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Genes for systemic vascular complications are differentially expressed in the livers of Type 2 diabetic patients

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Abstract

Aims/hypothesis. Type 2 diabetes is characterised by excessive hepatic glucose production and frequently leads to systemic vascular complications. We therefore analysed the relationship between the gene expression profile in the liver and the pathophysiology of Type 2 diabetes.

Methods. Liver biopsy samples were obtained from twelve patients with Type 2 diabetes and from nine non-diabetic patients. To assay gene expression globally in the livers of both groups, we made complementary DNA (cDNA) microarrays consisting of 1080 human cDNAs. Relative expression ratios of individual genes were obtained by comparing cyanine 5-labelled cDNA from the patients with cyanine 3-labelled cDNA from reference RNA from the liver of a non-diabetic patient.

Results. On assessing the similarities of differentially expressed genes, the gene expression profiles of the twelve diabetic patients formed a separate cluster from those of the non-diabetic patients. Of the 1080 genes assayed, 105 (9.7%) were up-regulated and 134 (12%) were down-regulated in the diabetic livers ($p<0.005$). The genes up-regulated in the diabetic patients included those encoding angiogenic factors such as vascular endothelial growth factor, endothelin and platelet-derived growth factor. They also included TGF superfamily genes such as *TGFA* and *TGFB1* as well as bone morphogenetic proteins. Among the down-regulated genes in the diabetic patients were molecules defending against stress, e.g. flavin-containing monooxygenase and superoxide dismutase.

Conclusions/interpretation. These findings suggest that livers of patients with Type 2 diabetes have gene expression profiles indicative of an increased risk of systemic vascular complications.

Keywords cDNA microarray · Liver · Type 2 diabetes · Vascular complications

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Abbreviations: aRNA, antisense RNA · BMP, bone morphogenetic protein · cDNA, complementary DNA · Cy, cyanine · FMO, flavin-containing monooxygenase · NASH, non-alcoholic steatohepatitis · PDGF, platelet-derived growth factor · SSC, standard saline citrate · SOD, superoxide dismutase · VCAM, vascular cell adhesion molecule · VEGF, vascular endothelial growth factor

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Introduction

Type 2 diabetes, the most common form of diabetes mellitus, results from the interaction of genetic and environmental factors. Although a few cases of Type 2 diabetes involve monogenic syndrome, in which the onset of disease in young patients is predictable, the majority of cases involve multiple genetic alterations [1]. The liver plays a central role in glucose homeostasis, and Type 2 diabetes is characterised by excessive hepatic glucose production. Due to its long duration, the disease causes systemic vascular complications such as micro- and macroangiopathy. Indeed, the liver is also a major source of angiogenic factors and cytokines involved in the development of atherosclerosis, e.g. vascular endothelial growth factor (VEGF) and TGF [2, 3]. For this reason, a comprehensive analysis of gene expression in the liver could deepen understanding of Type 2 diabetes and its related complications.

The recently developed complementary DNA (cDNA) microarray technology allows simultaneous, parallel analysis of the expression of hundreds to thousands of genes in cell lines or tissues, as well as the investigation of drug-induced changes in gene expression [4]. We have already used cDNA microarrays, consisting of 1080 human cDNAs, to characterise alterations that occur in various liver diseases [5, 6]. More recently, microarray profiling of skeletal muscle tissue of Type 2 diabetic patients [7, 8] and of healthy humans treated with insulin [9] has been reported. Although the liver is central for glucose homeostasis, microarray profiling of diabetic liver has been done only in Ob/Ob mice, a genetically obese rodent model of diabetes [10, 11, 12]. We have now used it to clarify the transcriptional alterations in the liver that are associated with the pathophysiology of Type 2 diabetes. We have also used this methodology to determine differentially expressed liver genes that are representative of increased risk for systemic diabetic vascular complications.

Subjects and methods

Patients. The subjects were twelve patients with Type 2 diabetes and nine non-diabetic patients serially recruited between 1998 and 2001 at Kanazawa University Hospital, Japan. There were no significant differences in age, BMI and liver function tests between diabetic and non diabetic patients (Table 1). Informed consent was obtained from all subjects, and the experimental protocol was approved by our institution.

All subjects tested negative for hepatitis B and C viruses. The diabetic patients were diagnosed according to criteria established by an expert committee on the diagnosis and classification of diabetes mellitus [1]. The diabetic patients (DM 1 to DM 12) were treated either with diet therapy alone (DM 7, 10 and 11), or with an α-glucosidase inhibitor (DM 6 and 8) or with insulin (DM 1 to 5, 9 and 12). None of them was taking any other oral hypoglycaemic agent. Pharmacological treatments other than anti-diabetic treatments included diltiazem hydrochloride (DM 3), nicorandil (DM 6) and nifedipine (DM 6) for angina pectoris. No statins, angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers were being taken.

