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Gene expression profiles of peripheral blood cells in type 2 diabetes and nephropathy in Asian Indians

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Research

Gene expression profiles of peripheral blood cells in type 2 diabetes and nephropathy in Asian Indians

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Abstract

Background

Asian Indians with type 2 diabetes mellitus (T2D) have higher susceptibility to diabetic nephropathy (T2DN), the leading cause of end-stage renal disease and morbidity in diabetes. Peripheral blood cells (PBCs) play an important role in diabetes, yet very little is known about the molecular mechanisms of PBCs regulated in insulin homeostasis. In this study we explored the global gene expression changes in PBCs in diabetes and diabetic nephropathy to identify the potential candidate genes and molecular networks regulated in diabetes and nephropathy.

Results

Gene expression profiling of mRNA from PBCs from 6 diabetics with nephropathy (T2DN), 6 diabetics without nephropathy (T2D) and 6 non-diabetic subjects (C), using 13,824 human sequence verified cDNA clones revealed significant differential expression of 420 genes. Hierarchical clustering of significant genes revealed distinct gene expression signatures for diabetes and diabetic nephropathy. Functional categories distinctly regulated in T2D vs. T2DN included, cell growth and maintenance (27 vs. 7%), enzymes (10 vs. 7%) and protein synthesis (13 vs. 18%). Pathway analysis of genes in glucose and fat metabolism were unremarkable, in contrast proteasome pathway involved in protein degradation is significantly regulated in T2D vs. T2DN.

Conclusion

Gene expression changes in PBCs could distinguish variable diabetic states. The data provides the opportunity to explore cellular processes in PBCs that may play a role in insulin homeostasis and insulin resistance that are distinct from target tissue such as skeletal muscle and pancreas. Identification of candidate genes in peripheral blood could provide easily accessible biomarkers to monitor diabetic nephropathy.

Background

In 2001, an estimated 177 million adults (20-79 years) worldwide have diabetes. Type 2 diabetes (T2D) constitutes 85-95% of all diabetics in developed countries and accounts for an even higher percentage in the developing countries [1] like India. Asian Indians with T2D, in particular, are more prone to develop diabetic nephropathy [2]. Type 2 diabetes has become the single most common cause of end-stage renal disease (ESRD), accounting for 40% of new cases of ESRD with an estimated cost in excess of US$15.6 billion [3] for treatment.

The role of greater glycemic exposure resulting in overt nephropathy in T2D is poorly understood. Genetic factors, which may increase the susceptibility to nephropathy in patients with diabetes, have been proposed. A range of linkage studies of familial aggregation of diabetic nephropathy in type 1 or 2 diabetes indicates that potential genetic determinants have larger effects not amenable for detection by conventional genetic techniques [4]. Major breakthroughs in finding genetic susceptibility factors in diabetic nephropathy remain elusive.

Type 2 diabetes is a polygenic and complex disease. Earlier studies to examine the molecular mechanisms that underlie the origin and progression of diabetic nephropathy have been limited, in part because conventional research tools have restricted investigators to focus on single genes or isolated pathways [5]. Several genes such as, *nephrin* [6], *NAD(P)H oxidase* and *RAGE (receptor for advanced glycation end product)* [7], *toll-like receptor TLR4 gene* [8]*, ACE (Angiotensin converting enzyme)* [9], *PPAR (peroxisome proliferator-activated receptor) gamma2* [10], *AKR1B1 (aldo-keto reductase)* [11], *solute carrier family 12 (sodium/chloride) member 3* [12]*, MTHFR (methylenetetrahydrofolate reductase gene)* [13], *co-inheritence of HSPG (heparan*

sulfate proteoglycan) and ApoE genes [14], *ecNOS (endothelial nitric oxide synthase), PRKCB1 (protein kinase C-beta1)* [15], *adrenomedullin* (AM) gene [16] and antioxidant genes [17] have been proposed as candidate genes in diabetic nephropathy in type 2 diabetes through these studies. Some of these genes were also identified in the present study.

Genomic approaches to determine differential expression profiles utilizing serial analysis of gene expression (SAGE) [18] and DNA microarrays [19] are now providing global views of the potential genes and pathways that are associated with diabetes. Utilizing these approaches, tissue-specific gene expressions in human pancreas, muscle and fat demonstrated differential regulation of approximately 800 genes in diabetes [19]. Gene expression profiling in diabetic nephropathy has previously been performed only in animal models [20, 21].

