mean signal intensities. Red and green represent increase and decrease in expression level relative to the median value, respectively. Black represents expression to same degree 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 1 and 2, and supplement.

Table 1. Genes Differentially Expressed in WBC of OLETF Rats under Fasting and Insulin Administration

Accession No.	Annotation	Log_{10} (OLETF/LETO) ratio
Top 20 genes that are up- or down-regulated in OLETF under fasting		
M59028	Rat NICER element (GL5-14) 5′ long terminal repeat	−1.2
AC009361	Mouse chromosome 10 clone rp21-12n20 strain 129S6/SvEvTac	−0.9
X62951	Rat mRNA (pBUS19) with repetitive elements	−0.8
D90005	Rat endogenous retroviral seaquence, 5′ and 3′ LTR	−0.8
M11896	Mouse pancreatic amylase B-1	−0.8
M23952	Rat carboxypeptidase B gene, exon 4 and 5	−0.7
J00703	Rat pancreatic amylase	−0.7
X82396	Rat mRNA for cathepsin B	−0.6
M26140	Moouse pancreatic amylase B-beta	−0.5
AJ010709	Rat gene encoding tyrosine aminotransferase	−0.5
U23776	Rat Eker rat-associated intracisternal-A-particle element	1.1
AAF55702	CG4664b gene product	0.9
M30692	Rat Ly6-A antigen gene, exon 2	0.9
BAA86298	Cbl-c	0.9
BAA25466	KIAA0540 protein	0.8
AAC50717	Sallvary peroxidase	0.8
AF237721	Mouse collagen type IX alpha 3	0.8
M57891	Mouse complement component C2	0.8
AB017446	Rat mRNA for organic anion transporter 3	0.7
AF076337	Rat strain Long Evans shaker myelin basic protein (Mbp) gene, intron 3	0.7
Top 20 genes that are up- or down-regulated in OLETF under insulin administration		
M59028	Rat NICER element (GL5-14) 5′ long terminal repeat	−1.2
AC009361	Mouse chromosome 10 clone rp21-12n20 strain 129S6/SvEvTac	−1.0
D90005	Rat endogenous retroviral seaquence, 5′ and 3′ LTR	−0.9
X62951	Rat mRNA (pBUS19) with repetitive elements	−0.6
AJ010709	Rat gene encoding tyrosine aminotransferase	−0.6
M23952	Rat carboxypeptidase B gene, exon 4 and 5	−0.6
L31894	Rat PACE4	−0.5
AAC50260	Zinc finger protein ZNF133	−0.5
AB002136	Mouse mRNA for glycosylphosphatidylinositol anchor attachment 1(GPAA1)	−0.5
Z18877	Rat mRNA for 2′5′ oligoadenylate synthetase	−0.5
U23776	Rat Eker rat-associated intracisternal-A-Particle element	1.2
AAC50717	Sallvary peroxidase	1.0
CAB71312	Proline racemase	1.0
AJ011005	Rat mRNA for Ptx3 protein	1.0
AF192499	Mouse putative secreted protein ZSIG37	1.0
AF076337	Rat strain Long Evans shaker myelin basic protein gene, intron 3	1.0
AAD40375	PTD012	0.9
X51974	Rat mRNA for pl 6.1 esterase	0.9
BAA31237	Mitochondrial methionyl-tRNA transformylase	0.9
CAA73095	Golgi-associated microtuble-binding protein	0.9

Twenty genes (top 10 down- and up-regulated genes) in WBC are shown. Blue- and red-letters represent the NIDDM-related genes that have been reported. The scale of genes alteration represent as $\log_{10}$ (OLETF/LETO) ratio value. All selected genes are listed in the supplement. p values of all genes <0.05 are presented.

tively (Table 2). The top 10 genes that were up- and down-regulated are shown in each list. In liver, under fasting conditions, 68 genes in which their expressions were altered by more than 2-fold were screened, 33 were down-regulated and 35 up-regulated (Table 2, upper list). In the liver, fasting conditions induced the down-regulation of the mouse complement component C2 and the rat apolipoprotein E genes which have been reported to be a constituent protein of very low-density lipoprotein (VLDL),[21] and fasting conditions also induced the up-regulation of BRG1-associated factor 250a, co-factor of the peroxisomal proliferators-activated receptor γ.[22] In the liver, under insulin administration, 316 genes in which their expressions were altered by more than 2-fold were screened. 211 genes were down-regulated and 105 genes were up-regulated (Table 2, lower list). Insulin administration induced down-regulation of the mouse complement component C2 gene,[18] the rat apolipoprotein E gene,[21] the rat c-fos gene[23] and the mouse Dvl-2 gene,[24] all of which have been reported to be NIDDM-related genes. Insulin administration also induced the up-regulation of the rat liver stearyl-CoA desaturase which function in lipid metabolism.[25]