Table 1. Characteristics of subjects

Characteristic	Type 2 diabetic patients	Non-diabetic patients
Men/women	11/1	5/4
Age (years)	47±11	55±11
BMI (kg/m^2)	28±4	25±3
FPG (μmol/l)	6.7±1.7*	5.3±0.6
HbA_1c (%)	7.1±1.5*	5.0±0.6
AST (IU/l)	40±25	27±13
ALT (IU/l)	55±38	29±14
HOMA-R	4.14±2.45	ND

Data are expressed as means ± SD. FPG, fasting plasma glucose; AST, aspartate transaminase; ALT, alanine aminotransferase; HOMA-R, homeostasis model insulin resistance index; ND, not determined. *$p<0.05$ vs non-diabetic patients

Informed consent was obtained from the diabetic patients both for this study and for the histolological examination of liver diseases, including non-alcoholic steatohepatitis (NASH), which is often associated with diabetes [13]. Liver biopsy specimens were obtained from all diabetic patients, immediately frozen in liquid nitrogen and stored at −80 °C until use. The nine non-diabetic subjects (Non-DM 1 to 9) were patients undergoing resection for colon cancer; for each, a tissue specimen was surgically removed from the non-cancerous part of the liver. The non-diabetic subjects were not taking medication, except anti-indigestion or anti-ulcer agents. Informed consent was also obtained from them for this study.

The study was approved by the relevant ethics committee and was carried out in accordance with the Declaration of Helsinki.

Pathology of the liver. All liver biopsy specimens were examined using haematoxylin-eosin and silver reticulin stain. A pathologist who was blinded to the patients' clinical condition and biochemical data scored each biopsy for steatosis, inflammation and fibrosis, using previously reported criteria [14, 15]. Steatosis was scored as 0 (no steatosis), 1 (in less than 5% of the lobular parenchyma), 2 (in 5–25% of the lobular parenchyma), 3 (in 25–75% of the lobular parenchyma) or 4 (in more than 75% of the lobular parenchyma). Inflammation was scored as 0 (no hepatocyte injury or inflammation), 1 (sparse injury and/or inflammation), 2 (mild focal injury and/or inflammation), 3 (noticeable injury and/or inflammation) or 4 (severe zone 3 hepatocyte injury and/or inflammation). Fibrosis was scored as 0 (normal connective tissue), 1 (focal pericellular fibrosis in zone 3), 2 (perivenular and pericellular fibrosis confined to zones 2 and 3 with or without portal and/or periportal fibrosis), 3 (bridging or extensive fibrosis with architectural distortion) or 4 (cirrhosis). No subjects had severely inflamed or fibrotic livers (score of 3 or higher).

Preparation of cDNA microarray slides. The protocol for preparing microarray slides containing 1080 cDNA clones has been described [5, 6]. The genes to be spotted were selected from the Clontech atlas membrane array and obtained from IMAGE Consortium libraries through its distributor, Research Genetics (Huntsville, Ala., USA). Each sequence-

verified clone was repeatedly sequenced in our laboratory to avoid using cross-contaminated clones. Sequence homology was confirmed using the advanced BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/). When sequencing failed to verify the purity of IMAGE clones for key genes, corresponding clones were obtained from RIKEN Gene Bank (Takaodai, Tukuba, Japan) or through personal communication. Of the 1080 cDNA clones used for analysis, 1001 were sequence-verified and 79 were sequence-unverified human expressed sequence tag clones. As negative controls for each assay, we used Firefly luciferase (Promega, Madison, Wis., USA) sequences, which have no homology to sequences in the human genome. Polymerase chain reaction products prepared from these clones were spotted on to glass slides with 16 pins using SPBIO 2000 (Hitachi Software, Fukuoka, Japan). To normalise varying efficiencies of labelling and detection, a series of housekeeping genes (encoding β-actin, ribosomal protein L32, GAPDH and albumin), as well as firefly luciferase sequences, were spotted in each of the 16 rectangles of DNA spots.

RNA isolation and antisense RNA amplification. Total RNA was isolated from frozen liver tissue samples using an RNA extraction kit [5], and first-strand cDNA was synthesised with Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg, Md., USA). Second-strand cDNA was synthesised with *Escherichia coli* DNA ligase and *E. coli* DNA polymerase (both from New England Biolabs, Beverly, Mass., USA) as described [5], and double-stranded cDNA was purified by phase lock gel (Eppendorf, Westbury, N.Y., USA) with phenol/chloroform extraction. We subsequently used double-stranded cDNA as a template for in vitro antisense RNA (aRNA) transcription and amplification using a MEGA script T7 kit (Ambion, Austin, Tex., USA) according to the manufacturer's protocol. The resulting aRNA was purified on an affinity resin column (RNeasy Mini Kit, Qiagen, Chatsworth, Calif., USA). All aRNAs were stored at −80 °C until use.