The relationship between white blood cells (WBCs) and diabetic vasculopathy is well described and elevated WBC count, even within the normal range, is strongly associated with both macro- and microvascular complications in type 2 diabetes. Immunological changes mediated by WBCs, such as chronic inflammation [22, 23], infiltration of lymphocytes and macrophages by advanced glycation endproducts (AGEs) [24] and autoantibodies [25, 26] were demonstrated to play a role in the pathogenesis of diabetic nephropathy. Peripheral blood mononuclear cells express enzymes such as manganese superoxide dismutase, CuZn superoxide-dismutase and glutathione peroxidase which can protect against oxidant damage. Dysregulation of antioxidant enzyme activity in blood cells was demonstrated to increase the flux of glucose through the polyol pathway and generation of excess reactive oxygen species (ROS), leading to tissue damage and contributing to early diabetic renal disease [17].

Even though extensive literature exists relating peripheral blood cells to diabetic complications, the association of gene expression changes in PBCs in insulin homeostasis

compared to target tissue such as pancreas is largely unknown. In this study, we examined gene expression profiles of peripheral blood cells in type 2 diabetes with and without nephropathy to identify potential gene signatures that could detect nephropathy. We compared the changes in PBCs with previous studies of global gene expression changes in endocrine pancreas and skeletal muscle to provide new insights into molecular mechanisms underlying diabetic nephropathy.

Results and discussion

Global gene expression changes in type 2 diabetes

Profiling of 13,824 human cDNAs using RNA extracted from peripheral blood cells of subjects with type 2 diabetes and nephropathy showed statistically significant changes in 420 genes (complete list in additional data file 1), less than 2% of the cDNAs profiled. Comparison of T2D and T2DN with control subjects revealed a similar magnitude of regulatory response with number of genes perturbed in each disease state, 142 genes in T2D vs. 158 genes in T2DN (table 1). As shown in figure 1a, 109 and 125 genes were uniquely regulated in type T2D and T2DN, respectively. Thirty three genes were commonly regulated in T2D and T2DN when compared to control subjects. The detection of significant number of genes uniquely changed in T2D or T2DN suggests that distinct subsets of genes are involved in these two variable states in diabetes. Hierarchical clustering [27] of the genes significantly regulated in diabetes (420 genes) showed distinct gene clusters that are unique to T2D and T2DN (figure 1b). This demonstrates that gene expression signatures in PBCs could potentially provide a mechanism to distinguish diabetic nephropathy state.

Functional analysis of genes regulated in diabetes

Two thirds of the 420 genes with significant changes in expression in type 2 diabetes and nephropathy in the present study were ESTs (expression sequence tags) or genes with unknown function (complete list in additional data file 1). All named genes with functional annotations in the Unigene database [28] as of January 14, 2004 were categorized by broad functions as shown in figures 2a and 2b. The cell growth and maintenance category was the largest functional category (27%) that was regulated in T2D. This functional category also showed distinct differences between T2D and T2DN, with a smaller number of changes in nephropathy (7% in T2DN compared to 27% in T2D). Other functional categories that showed significant differences between T2D and T2DN are nucleic acid binding (13 vs. 22%), protein biosynthesis (13 vs. 18%) and signal transduction (10 vs.16%). For descriptive purposes, enzymes involved in various cellular processes such as cell cycle, growth and others are represented under the enzymes and metabolism category (table 2).

Changes in genes regulating energy metabolism/enzymes

Metabolic homeostasis is long considered a major component in pathophysiology of diabetes. In PBCs, of the 99 genes encoding for enzymes regulating carbohydrate and fat metabolism, surprisingly only 3 enzymes showed significant differential expression greater than 2 fold change in diabetes or diabetic nephropathy (additional data files 2 and 3). This shows that insulin regulation of energy homeostasis in PBCs is distinctly different from target tissue such as skeletal muscle and pancreas. The genes encoding metabolic enzymes (i.e., protein phosphatase 2A, glutamic-oxaloacetic transaminase and calmodulin 3) that showed significant differential expression (table 2) are well known to play a role in insulin homeostasis. The *serine/threonine-specific protein phosphatase type 2A (PP2A)* that is upregulated in T2DN is one of the most abundant of the

phosphatases that globally regulates several enzymes associated with insulin regulation of glucose uptake and glycogen synthesis. Insulin downregulates PP2A for its normal action on glucose metabolism and homeostasis. Impaired PP2A regulation is shown to be associated with insulin resistance which is a contributing factor in the pathogenesis of type 2 diabetes [29]. *Calcium/calmodulin-dependent protein kinase II (CaMKII)/ the delta subunit of phosphorylase kinase* upregulated in T2D is known to mediate insulin release and was implicated in diabetic vascular dysfunction and weight loss during diabetes [30]. Other protein kinases that were downregulated in T2DN were, *dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A*, *AMP-activated protein kinase (AMPK)* and *guanylate kinase 1,* but their association with pathogenesis of diabetes is unknown.