Screening of Concurrently Regulated Genes in WBC and Liver of OLETF Rats In the following analysis, genes that were concurrently altered by more than 2-fold in WBC and liver of OLETF were screened. Fifty-five genes were screened under insulin administration (Fig. 3). In cluster A, 26 out of 55 genes were up-regulated in both WBC and liver (colored red in column WI, LI). In cluster B, 21 genes were up- and down-regulated in the WBC and liver, respectively (colored red and green in columns WI and LI). In cluster C, 7 genes were down-regulated in both tissues. One gene, rat thymidine kinase, was down- and up-regulated under insulin administration in WBC and liver, respectively. Under fasting conditions, 4 genes were screened as concurrently expressed genes in WBC and liver. Two out of 4 genes were up- and down-regulated in WBC and liver, respectively (colored red and green in column WF and LF, lower panel).

Table 2. Genes Differentially Expressed in Liver of OLETF Rats under Fasting and Insulin Administration

Accession No.	Annotation	Log_{10} (OLETF/LETO) ratio
Top 20 genes that are up- or down-regulated in OLETF under fasting		
X95096	Rat mRNA for macrophage stimulating protein	−0.8
AB022086	Mouse Cctz-2 gene for chaperonin containing TCP-1 zeta-2 subunit	−0.6
M57891	Mouse complement component C2	−0.6
M17083	Rat major alpha-globin	−0.6
J02582	Rat apolipoprotein E	−0.4
AAC37550	Bile acid CoA: Aminoacid N-acyltransferase	−0.4
U05675	Rat Sprague-Dawly fibrinogen B beta chain	−0.4
AAF47298	CG2803 gene product	−0.4
X62145	Rat mRNA for ribosomal protein L8	−0.4
AAA60043	Endothelial cell growth factor	−0.4
AF146569	Mouse GRIN1 (Z16)	0.6
AF075704	Rat neuronal glutamine transporter	0.6
AF093671	Mouse peroxisomal biogenesis factor (Pex11b)	0.6
AC034109	Mouse chromosome 1 clone rp23-88k7 strain C57BL/6J	0.5
AF289207	Mouse aspartyl beta-hydroxylase (ASPH) gene, exon 4 through 13, and alternative exon 14a; and aspartyl beta-hydroxylase Humbug splice variant	0.5
X06984	Rat brain mRNA for aldolase C	0.5
AF101779	Mouse Ring1 interactor RYBP	0.5
AB027568	Mouse mTPK1 mRNA for thiamin pyrophosphokinase	0.5
AAG33967	BRG1-Associated Factor 250a	0.5
BAA11018	Histone H1x	0.5
Top 20 genes that are up- or down-regulated in OLETF under insulin administration		
AF143543	Mouse alpha-dystrobrevin 2a	−0.9
X95096	Rat mRNA for macrophage stimulating protein	−0.9
U07619	Rat Sprague-Dawley tissue factor protein	−0.8
M57891	Mouse complement component C2	−0.8
CAC00659	bA39402.1 (CGI-15 protein)	−0.8
AF276775	Rat survivin	−0.8
J02582	Rat apolipoprotein E	−0.8
X06769	Rat c-fos	−0.7
U24160	Mouse Dvl-2	−0.7
AF276998	Rat junctional adhesion molecule JAM	−0.7
M29302	Rat senescence marker protein 2B gene, exon 1 and 2	1.1
AF093671	Mouse peroxisomal biogenesis factor (Pex11)	0.8
AAF54638	CG5276 gene product	0.8
U53855	Rat prostacyclin synthase (ratpgis)	0.8
AJ251363	Mouse mRNA for 15 kD Interferon alpha responsive protein	0.7
M80456	Mouse Int-3	0.6
U67081	Rat C2-HC type zinc finger protein r-MyT2	0.6
AF065161	Rat cytokine-inducible SH2-containing protein partial cds.	0.6
J02585	Rat liver stearyl-CoA desaturase	0.6
M64817	Mouse folate-binding protein 2 (FBP2)	0.6

Twenty genes (top 10 down- and up-regulated genes) in liver are shown. Blue- and red-letters represent the NIDDM related genes that have been reported. The scale of genes alteration represent as $\log_{10}$ (OLETF/LETO) ratio value. All selected genes are listed in the supplement. p values of all genes <0.05 are presented.