Preparation of fluorescence-labelled cDNA and microarray hybridisation. To label the probes, approximately 2 to 3 μg of aRNA was used as a template for first-strand cDNA synthesis with cyanine (Cy) 3- or Cy5-deoxyuridine triphosphate (Amersham Biosciences, Piscataway, N.J., USA) and Superscript II reverse transcriptase [5]. As a reference for each microarray analysis, we used aRNA samples prepared from the normal liver tissue of a 72-year-old man with a single liver tumour. Reference aRNAs were labelled with Cy3 and test sample aRNAs with Cy5. The labelled probes were purified on Microcon 30 columns (Millipore, Bedford, Mass., USA), and then the blocking reagents 10 μg yeast transfer RNA, 4 μg poly(dA) and 15 μg human Cot DNA were added to each probe and each mixture was concentrated to 12 μl. We then added 2.55 μl 20× standard saline citrate (SSC) and 0.45 μl 10% sodium dodecyl sulfate to each mixture, and each 15 μl aliquot was used as a hybridisation probe for each cDNA-spotted slide. The slides were covered with glass coverslips and fixed in a hybridisation cassette (TeleChem, Sunnyvale, Calif., USA), then hybridisation was performed for 12 hours at 65 °C. Slides were washed in 2× SSC and 0.03% sodium dodecyl sulfate, in 1× SSC and in 0.2× SSC; each washing lasted 5 minutes.

Image analysis. Quantitative assessment of the signals on the slides was done by scanning on a ScanArray 5000 (General Scanning, Watertown, Mass., USA), followed by image analysis using ImaGene 3.0 software (Bio Discovery, Los Angeles, Calif., USA). The signal intensity of each spot was corrected by subtracting adjacent background signals. To normalise the data, we averaged the intensities of all spots obtained with Cy3 and Cy5 in each of the 16 rectangles and adjusted the intensity of each corrected DNA spot by the average intensity ratio of Cy5:Cy3 (=1.0). This global normalisation of intensity provided a smaller variance of the Cy5:Cy3 ratio and almost the same results as normalisation using the housekeeping genes. Because signal values of approximately 500 to 600 were obtained for luciferase genes, which have no homology to any human gene sequence, all values below 600 were set as background values.

Hierarchical clustering of the gene expression of the patients was assessed by calculating Pearson's product–moment correlation coefficient using the program Cluster, and by visualisation using 'Tree View' (http://genexpress.stanford.edu). The data were log-transferred, normalised, mean-centred and applied to the average linkage clustering. The resulting dendrogram indicated the order in which patients were grouped according to similarities in their gene expression patterns. The gene cluster data were presented graphically as coloured images, and the genes that had been analysed were arranged as ordered by the clustering algorithm, so that genes with the most similar expression patterns were adjacent to each other.

Real-time quantitative PCR. Double-strand cDNA was used as a template for real-time quantitative PCR of bone morphogenetic protein-4 (*BMP4*), *TGFB1*, endothelin and glutathione S-transferase theta 1. To do this we used the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). The sets of primers and TaqMan probes were proprietary to Applied Biosystems (Assays-on-Demand gene expression product). To control for variation in the amount of DNA available for PCR in the different samples, gene expression of the target sequence was normalised relative to expression of an endogenous control, 18S ribosomal RNA (18S rRNA TaqMan Control Reagent Kit, Applied Biosystems). The PCR conditions were 1 cycle at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min.

Statistical analysis. All data are expressed as means ± SEM. To test the significance of numbers and frequencies of genes or Cy5:Cy3 ratios, we used supervised analyses with the permutation-based method (BRB-ArrayTools, http://linus.nci.nih.gov/BRB-ArrayTools.html) [16]. This software for the statistical analysis of cDNA microarray gene expression data was developed by the Biometric Research Branch of the National Cancer Institute (USA). It contains a class comparison tool based on univariate F tests to find genes differentially expressed between predefined clinical groups. The permutation distribution of the F statistic, based on 2000 random permutations, was also used to confirm statistical significance. A p value of less than 0.005 was considered significant.

Results

Histological findings in the livers of Type 2 diabetic patients. The clinical characteristics of the diabetic and non-diabetic patients were similar, except for diabetic markers (Table 1). Histological examination of the livers of Type 2 diabetic patients revealed various degrees of steatosis, inflammation and fibrosis (Table 2), although all had normal liver function (Table 1). We did not identify any patients with advanced NASH, or whose livers revealed severe inflammation or fibrosis

Fig. 1. Hierarchical clustering analysis of gene expression profiles in the livers of twelve diabetic and nine non-diabetic patients. Genes up-regulated in diabetic livers are shown in red; genes down-regulated in diabetic livers are in green. DM, Type 2 diabetic patient; Non-DM, non-diabetic patient

Table 2. Histological scoring of steatosis, inflammation and fibrosis in the livers of non-diabetic and Type 2 diabetic patients