As shown in table 2, a number of important enzymes involved in general metabolic functions that maintain normal cell growth were regulated in T2D and T2DN. Genes such as, *methylenetetrahydrofolate reductase (MTHFR)* gene upregulated in T2DN, has been shown to be associated with diabetic nephropathy in specific populations [13] with a predisposition to ESRD. *Cytochrome P450* that is significantly downregulated in T2DN, is an important member in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens and estrogens with important implications in metabolic regulation of insulin homeostasis.

Genes related to signal transducer and cell cycle

Immunoregulation plays a significant role in diabetes and pathogenesis of nephropathy. Regulation of *interleukin 2 (IL2) receptor gamma chain (IL2RG), interleukin 17 receptor B* (*IL-17BR)* and *chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)* detected in PBCs correlates well with previous studies on their role in T2D and particularly T2DN. *Interleukin 2 (IL2) receptor gamma chain (IL2RG)* in T2DN*,* is an important signaling component of interleukin receptors (IL2, IL4, IL7, IL9 and IL15) and a target for

therapeutics in ESRD [31]. Similarly, *interleukin 17 receptor B* (*IL-17BR)* gene encodes a proinflammatory cytokine receptor on a restricted set of target cell types including human kidney and pancreas [32]. Increased excretion of urinary IL-17 was shown to be associated with minimal-change nephrotic syndrome [33]. Regulation of *chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)* causes glomerular immune complex deposition in prolonged hyperglycemia and macrophage accumulation in diabetic db/db kidneys [34]. Other important signal transducers, *tumor necrosis factor (ligand), brain-derived neurotrophic factor* and *ephrin-B2* regulated in T2DN play an important role in cell proliferation and apoptosis. Dysregulation of these factors have been implicated in the etiopathogenesis of diabetic nephropathy [35].

Genes related to transporter/ligand binding or carrier

In this class, important molecules (table 2) mediating growth and cell maintenance such as *insulin-like growth factor binding protein 2 (IGFBP-2)* gene (upregulated) that is linked to potential nephromegaly and microalbuminuria in diabetic nephropathy [36] was detected. Interestingly, low serum IGFBP-2 concentration was also shown as a good indicator for overall good physical functional status, inversely reflecting the integrated sum of nutrition and the biological effects of growth hormone, IGF-I and insulin [37]. *Galactoside-binding lectin soluble 3 (galectin 3, gal-3)* gene upregulated in diabetic nephropathy, a multifunctional lectin with (anti)adhesive and growth-regulating properties, is an advanced glycation end product (AGE) receptor (AGER, RAGE) and contributes to the development of diabetic glomerular disease [38]. *Solute carrier family 11 (proton-coupled divalent metal ion transporters)* gene downregulated in diabetic nephropathy is a member in *solute carrier (SLC) family (sodium/chloride transporters). SLC12A3* gene was recently identified by genome-wide analyses of 56,648 single nucleotide polymorphisms as a good candidate for the susceptibility to diabetic nephropathy [12].

Regulation of cell adhesion molecules plays an important role in vascular complications in diabetes. *Vascular cell adhesion molecule 1* gene downregulated in diabetic nephropathy is a member of the Ig superfamily and encodes a cell surface sialoglycoprotein expressed by cytokine-activated endothelium. This type I membrane protein mediates leukocyte-endothelial cell adhesion and signal transduction, and may play a role in the development of atherosclerosis. Plasma concentrations of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), in patients with type 2 diabetes were more reflective of hyperglycemia than hyperinsulinemia or insulin resistance [39].

Other genes related to cell cycle regulation with significant differential expression in diabetes in the present study were, *activated leukocyte cell adhesion molecule, chaperonin containing TCP1 subunit 4 (delta), chromosome 1 open reading frame 38, cyclin G1, cyclin T1* and *telomeric repeat binding factor 2*. Two genes, *cadherin 8 type 2* gene and *E2F transcription factor 4 p107/p130-binding* gene, associated with cell proliferation, oncogenesis and kidney morphogenesis [40] were downregulated in diabetes.

Genes regulated in protein catabolism, ubiquitin-proteasome pathway

Ubiquitin-proteasome regulating protein degradation mediates several biological processes such as transcriptional regulation, cell cycle control, antigen processing, apoptosis and DNA repair. Insulin is known to influence many of these mechanisms potentially through proteasome inhibition or activation. Insulin and IGF-1 promote ubiquitin-proteasome mediated degradation of insulin receptor substrates-1 and 2, an important complex system involved in insulin action and B-cell survival [41, 42]. Direct interaction of the glucose transporters (Glut) 1 and 4 and Glut4 with members of ubiquitin family, has been shown to play an important role in the control of glucose

uptake [43]. Ubiquitin-proteasome also plays a major role in diabetes induced protein wasting and skeletal muscle loss [19].