Only one gene, BRG1-Associated Factor 250a,[22] was up-regulated and only one gene, mouse ATFs,[26] was down-regulated in both tissues. The expression of a total of 59 out of 1080 genes were simultaneously altered in WBC and liver under the same conditions. Alterations in gene expression of mouse complement component C2 and mouse ATFs occurred under both fasting and insulin administration. These two genes were selected for use in both stimulations, therefore a total of 57 genes are included (Fig. 3). The selected genes in Fig. 3 with the expression ratios on a $\log_{10}$ scale and the putative biological functions of their encoded proteins are also summarized (Table 3). Thirty, out of 57 genes reported to be NIDDM related genes were screened, while there was no information related to NIDDM for the remaining 27 genes.

DISCUSSION

Gene expression was analyzed comprehensively by cDNA-microarrays and candidates for a gene diagnosis of NIDDM before onset were identified. To date, an analysis of SNPs, inherent sequence alterations, is the method of choice for the identification of disease-related genes. However, the cases where complicating factors such as heredity and environment are involved, as in NIDDM, it is difficult to predict their onset based on only altered nucleotide sequences. On the other hand, a comprehensive examination of amount of mRNA enables the detection of dynamic changes in transcription levels caused by altered signal transduction and/or epigenetic silencing, alteration that are affected by environmental factors. This is a major advantage of a cDNA-microarray analysis compared to an SNP analysis. We thus planned to determine candidates for the gene diagnosis of NIDDM before onset by comprehensive cDNA-microarray

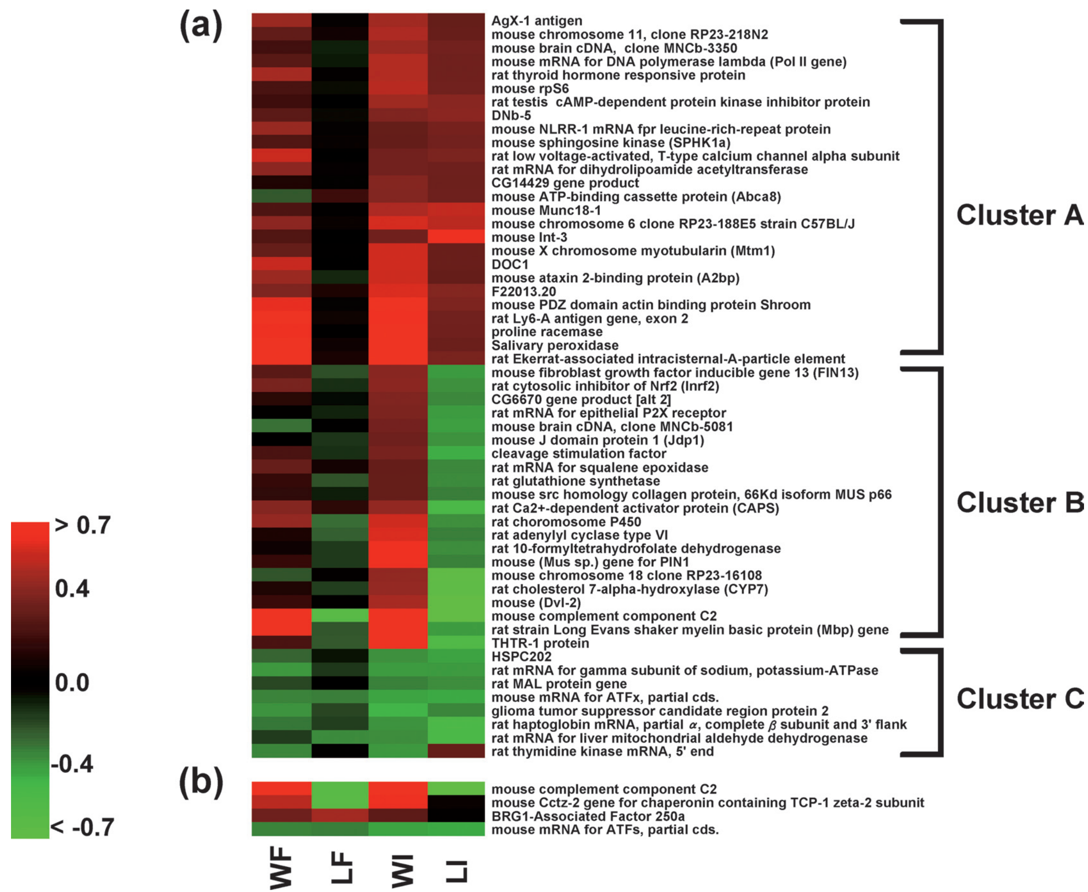

Fig. 3. Genes Whose Expressions Were Concurrently Altered in WBC and Liver Were Clustered Conditions Described in Materials and Methods