	Steatosis	Inflammation	Fibrosis		Steatosis	Inflammation	Fibrosis
Non-DM 1	0	0	0	DM 1	3	2	2
Non-DM 2	0	0	0	DM 2	1	0	2
Non-DM 3	0	0	0	DM 3	1	1	1
Non-DM 4	0	0	0	DM 4	1	1	1
Non-DM 5	0	0	0	DM 5	3	2	2
Non-DM 6	0	0	0	DM 6	1	1	1
Non-DM 7	0	0	0	DM 7	2	1	1
Non-DM 8	0	0	0	DM 8	3	2	0
Non-DM 9	0	0	0	DM 9	2	0	2
				DM 10	1	0	0
				DM 11	4	1	1
				DM 12	2	1	1
Mean	0.0	0.0	0.0	Mean	2.0	1.0	1.2
SD	0.0	0.0	0.0	SD	1.0	0.7	0.7

Scoring was on a scale of 0 to 4. Non-DM, liver of non-diabetic patient; DM, liver of Type 2 diabetic patient

(score of 3 or more). The livers of all non-diabetic patients were histologically normal (Table 2).

Hierarchical clustering of gene expression. Hierarchical clustering of gene expression in the livers of all 21 patients was assessed by calculating Pearson's product–moment correlation coefficient (Fig. 1). For differentially expressed genes, the patients clustered into two groups, with one cluster being the twelve diabetic patients and the second cluster being the nine non-diabetic patients. The presence of diabetes was the only clinical determinant of gene expression contributing to clustering. In contrast, age, sex and BMI were not clinical determinants of gene expression profiling (data not shown). Differences in the clustering of diabetic and non-diabetic patients was observed using three subsets of genes: (i) transcription factors (95 genes); (ii) cytokines and growth factors (154 genes); and (iii) apoptosis-related genes (114 genes) (data not shown). These results suggest that the livers of diabetic patients have different patterns of gene expression than livers of non-diabetic persons.

Identification of differentially expressed genes in the livers of Type 2 diabetic patients. In characterising the differentially expressed genes in the livers of patients with Type 2 diabetes, we found that of the 1080 sequences assayed 105 (9.7%) were significantly up-

Table 3. Representative genes significantly up-regulated in the livers of Type 2 diabetic patients

GenBank Ref. Seq. ID	Gene	DM/ Non-DM	Parametric *p* value
Signal transduction			
NM_002747	Mitogen-activated protein kinase 4 (MAPK4; p63)	1.36	0.0000001
NM_000924	Calmodulin-dependent phosphodiesterase PDE1B1	1.81	0.0000017
NM_005512	Garp gene	1.56	0.0000036
NM_000835	Glutamate receptor, ionotropic, *N*-methyl D-aspartate 2C	1.32	0.0001347
NM_000675	Adenosine receptor	1.60	0.0001912
NM_004878	Homo sapiens Pig12 (PIG12)	1.39	0.0003145
NM_130850	Bone morphogenetic protein 4	1.38	0.0003244
NM_002045	Growth-associated protein 43	1.67	0.0005587
NM_001719	Bone morphogenetic protein 7 (osteogenic protein 1)	1.45	0.0006087
NM_145751	TRAF2	1.40	0.0008602
NM_145803	TNF receptor-associated factor 6	1.59	0.0015876
NM_024408	Notch 2	1.52	0.0033336
Cell adhesion/cell–cell signal			
NM_004932	Cadherin 6, K-cadherin (fetal kidney)	1.45	0.0000026
NM_001850	Collagen, type VIII, alpha 1	1.50	0.0000038
NM_002206	Integrin, alpha 7	1.32	0.0000111
NM_000887	Integrin, alpha X (antigen CD11C [p150], alpha polypeptide)	1.30	0.0000127
NM_002286	Lymphocyte-activation gene 3	1.22	0.0000212
NM_005562	Nicein B2 chain	1.36	0.0000351
NM_005562	Integrin, beta 7	1.38	0.0000532
NM_032966	Burkitt lymphoma receptor 1, GTP-binding protein	1.28	0.0000604
NM_002658	Plasminogen activator, urokinase	1.40	0.0001332
NM_001264	Corneodesmosin	1.47	0.0002728
NM_080679	Collagen, type XI, alpha 2	1.49	0.0002958
NM_033641	Collagen, type IV, alpha 6	1.25	0.0004334
NM_004061	Cadherin 12 (*N*-cadherin 2)	1.37	0.0004492
NM_002214	Integrin, beta 8	1.28	0.0012977
NM_002428	Matrix metalloproteinase 15 (membrane-inserted)	1.26	0.0029267
Cytokine/growth factor-related			
NM_001956	Endothelin 2	1.56	0.0000030
NM_000575	Interleukin 1, alpha	1.68	0.0000069
NM_002009	Fibroblast growth factor 7 (keratinocyte growth factor)	1.87	0.0000080
NM_003236	Transforming growth factor, alpha	1.52	0.0000319
NM_005429	Vascular endothelial growth factor C	1.38	0.0000673
NM_000601	HGF agonist/antagonist	1.24	0.0002486
NM_002045	Growth-associated protein 43	1.67	0.0005587
NM_023105	Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2)	1.31	0.0007361
NM_033016	Platelet-derived growth factor beta	1.37	0.0007417
NM_002506	Nerve growth factor, beta polypeptide	1.26	0.0012522
NM_147187	Tumor necrosis factor receptor superfamily, member 10b	1.31	0.0012870
NM_001957	Endothelin receptor type A	1.22	0.0016449
NM_004447	Epidermal growth factor	1.25	0.0017034
NM_006207	Platelet-derived growth factor receptor-like	1.27	0.0022775
NM_003247	Thrombospondin 2	1.24	0.0045472
Transcription/translation factor			
NM_002934	Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	1.37	0.0000369
NM_005522	Human HOXA1	1.39	0.0001436
Stress response			
NM_007294	BRCA1	1.64	0.0000002
NM_000465	BRCA1-associated RING domain 1	1.94	0.0000078
NM_000852	Glutathione S-transferase pi	1.58	0.0000842
NM_000853	Glutathione S-transferase T1	2.84	0.0001879
NM_004134	Heat shock 70kD protein 9B (mortalin-2)	1.22	0.0002101
NM_006904	Human DNA-dependent protein kinase catalytic subunit (DNA-PKcs)	1.26	0.0006155
NM_002542	8-oxoguanine DNA glycosylase	1.24	0.0033415