In this study, significant number (8) of ubiquitin-proteasome genes were regulated in T2D and T2DN. The related components of the ubiquitin-proteasome degradation pathway in type 2 diabetes with or without nephropathy are mapped in GeneMAPP-derived proteasome pathway [44] modified by Glickman and Ciechanover [45] with updated gene symbols from Locuslink [46], as represented in figure 3. In the present study, the expression level of 2 mRNAs of the ubiquitin-conjugating enzymes increased and 6 mRNAs of the proteasome components decreased in diabetic nephropathy (additional data file 4). The *ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)* gene known to catalyze the ubiquitination of histones, plays a major role in transcription regulation. In chronic renal failure (CRF), the ATP-dependent, ubiquitin-proteasome proteolytic pathway is activated with increases in the transcription of genes encoding proteins of this pathway. Endocrine abnormalities in CRF (e.g., insulin resistance) could also upregulate proteasome activity and contribute to muscle protein wasting in CRF [47]. Ubiquitin-proteasome pathway was also shown to be significantly regulated in gene profiling study of human skeletal muscle [19].

Genes with significant regulation in diabetic nephropathy

Diabetic nephropathy is the leading cause of ESRD, yet the molecular mechanisms underlying this diabetic complication are poorly understood. To identify potential genes reflective of diabetic nephropathy state, first we compared expression profiles of T2DN with control normal subjects. This direct comparison revealed significant differential expression of a set of 158 genes (table 1). In order to subset genes that are associated with diabetes and shared in T2DN, we compared diabetics with nephropathy and diabetics without nephropathy and identified that 262 genes were regulated in this comparison (table 1 and figure 1a). As shown in figure 1a, 48 genes were commonly

regulated in both comparisons (T2DN vs. C and T2DN vs. T2D) suggesting that they were uniquely regulated in nephropathy. Among these 48 genes, 16 were ESTs with unknown functions; others with known function are listed in table 3. Of the 32 known genes, 9 were described in literature as associated with diabetes or related kidney disease.

Two of the potential genes upregulated in T2DN, *endosulfine alpha* and *dipeptidylpeptidase IV (DPPIV)* are known to modulate insulin secretion. *Endosulfine alpha* gene is known to express in a wide range of tissues including muscle, brain and endocrine tissues. Its recombinant protein displaces binding of the sulfonylurea to beta cell membranes, inhibits cloned KATP channel currents and stimulates insulin secretion [48]. *DPPIV* gene encodes a cell surface serine protease that modulates biological activity of glucose-dependent insulinotropic polypeptide (GIP) by removing the N-terminal tyr(1)-ala(2) dipeptide from GIP [49]. This could potentially explain the glycemic differences and insulin resistance often noted in nephropathy.

UDP-GlcNAc:betaGal beta-1 3-N-acetylglucosaminyltransferase 5 gene located at 3q28 encodes an enzyme that is a member of the beta-1 3-N-acetylglucosaminyltransferase family, which is another target that is dysregulated in T2DN. It has been demonstrated that acetylglucosaminyltransferases induced in the heart by diabetes or hyperglycemia, were responsible for the increase in the deposition of glycoconjugates and the abnormal functions found in the hearts of diabetic rats [50]. Elevated glucose increased the activity of core 2 GlcNAc-T and adhesion of human leukocytes to retinal capillary endothelial cells, in a dose-dependent manner, through diabetes-activated serine/threonine protein kinase C beta2 (PKCbeta2)-dependent phosphorylation. This regulatory mechanism, involving phosphorylation of core 2 GlcNAc-T, is also present in polymorphonuclear leukocytes isolated from type 1 and type 2 diabetic patients [51].

Lipid abnormalities are known to contribute to the development and progression of diabetic nephropathy. Increased expression of *apolipoprotein C-III (apo C3)* gene

observed in this study is closely associated with hypertriglyceridemia phenotype. Recently a rare S2 allele of this gene was reported twice more prevalent in Asian Indians with hypertriglyceridemia [52]. Polymorphisms in *apo E* were documented in the development of nephropathy in type 2 diabetes in Asians [53].

Other genes that are regulated in T2DN such as *fucosyltransferase 8 (alpha 1,6)* gene, *T cell receptor (TCR) alpha*, *adenylosuccinate lyase* gene, *peripheral myelin protein 22* gene and *glutathione S-transferase A2* gene were implicated in mechanisms of glycosylation of specific urinary proteins [54], immune damage to renal basement membrane [55], AMP catabolism [56] and production of reactive metabolites [57], which could mediate renal damage. Known phenotypes associated with regulated genes in PBCs in diabetic nephropathy, are listed in additional data file 5.