57 genes were screened as candidate genes for a gene diagnosis of NIDDM under insulin administration (a) and fasting (b).

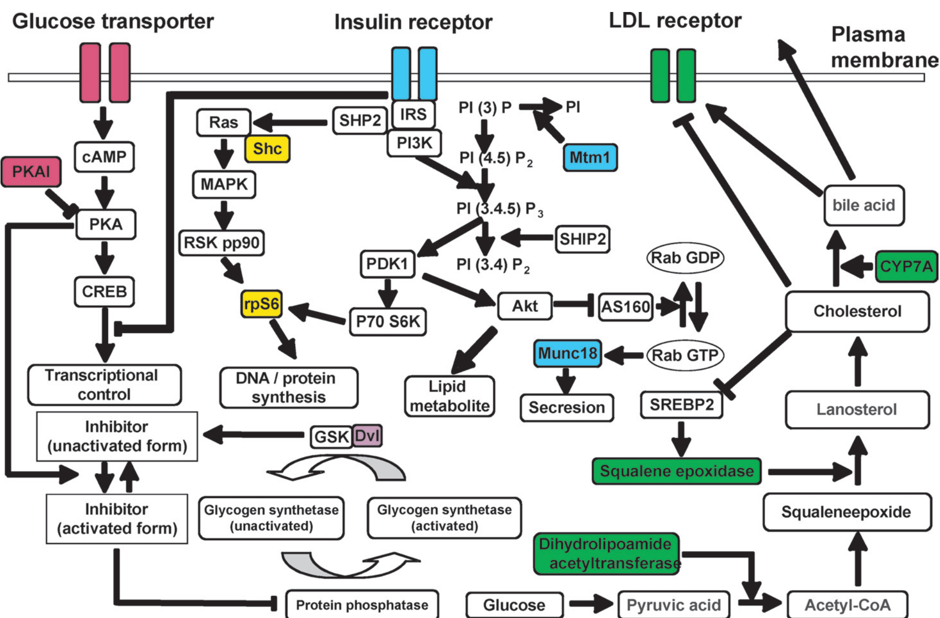

Fig. 4. Schematic Representation of NIDDM Related Signals in Cells

Representative proteins were selected from Table 3, and shown as colored.

Table 3. Concurrent Expressed Genes in WBC and Liver of OLETF Rats under Insulin Administration and Fasting