Table 3. (continued)

GenBank Ref. Seq. ID	Gene	DM/ Non-DM	Parametric *p* value
Metabolism			
NM_015865	RACH1 (RACH1)	1.41	0.0000333
NM_000022	Adenosine deaminase	1.21	0.0001155
Cell cycle/apoptosis/oncogenesis			
NM_001254	CDC18 (cell division cycle 18, *S. pombe*, homolog)-like	1.44	0.0000060
NM_001168	Apoptosis inhibitor 4 (survivin)	1.86	0.0000177
NM_001786	Cell division cycle 2, G1 to S and G2 to M	1.29	0.0002815
NM_022809	Cell division cycle 25C	1.29	0.0002819
NM_005521	HOX11=HOX11 homeodomain {homeobox}	1.20	0.0005101
NM_004397	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 6 (RNA helicase, 54kD)	1.30	0.0006865
NM_000546	p53	1.39	0.0020913

We identified 105 genes that were up-regulated in the livers of Type 2 diabetic patients ($p<0.005$). Expressed sequence tags and genes with unknown function are not listed. DM, livers from Type 2 diabetic patients; Non-DM, livers from non-diabetic patients

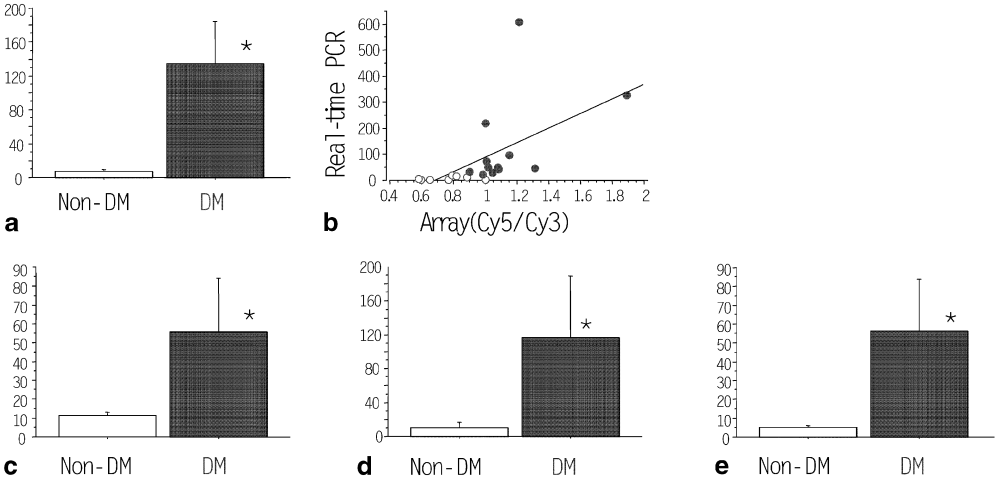

Fig. 2. Expression of gene transcripts by real-time quantitative PCR (**a–e**) and (**b**) gene expression levels assessed by cDNA microarray and real-time quantitative PCR. Double-strand complementary DNAs (cDNA) were used as templates for expression of bone morphogenetic protein 4 (*BMP4*) (**a, b**), endothelin (**c**), glutathione S-transferase theta 1 (**d**) and *TGFB1* (**e**) mRNAs. Expression of each target sequence was normalised relative to the expression of 18S ribosomal RNA. Each bar represents means ± SD. The correlation (**b**) between *BMP4* gene expression levels assessed by cDNA microarray and real-time quantitative PCR was significant ($r=0.58$, $p=0.0053$). ●, Type 2 diabetic patients; ○, non-diabetic patients. Non-DM, liver of non-diabetic patients; DM, liver of Type 2 diabetic patients; Cy, cyanine. * $p<0.05$ vs Non-DM