Of importance, genes with known function that are not attributed to T2D are novel candidate genes that may provide new mechanistic insights into diabetes. *Nuclear receptor co-repressor 2* in this category is known to have a significant role in modulating androgen receptor transcriptional activity, as a coactivator for thyroid hormone receptor and interacts strongly with peroxisome proliferator-activated receptor alpha (PPARalpha) [58]. Differential expression of this gene was also reported significant in insulin induced human skeletal muscle and human pancreas [18, 19] profiling studies. Regulation of *zinc finger protein subfamily 1A 1 (Ikaros)* detected in this study is a key member of the Ikaros (ZNFN1A1) family of transcription factors that are implicated in the control of lymphoid development. Another regulated gene, *ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)*, a member of the ubiquitin-conjugating enzyme family that catalyzes the ubiquitination of histones was also known as an important regulator of several key transcriptional factors. Similarly, *HT002 protein (hypertension-related calcium-regulated)* gene, a novel gene that is negatively regulated by extracellular calcium concentration with higher levels of transcripts in hypertensive animals [59] is also a likely candidate gene for diabetic nephropathy.

Among the ESTs, *hypothetical protein FLJ21820* gene on chromosome 2p24.2 expressed in kidney and islets of Langerhans, was significantly elevated in both T2DN vs. C (3.7 fold change) and T2DN vs. T2D (6.7 fold change).

Comparison with other insulin gene expression profiling studies of muscle and pancreas

Insulin was shown to modulate mRNA levels of 757 genes in normal human skeletal muscle [19]. Present microarray analysis included 183 of these genes and 171 of them were detectable (by decile rank of normalized mean signal intensity of a gene equaling or greater than 3 in at least one of the two groups of diabetes or diabetic nephropathy vs. controls) in peripheral blood cells. The direction of mRNA expression matched in 106 genes in peripheral blood cells and normal human skeletal tissue, of which 85 were upregulated and 21 downregulated. In this subset of genes, 10 had significant differential mRNA expression in diabetes in peripheral blood cells (additional data file 6).

SAGE analysis for human pancreatic islet mRNAs revealed 253 genes with significant expression [18], 65 of them were represented on our array and 59 genes were detectable in peripheral blood cells. This showed a significant overlap of genes detected in pancreas and peripheral blood cells (additional data file 7). Six genes, *peroxiredoxin 6*, *chromosome 1 open reading frame 21*, *pancreatic elastase 3B*, *nascent-polypeptide-associated complex alpha polypeptide*, *cytoplasmic dynein light polypeptide 1* and *coated vesicle membrane protein* were detected in all 3 studies. Of importance, greater than 80% (230 out of 248 genes) of the genes on our array representative of genes identified in pancreas and skeletal muscle studies, were also detectable in PBCs. This suggests identification of key molecules in PBCs that are also regulated in target tissue such as pancreas and skeletal muscle, provides a new opportunity to monitor diabetic states through easily accessible peripheral blood.

Whole genome scanning of families with multiple members affected with diabetes has identified chromosome regions 1q, 12q and 20q to likely harbor type 2 diabetes gene. [60]. In the present analysis, there were 45 genes, which were preferentially expressed in either of the groups of diabetes vs. controls in peripheral blood cells that were mapped to these chromosomal regions (additional data file 8). Further investigations on some of these target genes could provide new evidence for genetic contribution in diabetes and underlying mechanisms.

Conclusion

During the past few years, microarrays have greatly facilitated obtaining global views of gene expression changes to compare phenotypic changes or response to stimulus. Although these arrays were not specifically geared to represent tissues and pathways known to be affected by diabetes, they have been used in both type 1 and type 2 diabetes research [61]. This is the first study to examine the changes in peripheral genome to identify novel candidates in the development of diabetes and diabetic nephropathy. The present investigation examined gene expression at a single time-point and it is likely that the nature and extent of gene expression vary depending on many other parameters during the progression of diabetes. Moreover, expression data in microarray study, i.e., mRNA levels, may not accurately reflect protein levels, and expression of a protein may not always have a physiological or pathological consequence. However, the identification of candidate genes on the basis of quantification of their expression level and the subsequent application of this knowledge to disease gene identification and target manipulation, is a logical step toward realization of the new genes-to-mechanisms paradigm [62].

Although peripheral blood cells are long implicated in the etiopathogenesis of diabetes mellitus, little is known of genes expressed in blood cells and their regulatory effects. This study established that significant number of genes in PBCs change in response to variable diabetic states and demonstrated that changes observed in insulin target tissues such as skeletal muscle or those expressed in pancreas, can also be correlated with changes in PBCs. Global gene expression profiling of peripheral blood cells in diabetes and diabetic nephropathy identified distinct gene signatures for these variable diabetic states and raises the possibility to use measurements of gene expression differences in peripheral blood to monitor diabetes progression and complications. Further studies on important known and novel targets regulated in diabetic nephropathy in peripheral blood cells identified in this study will provide new insights in the role of peripheral blood cells in insulin action, insulin resistance and interactions with key target tissues such as skeletal muscle and endocrine pancreas.