Accession No.	Annotation	Log_{10} (OLETF/LETO) ratio		NIDDM related function
		WBC	Liver	
Insulin injection				
AAB31210	AgX-1 antigen	0.5	0.3	Not reported
AAF46232	CG14429 gene product	0.4	0.3	Not reported
AAD27583	DNb-5	0.4	0.4	Not reported
AAA98972	DOC1	0.6	0.3	Not reported
AAF99762	F22013.20	0.6	0.4	Not reported
AF107204	Mouse ataxin 2-binding protein (A2bp)	0.6	0.3	Not reported
AF213393	Mouse ATP-binding cassette protein (Abca8)	0.4	0.3	Insulin secretion
AB041616	Mouse brain cDNA, clone MNCb-3350	0.4	0.3	Not reported
AC011013	Mouse chromosome 11, clone RP23-218N2	0.5	0.3	Not reported
AC019026	Mouse chromosome 6 clone RP23-188E5 strain C57BL6/J	0.6	0.5	Not reported
M80456	Mouse Int3	0.3	0.6	Not reported
AJ131889	Mouse mRNA for DNA polymerase lambda (Pol II gene)	0.5	0.3	Not reported
AB012584	Mouse Munc18-1	0.5	0.5	GLUT4 translocation
D45913	Mouse NLRR-1 mRNA for leucine-rich-repeat protein	0.3	0.4	Not reported
AF199421	Mouse PDZ domain actin binding protein Shroom	0.8	0.4	Insulin secresion
Z54209	Mouse rpS6	0.5	0.3	Insulin signaling
AF068748	Mouse sphingosine kinase (SPHK1a)	0.3	0.3	Nephropathy
AF125314	Mouse X chromosome myotubularin (Mtm1)	0.6	0.3	Insulin signaling
CAB71312	Prolin racemase	1.0	0.3	Not reported
U23776	Rat Eker rat-associated intracisternal-A-particle element	1.2	0.4	Not reported
AF027984	Rat low voltage-activated, T-type calcium channel alpha subunit	0.4	0.4	Insulin secretion
M30692	Rat Ly6-A antigen gene, exon 2	0.7	0.3	Not reported
D10655	Rat mRNA for dihydrolipoamide acetyltransferase	0.3	0.3	Lipid metabolism
M64092	Rat testis cAMP-dependent protein kinase inhibitor protein	0.5	0.4	Insulin secretion
U94904	Rat thyroid hormone responsive protein	0.5	0.3	GLUT4 translocation
AAC50717	Salivary peroxidase	1.0	0.3	Oral dryness
AAF55933	CG6670 gene product [alt2]	0.4	−0.3	Not reported
AAA35691	Cleavage stimulation factor	0.4	−0.4	Not reported
U24160	Mouse (Dvl-2)	0.5	−0.7	Insulin signaling
AB009692	Mouse (Mus sp.) gene for PIN1	0.7	−0.3	Not reported
AB041604	Mouse brain cDNA, clone MNCb-5081	0.4	−0.4	Not reported
AC020967	Mouse chromosome 18 clone RP23-16108	0.4	−0.7	Not reported
M57891	Mouse complement component C2	0.8	−0.8	Type1 diabetes marker
U42383	Mouse fibroblast growth factor inducible gene 13 (FIN13)	0.4	−0.4	Not reported
AF132906	Mouse J domain protein 1 (Jdp1)	0.3	−0.3	Not reported
U46956	Mouse src homology collagen protein, 66Kd isoform MUS p66	0.3	−0.3	Insulin signaling
M59861	Rat 10-formyltetrahydrofolate dehydrogenase	0.7	−0.3	Not reported
L01115	Rat adenylyl cyclase type VI	0.6	−0.3	Insulin signaling
U16802	Rat Ca^{2+}-dependent activator protein (CAPS)	0.4	−0.5	Insulin secretion
J05430	Rat cholesterol 7-alpha-hydroxylase (CYP7)	0.4	−0.7	Glycogenesis
M37828	Rat cytochrome P450	0.6	−0.3	Mitochondria damage
AF304364	Rat cytosolic inhibitor of Nrf2 (Inrf2)	0.4	−0.3	Not reported
L38615	Rat glutathione synthetase	0.3	−0.3	Oxidative stress
X87763	Rat mRNA for epithelial P2X receptor	0.4	−0.4	Renal injury
D37920	Rat mRNA for squalene epoxidase	0.3	−0.3	Lipid metabolism
AF076337	Rat strain Long Evans shaker myelin basic protein (Mbp) gene	1.0	−0.4	Neuropathy
CAB50771	THTR-1 protein	0.9	−0.5	Glucose transport
M22642	Rat thymidine kinase mRNA, 5′ end	−0.4	0.3	Insulin signaling
AAF36122	HSPC202	−0.3	−0.4	Not reported
AB012276	Mouse mRNA for ATFx, partial cds.	−0.4	−0.4	β-cell disfunction
AAF62873	Glioma tumor suppressor candidate region protein 2	−0.4	−0.3	Not reported
K01933	Rat haptoglobin mRNA, partial α, complete β subunit and 3′ flank	−0.4	−0.5	Adipokine
X82557	Rat MAL protein gene	−0.3	−0.3	Not reported
X70062	Rat mRNA for gamma subunit of sodium, potassium-ATPase	−0.4	−0.4	Insulin secretion
X14977	Rat mRNA for liver mitochondrial aldehyde dehydrogenase	−0.3	−0.5	Mitochondria damage
Fasting				
AAG33967	BRG1-Associated Factor 250a	0.3	0.5	Adipocyte differentiation
AB022086	Mouse Cctz-2 gene for chaperonin containing TCP-1 zeta-2 subunit	0.5	−0.6	β-Cell disfunction
M57891	Mouse complement component C2	0.8	−0.6	Type1 diabetes marker
AB012276	Mouse mRNA for ATFx, partial cds.	−0.3	−0.3	β-Cell disfunction

Genes shown in Fig. 3 are listed. Accession No., annotation, scale of alteration, and function of genes are described. p values of all genes <0.05 are presented.

analysis.