regulated and 134 (12%) were significantly down-regulated, compared with those of non-diabetic patients ($p<0.005$ by univariate F tests) (Tables 3 and 4). Although our cDNA microarrays did not contain many genes associated with glucose and lipid metabolism, expression of other genes was differentially altered in patients with Type 2 diabetes.

Interestingly, the genes significantly up-regulated included: (i) those encoding angiogenic factors, such as VEGF, endothelin and platelet-derived growth factor (PDGF); (ii) members of the TGF superfamily, including TGF-α and BMP-4 and –7; and (iii) collagens IV and VIII (Table 3). Also up-regulated were genes coding for pro-inflammatory cytokines or their receptors, e.g. interleukin 1 alpha, fibroblast growth factor and tumour necrosis factor receptor, and stress-response proteins, including glutathione S-transferase T1 and 8-oxoguanine DNA glycosylase (Table 3).

In contrast, the following genes were down-regulated in diabetic compared with non-diabetic livers: genes coding for general transcription factors, such as TFII and Jun; genes coding for lipogenic enzymes, including fatty acid desaturase and CD36 (fatty acid translocase); and genes coding for the ketogenic enzyme, acetoacetyl coenzyme A thiolase (Table 4). Also down-regulated in the livers of diabetic patients were genes encoding cell–cell interaction molecules, such as fibronectin, vascular cell adhesion molecule (VCAM)-1, selectin L, ficolin, decorin and MHC classes I and II, as well as stress defence molecules

Table 4. Representative genes significantly down-regulated in the livers of Type 2 diabetic patients

GenBank Ref. Seq. ID	Gene	DM/ Non-DM	Parametric *p* value
Transcription/translation factor			
NM_003407	Zinc finger protein homologous to Zfp-36 in mouse	0.37	0.0000096
NM_002126	Hepatic leukaemia factor	0.49	0.0000119
NM_001514	General transcription factor IIB (TFIIB)	0.46	0.0000227
NM_002228	c-jun proto oncogene (JUN)	0.45	0.0000238
NM_002052	GATA-binding protein 4	0.57	0.0000286
NM_002568	Poly(A)-binding protein, cytoplasmic 1	0.49	0.0000295
NM_004514	Interleukin enhancer binding factor 1	0.56	0.0000567
NM_003998	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	0.76	0.0001071
NM_006756	TRANSCRIPTION ELONGATION FACTOR S-II	0.59	0.0001304
NM_006195	Pre-B-cell leukaemia transcription factor 3	0.69	0.0001337
NM_001806	CCAAT/enhancer binding protein (C/EBP), gamma	0.79	0.0001430
NM_139276	Signal transducer and activator of transcription 3	0.61	0.0001961
NM_134447	RPB5-mediating protein (RMP)	0.53	0.0002762
NM_139266	STAT1	0.69	0.0003492
NM_001416	eIF4A	0.51	0.0004738
NM_006565	Transcriptional repressor (CTCF)	0.76	0.0005205
NM_080648	Ref-1	0.73	0.0005552
NM_004094	eIF2A	0.69	0.0006909
NM_003750	eIF3-p170	0.49	0.0007163
NM_005194	NF-IL6	0.50	0.0016377
Cell adhesion/cell-cell signal			
NM_080682	VCAM 1	0.51	0.0000012
NM_021983	MHC class II DR beta 4	0.39	0.0000013
NM_002026	Fibronectin	0.54	0.0000055
NM_004615	Transmembrane 4 superfamily member 2	0.61	0.0000157
NM_000655	Selectin L (lymphocyte adhesion molecule 1)	0.63	0.0000192
NM_004415	Desmoplakin I and II	0.57	0.0000556
NM_004559	MHC class II, Y box-binding protein I; DNA-binding protein B	0.56	0.0002113
NM_004530	Matrix metalloproteinase 2 (gelatinase A, 72kD type IV collagenase)	0.82	0.0002275
NM_003665	Ficolin	0.52	0.0002861
NM_005514	MHC class IB	0.52	0.0005335
NM_033666	Integrin, beta 1 (fibronectin receptor, beta polypeptide)	0.40	0.0007798
NM_133503	Decorin	0.68	0.0011217
Signal transduction			
NM_004040	Ras homolog gene family, member B	0.25	<0.0000001
NM_003010	SAPK/Erk kinase 1	0.66	0.0000008
NM_000591	CD14 antigen	0.46	0.0000095
NM_004447	Epidermal growth factor receptor kinase substrate (Eps8)	0.64	0.0000166
NM_001175	Rho GDP dissociation inhibitor (GDI) beta	0.66	0.0000191
NM_001665	Ras homolog gene family, member G (rho G)	0.65	0.0000242
NM_002759	PKR	0.62	0.0000251
NM_080921	Protein tyrosine phosphatase, receptor type, c polypeptide	0.54	0.0001131
NM_000560	CD53 antigen	0.66	0.0001768
NM_033423	Granzyme H	0.77	0.0001909
NM_000064	Complement 3	0.52	0.0002377
NM_002576	p21-activated protein kinase (Pak1)	0.81	0.0004081
NM_000609	Intercrine-alpha (hIRH)	0.69	0.0004506
NM_006208	Phosphodiesterase I/nucleotide pyrophosphatase 1	0.70	0.0008048
NM_002659	Plasminogen	0.70	0.0035258
Metabolism			
NM_005891	Cytosolic acetoacetyl-coenzyme A thiolase	0.58	0.0000133
NM_000072	Fatty acid translocase (FAT/CD36)	0.69	0.0002073
NM_000175	Glucose phosphate isomerase	0.67	0.0004559
NM_000024	Adrenergic beta-2 receptor	0.63	0.0008699
NM_006430	Aspartylglucosaminidase	0.63	0.0015513
NM_013402	Fatty acid desaturase	0.60	0.0046714