Methods

Subjects

Peripheral blood cells collected from 6 subjects with type 2 diabetes, 6 subjects with diabetic nephropathy and 6 control subjects were used to prepare pooled samples for each group. Selected subjects with T2DN had serum creatinine more than 2.5mg/dL, glomerular function rate reduced by 50% and no other renal pathologies. They were matched with subjects with T2D without nephropathy, who had urine albumin less than 20mg per 1g of creatinine. Control subjects (n=6) without diabetes and renal disease were selected by matching for age and gender with type 2 diabetics. Informed consent was obtained from the subjects following the institution (Nizam's Institute of Medical Sciences, Hyderabad) review board guidelines for human subjects.

cDNA microarray processing

13,824 human sequence verified cDNAs were amplified using universal forward and reverse primers that were amino modified with a 5' C_{12} spacer. PCR products were purified using Telechem PCR cleanup plates, dried down, re-suspended in 20μl of s Telechem spotting solution, and printed on Telechem SuperAldehyde Substrates using a Cartesian Pixsys printer with quill pins from Telechem.

Total RNA was extracted using TriReagent™ (Molecular Research Corp.) and purified using RNAeasy columns (Qiagen) according to the manufacturer's protocol. Six individual samples from each group (T2D, T2DN, C) were pooled to prepare labeled cRNA probes. Total RNA (10 μg) was reverse transcribed with Superscript II (Invitrogen) using poly-T primer and labelled with Cy5 by an amino-allyl labeling protocol. Each sample was hybridized to two individual arrays. Arrays were scanned using SA5000 fluorescent scanner (Perkin Elmer) and the data collected and analyzed with QuantArray™ software (Perkin Elmer). The detailed microarray protocols and the full data sets are available on our supplemental website [63]. Single channel method was used to avoid dye bias following the Affymetrix and recent Agilent and Codelink protocols. Quality control of duplicate arrays was set to r >0.94 (additional data file 9). The dataset is MIAME compliant; raw and processed data files in MAGE-ML format are available for depositing in a public data repository.

Data analysis

Mean signal intensity was adjusted for local background by subtracting the median background intensity. Data was normalized via intensity dependent procedure (loess function) by pin group [64]. Normalized data was exported to Arraystat™ statistical software (Imaging Research, Version 1.0, Revision 2.0). Modified ANOVAs (Arraystat F*

tests) and significance of differences between means (z tests) were determined using a pooled error model. Data set adjusted for multiple testing was done on the p values of the statistical tests using the False Discovery Rate (FDR) correction with the level of acceptable false positives set at 0.05 for each statistical test [65].

Abbreviations used

ACE	Angiotensin converting enzyme
AGER, RAGE	Receptor for advanced glycation end product
AGEs	Advanced glycation endproducts
AKR	Aldo-keto reductase
AM	Adrenomedullin
AMPK	AMP-activated protein kinase
Apo	Apolipoprotein
C	Controls
CaMKII	Calcium/calmodulin-dependent protein kinase II
CRF	Chronic renal failure
DPPIV	Dipeptidylpeptidase IV
EcNOS	Endothelial nitric oxide synthase
ESRD	End-stage renal disease
ESTs	Expression sequence tags
Galectin 3, gal-3	Galactoside-binding lectin soluble 3
GH	Growth hormone
GIP	Glucose-dependent insulinotropic polypeptide
Glut	Glucose transporters
HSPG	Heparan sulfate proteoglycan
ICAM-1	Intracellular adhesion molecule-1
IGFBP-2	Insulin-like growth factor binding protein 2
IGF-I	Insulin-like growth factor 1
IL-17BR	Interleukin 17 receptor B
IL2	Interleukin 2
IL2RG	Interleukin 2 receptor gamma chain
MTHFR	Methylenetetrahydrofolate reductase gene
PBCs	Peripheral blood cells
PKCbeta2	Protein kinase C beta2
PP2A	Protein phosphatase type 2A
PPAR	Peroxisome proliferator-activated receptor
PRKCB1	Protein kinase C-beta1
ROS	Reactive oxygen species
SAGE	Serial analysis of gene expression
SLC	Solute carrier family
T2D	Type 2 diabetes mellitus
T2DN	Nephropathy in type 2 diabetes mellitus
TCR	T cell receptor
TLR	Toll-like receptor
UIL-17	Urinary IL-17
VCAM-1	Vascular cell adhesion molecule-1
WBCs	White blood cells

Tables

Table 1

Global view of regulated genes in diabetes in peripheral blood cells

Table 2

Diabetes regulated genes in peripheral blood cells

Table 3

Genes associated with nephropathy in type 2 diabetes mellitus

Figures and legends

Figure 1a. Venn Diagram of overlap in regulated genes in type 2 diabetes mellitus with and without nephropathy.