It is not easy to use parenchymal tissues such as liver in a clinical genetic diagnosis before onset. In contrast, blood samples are easily obtained and may be useful for such diagnostics. However, whether altered expression of genes related to NIDDM that reflect pathologic conditions can be detected in blood cells is unclear. We previously showed this possibility by a reverse transcription-PCR analysis of calpain 10 gene expression in WBC.[9] In this study, a cDNA-microarray analysis using total RNA pools from WBC and hepatic cells was employed to comprehensively analyze gene expression in both tissues, to select alternate candidates for the genetic diagnosis.

We observed that more genes were transcriptionally changed after insulin administration than under fasting in OLETF rats in comparison with LETO rats (Fig. 2). It would be reasonable to hypothesize that the signal transduction pathway under the insulin receptor has been altering in 6-week OLETF rats, although blood glucose concentrations were similar in OLETF and LETO rats at this stage. Since the insulin administration directly affects the insulin signal pathway, many genes including ones related to the insulin signal transduction pathway might differently respond to the insulin administration.

We found that a total of 57 genes were expressed differently in both WBC and liver of the two rat strains (Table 3). This novel result indicates that the expression of some genes was actually altered in common in WBC and liver, supporting our hypothesis. These 57 genes may be used in the gene diagnosis of NIDDM before onset by analyzing their expression in WBC. These genes include 30 which are known to be related to NIDDM. The fact that nearly 53% of the genes that are differently expressed between the LETO and OLETF rats in both WBC and liver are known NIDDM-related genes suggests that some known (and unknown) NIDDM-related genes were expressed in WBC reflecting an altered internal environment prior to onset in OLETF rats. This fact also supports the view that a genetic diagnosis using WBC would be useful.

The 30 genes of the 57 genes that are expressed differently in both WBC and liver are known to be related to insulin-resistance, gluconeogenesis and lipid-metabolism (Table 3, Fig. 4). The protein kinase A inhibitor gene was included in the 30 genes and its product is involved in the glycogen synthesis pathway, in which is activated by glucose uptake mediated by glucose transporters. The protein kinase A inhibitor may decrease fatty acid synthesis, energy consumption, and glucose uptake in the liver.[27] The Dvl gene product activates glycogen synthetase kinase 3, which regulates glycogen production in the liver (Fig. 4).[24]

The src homology collagen gene product is involved in insulin signaling and an adapter protein that forms a complex with the Ras protein (Fig. 4).[28] This gene has recently been reported to be expressed at accelerated leaves in monocytes and lymphocytes in NIDDM patients after onset.[29] This report supports our hypothesis that the expression of NIDDM-related genes is altered in blood cells before onset. Mouse X chromosome myotubularin regulates signal transduction through the phosphatidylinositol 3-phosphate dependent regulation, and may induce insulin resistance.[30] The ribosomal protein S6 kinase gene product is located downstream of the insulin signal, and regulates DNA/protein synthesis and homeostasis in cells (Fig. 4).[31]

The Munc18 gene, which is related to gluconeogenesis, was included in the 30 genes. The Munc18 protein is involved in the translocation of the glucose transporter from the intracellular compartment to the plasma membrane, thus facilitating the uptake of glucose by cells (Fig. 4).[32]

For genes involved in lipid metabolism, dihydrolipoamide acetyltransferase, squalene epoxidase, and cholesterol 7-α-hydroxylase genes were found. Dihydrolipoamide acetyltransferase catalyzes the production of acetyl-CoA from pyruvic acid.[33] Squalene epoxidase and CYP7A catalyze the syntheses of lanosterol from squalene epoxide and bile acid from cholesterol, respectively.[34,35] Interestingly, the expression of some enzymes involved in lipid metabolism in liver was altered in WBC of NIDDM model rats (Fig. 4).

No relationship between the expression patterns and functions of the 57 genes containing the known 30 genes could be found in the clustering analysis in this study, and it is unclear whether they are involved in the onset of NIDDM. Disease-causing genes have recently been searched by the construction of a network of genome sequences of various organisms, and large-scale transcriptome, proteome, interactome, and metabolome analyses. Hereafter, the huge amounts of information related to gene expression obtained by comprehensive analysis using microarrays will be interpreted by a pattern analysis similar to that used in bioinformatics. The concept of systems biology will be necessary for the diagnosis of many diseases.

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