Table 4. (continued)

GenBank Ref. Seq. ID	Gene	DM/ Non-DM	Parametric *p* value
Cytokine/growth factor-related			
NM_005228	Epidermal growth factor receptor (v-erb-b oncogene homolog)	0.64	0.0000001
NM_002087	Granulin	0.56	0.0000010
NM_000596	Insulin-like growth factor binding protein 1	0.16	0.0000013
NM_003242	Transforming growth factor, beta receptor IIB	0.50	0.0001222
NM_000416	Interferon gamma receptor 1	0.58	0.0001499
NM_000598	Insulin-like growth factor binding protein 3 precursor	0.58	0.0002442
NM_133484	TNF receptor-associated factor 2	0.65	0.0003767
Stress response			
NM_001461	Flavin-containing monooxygenase 5	0.41	0.0000081
NM_000940	Paraoxonase 3	0.65	0.0000222
NM_000636	Superoxide dismutase 2, mitochondrial	0.40	0.0001703
NM_001618	Poly(ADP-ribose) synthetase	0.75	0.0017561
Cell cycle/apoptosis/oncogenesis			
NM_006495	Ecotropic viral integration site 2B (EVI2B)	0.72	0.0000015
NM_001166	Baculoviral IAP repeat-containing protein 2	0.57	0.0000019
NM_003339	E2D2	0.66	0.0000081
NM_021960	Myeloid cell leukaemia sequence 1 (BCL2-related)	0.37	0.0000521
NM_002467	c-myc oncogene	0.61	0.0000620
NM_033355	FADD-homologous ICE/CED-3-like protease (FLICE)	0.69	0.0001103
NM_006826	14-3-3 protein TAU	0.65	0.0000472
NM_003932	Suppression of tumorigenicity 13 (colon carcinoma, Hsp70-interacting protein)	0.63	0.0003040

We identified 134 genes that were down-regulated in the livers of diabetic patients ($p<0.005$). Expressed sequence tags and genes with unknown function are not listed. DM, livers from Type 2 diabetic patients; Non-DM, livers from non-diabetic patients

like flavin containing monooxygenase (FMO) 5 and superoxide dismutase 2 (SOD2) (Table 4).

Real-time PCR analyses. Real-time PCR confirmed that expression of *BMP4* was significantly up-regulated in the livers of diabetic patients (Fig. 2a), with expression levels of *BMP4* as assessed by real-time PCR and cDNA microarray well correlated with each other (r=0.58, p=0.0053) (Fig. 2b). Real-time PCR also confirmed that the expression of endothelin and glutathione S-transferase T1 were up-regulated in the livers of diabetic patients (Fig. 2c, d). Because the microarray analysis was not sufficiently sensitive to detect *TGFB1* gene expression, we used real-time PCR to assay expression of this gene. As with other members of the TGF superfamily, expression of *TGFB1* message was up-regulated in the liver of diabetic patients, relative to that of non-diabetic patients (Fig. 2e).

Discussion

In assessing gene expression profiles in the livers of patients with and without Type 2 diabetes using cDNA microarray analyses and real-time quantitative PCR, we found that the twelve diabetic patients formed a separate cluster from the nine non-diabetic patients. This was especially evident in three subsets of differentially expressed genes encoding (i) transcription factors, (ii) cytokines and growth factors, and (iii) proteins associated with apoptosis. Diabetes is known to cause histological changes in the liver including NASH [13], and the difference in expression levels between diabetic and non-diabetic livers could therefore be related to these histological changes.