Figure 1b. Global Gene Expression changes in type 2 diabetes mellitus with and without nephropathy

Genes (n=420) with significant expression were normalized by the absolute value of the maximum fold change for the gene and grouped by hierarchical clustering using Euclidean distances. Genes included were statistically significant by z test using a pooled error model, cut-offs were adjusted for multiple comparisons using a false detection rate (FDR) of 0.05.

Figure 2A. Functional classes of regulated genes in type 2 diabetes mellitus

Figure 2B. Functional classes of regulated genes in type 2 diabetes mellitus with nephropathy

All named genes with functional annotations in the Unigene database were categorized by broad functional class and broad process class

Figure 3. Regulation of Proteasome Pathway in type 2 diabetes mellitus with nephropathy or without nephropathy

The gene expression of components of the ubiquitin-proteasome degradation pathway in type 2 diabetes mellitus with nephropathy or without nephropathy. Color scale represents relative fold change of type 2 diabetes mellitus with nephropathy compared to diabetes without nephropathy. Dark red = T2DN/T2D $\geq$ 2; Light red = T2DN/T2D > 1; Dark green = T2DN/T2D $\leq$ -2; Light Green = T2DN/T2D < -1. The GenMAPP-derived proteasome pathway was modified utilizing information from Glickman and Ciechanover and updated with gene symbols from Locuslink.

Additional data files

Additional data file 1 - list of diabetes regulated genes with significant levels of mRNA expression in type 2 diabetes and diabetic nephropathy

Additional data file 2 - table with genes regulating enzymes in carbohydrate metabolism in type 2 diabetes mellitus

Additional data file 3 - table with genes regulating enzymes in fat metabolism in type 2 diabetes mellitus

Additional data file 4 – two figures of GeneMAPP-derived proteasome pathway representing genes regulating ubiquitin-proteasomes in type 2 diabetes mellitus and type 2 diabetes mellitus with nephropathy, as compared to controls.

Additional data file 5 – table showing regulation of known genes associated with phenotypes in diabetic nephropathy

Additional data file 6 - table with diabetes regulated genes in peripheral blood cells which expressed in normal human muscle tissue

Additional data file 7 - table with diabetes regulated genes in peripheral blood cells which expressed in normal human pancreas tissue

Additional data file 8 -table with diabetes regulated genes on chromosome 1q, 12q, 20q in peripheral blood cells.

Additional data file 9 – quality control assessment with replicate agreement of human arrays examined for type 2 diabetes with or without nephropathy.

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References

1. **International Diabetes Federation** [http://www.idf.org/e-atlas/home/index.cfm]
2. Allawi J, Rao PV, Gilbert R, Scott G, Jarrett RJ, Keen H, Viberti GC, Mather HM: **Microalbuminuria in non-insulin-dependent diabetes: its prevalence in Indian compared with Europid patients**. *British Medical Journal* 1988, **296:**462-4.
3. Brandle M, Zhou H, Smith BR, Marriott D, Burke R, Tabaei BP, Brown MB, Herman WH: **The direct medical cost of type 2 diabetes**. *Diabetes Care* 2003, **26:**2300-4.
4. The Family Investigation of Nephropathy and Diabetes Research Group: **Genetic determinants of diabetic nephropathy: the family investigation of nephropathy and diabetes (FIND)**. *J Am Soc Nephrol* 2003, **Suppl 2:**s202-4.
5. Susztak K, Sharma K, Schiffer M, McCue P, Ciccone E, Bottinger EP: **Genomic strategies for diabetic nephropathy**. *J Am Soc Nephrol* 2003, **14:**271-8.
6. Toyoda M, Suzuki D, Umezono T, Uehara G, Maruyama M, Honma M, Sakai T, Sakai H: **Expression of human nephrin mRNA in diabetic nephropathy**. *Nephrol Dial Transplant* 2004, **19:**380-5.
7. Matsunaga-Irie S, Maruyama T, Yamamoto Y, Motohashi Y, Hirose H, Shimada A, Murata M, Saruta T: **Relation Between Development of Nephropathy and the p22phox C242T and Receptor for Advanced Glycation End Product G1704T Gene Polymorphisms in Type 2 Diabetic Patients**. *Diabetes Care* 2004, **27:**303-7.
8. Rudofsky G Jr, Reismann P, Witte S, Humpert PM, Isermann B, Chavakis T, Tafel J, Nosikov VV, Hamann A, Nawroth P, Bierhaus A: **Asp299Gly and Thr399Ile genotypes of the TLR4 gene are associated with a reduced prevalence of**

diabetic neuropathy in patients with type 2 diabetes. *Diabetes Care* 2004, **27:**179-83.