In the diabetes group, expression of 105 genes was significantly up-regulated, while expression of 134 genes was significantly down-regulated. Of those up-regulated, several belong to the TGF superfamily, including *BMP4*, *BMP7*, *TGFA* and *TGFB1*. Angiogenesis is an essential biological process, not only in embryogenesis but also in the progression of diabetic vascular complications. One member of the TGF superfamily, TGF-β, plays a critical role in angiogenesis [2], and also mediates expression of angiogenic factors such as *VEGF* [3], which we also found to be up-regulated in livers of diabetic patients. Another member, BMP-4, was recently found to stimulate VEGF synthesis [17, 18]. In development, VEGF acts upstream of the Notch pathway to determine arterial cell fate [19]. Notch signalling plays a critical role in vasculogenesis and angiogenesis [20]. In the absence of VEGF signalling, activation of the Notch pathway can rescue gene expression of arterial markers such as ephrin [20].

We also found that other cytokines involved in angiogenesis and the development of diabetic angi-

opathy, including endothelin [21] and *PDGF* [22], were up-regulated in diabetic livers. High concentrations of glucose enhance endothelin-1 expression, partly due to activation of protein kinase C, and may act to reduce retinal blood flow during the development of diabetic retinopathy [23]. In addition, receptors for PDGF and endothelin are up-regulated in the skeletal muscle of insulin-resistant Pima Indians [7]. Together, these alterations in gene expression in the diabetic liver could increase the risk for the systemic vascular complications associated with Type 2 diabetes, including diabetic retinopathy and ischaemic heart disease.

Plasmin is responsible for the proteolysis of extracellular matrix components, which prevent fibrosis and angiogenesis [24]. The down-regulation of plasminogen observed by us in the diabetic liver could thus result in reduced fibrinolysis, leading to thrombosis and atherosclerosis. Down-regulation of plasminogen has also been reported in the livers of ob/ob mice [11].

Recently, thrombospondin 2 gene polymorphism [25] and serum paraoxonase activity [26] were reported to predict coronary events. Mice deficient in thrombospondin 2 have a phenotype that could reduce the risk of myocardial infarction [27]. Thus the up-regulation of thrombospondin 2 and down-regulation of paraoxonase in the diabetic liver, as observed in this study, might be associated with increased risk of coronary artery disease.

Hyperglycaemia increases the production of reactive oxygen species, which may activate protein kinase C, induces advanced glycation end-product formation and activates the pleiotropic transcription factor, nuclear factor-kappa B [28]. Increases in reactive oxygen species are prevented by SOD2 [28]. Impaired expression of *SOD2* or other genes associated with a stress defence system, including *FMO5*, as observed here and in ob/ob mice [11], could increase oxidative stress in the diabetic state. Similar alterations in gene expression in blood vessels may increase the risk of atherosclerosis.

Several of the genes we found aberrantly expressed in livers of patients with Type 2 diabetes can also be altered in other organs of diabetic patients. For example, up-regulation of Rad (a Ras-oncogene associated with diabetes), heat shock 70kD protein, and receptors for PDGF and endothelin, and down-regulation of general transcription and translation factors, and of cadherin and *MHC* have been observed in the skeletal muscle of insulin-resistant Pima Indians [7] and in Type 2 diabetic patients [8]. In addition, up-regulation of epidermal growth factor receptor and down-regulation of fatty acid-associated enzymes, fibronectin, *VCAM1*, *MHC*, plasminogen, *SOD2*, *FMO5* and complement C3 have been observed in the liver of ob/ob mice [10, 11, 12].

We also observed, in the livers of Type 2 diabetic patients, reduced expression of the genes encoding some enzymes associated both with glucose metabolism (glucose phosphate isomerase, aspartylglucosaminidase and cytosolic acetoacetyl-coenzyme A thiolase) and with fatty acid metabolism (fatty acid desaturase and fatty acid translocase). Whilst this paper was being prepared, two other groups independently found a coordinated reduction of PGC1-responsive genes involved in oxidative metabolism in the skeletal muscle of Type 2 diabetic patients [29, 30]. A large-scale cDNA microarray containing comprehensive genes associated with metabolism could be used to find out whether these alterations also exist in the Type 2 diabetic liver.

In summary, the gene expression profile of Type 2 diabetic livers is different to that of the normal liver. Caution is necessary when generalising on the relevance of this observation for global cardiovascular complications. And while the direct contribution of these differential genes to systemic complications will, no doubt, be explained in the future, we suggest from our findings that clustering analyses and the identification of differentially expressed genes in the liver could be useful for clarifying the pathogenesis of Type 2 diabetes and its related complications.

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