9. Crook ED, Genous L, Oliver B: **Angiotensin-converting enzyme genotype in blacks with diabetic nephropathy: effects on risk of diabetes and its complications**. *J Investig Med* 2003, **51:**360-5.
10. Caramori ML, Canani LH, Costa LA, Gross JL: **The human peroxisome proliferator-activated receptor gamma2 (PPARgamma2) Pro12Ala polymorphism is associated with decreased risk of diabetic nephropathy in patients with type 2 diabetes**. *Diabetes* 2003, **52:**3010-3.
11. Makiishi T, Araki S, Koya D, Maeda S, Kashiwagi A, Haneda M: **C-106T polymorphism of AKR1B1 is associated with diabetic nephropathy and erythrocyte aldose reductase content in Japanese subjects with type 2 diabetes mellitus**. *Am J Kidney Dis* 2003, **42:**943-51.
12. Tanaka N, Babazono T, Saito S, Sekine A, Tsunoda T, Haneda M, Tanaka Y, Fujioka T, Kaku K, Kawamori R, Kikkawa R, Iwamoto Y, Nakamura Y, Maeda S: **Association of solute carrier family 12 (sodium/chloride) member 3 with diabetic nephropathy, identified by genome-wide analyses of single nucleotide polymorphisms**. *Diabetes* 2003, **52:**2848-53.
13. Moczulski D, Fojcik H, Zukowska-Szczechowska E, Szydlowska I, Grzeszczak W: **Effects of the C677T and A1298C polymorphisms of the MTHFR gene on the genetic predisposition for diabetic nephropathy**. *Nephrol Dial Transplant* 2003, **18:**1535-40.
14. Liu L, Xiang K, Zheng T, Zhang R, Li M, Li J: **Co-inheritance of specific genotypes of HSPG and ApoE gene increases risk of type 2 diabetic nephropathy**. *Mol Cell Biochem* 2003, **254:**353-8.
15. Araki S, Ng DP, Krolewski B, Wyrwicz L, Rogus JJ, Canani L, Makita Y, Haneda M, Warram JH Krolewski AS: **Identification of a common risk haplotype for diabetic nephropathy at the protein kinase C-beta1 (PRKCB1) gene locus**. *J Am Soc Nephrol* 2003, **14:**2015-24.

16. Ishimitsu T, Tsukada K, Minami J, Ono H, Ohrui M, Hino J, Kangawa K, Matsuoka H: **Microsatellite DNA polymorphism of human adrenomedullin gene in type 2 diabetic patients with renal failure**. *Kidney Int* 2003, **63:**2230-5.

17. Hodgkinson AD, Bartlett T, Oates PJ, Millward BA, Demaine AG: **The response of antioxidant genes to hyperglycemia is abnormal in patients with type 1 diabetes and diabetic nephropathy**. *Diabetes* 2003, **52:**846-51.

18. Cras-Meneur C, Inoue H, Zhou Y, Ohsugi M, Bernal-Mizrachi E, Pape D, Clifton SW, Permutt MA: **An expression profile of human pancreatic islet mRNAs by Serial Analysis of Gene Expression (SAGE)**. *Diabetologia* 2004, **Jan 13 [Epub ahead of print]**.

19. Rome S, Clement K, Rabasa-Lhoret R, Loizon E, Poitou C, Barsh GS, Riou JP, Laville M, Vidal H: **Microarray profiling of human skeletal muscle reveals that insulin regulates approximately 800 genes during a hyperinsulinemic clamp**. *J Biol Chem* 2003, **278:**18063-8.

20. Fan Q, Shike T, Shigihara T, Tanimoto M, Gohda T, Makita Y, Wang LN, Horikoshi S, Tomino Y: **Gene expression profile in diabetic KK/Ta mice**. *Kidney Int* 2003, **64:**1978-85.

21. Wada J, Zhang H, Tsuchiyama Y, Hiragushi K, Hida K, Shikata K, Kanwar YS, Makino H: **Gene expression profile in streptozotocin-induced diabetic mice kidneys undergoing glomerulosclerosis**. *Kidney Int* 2001, **59:**1363-73.

22. Tong PC, Lee KF, So WY, Ng MH, Chan WB, Lo MK, Chan NN, Chan JC: White blood cell count is associated with macro- and microvascular complications in chinese patients with type 2 diabetes.: **White blood cell count is associated with macro- and microvascular complications in chinese patients with type 2 diabetes**. *Diabetes Care* 2004, **27:**216-22.

23. Erlinger TP, Tarver-Carr ME, Powe NR, Appel LJ, Coresh J, Eberhardt MS, Brancati FL: **Leukocytosis, hypoalbuminemia, and the risk for chronic kidney disease in US adults**. *Am J Kidney Dis* 2003, **42:**256-63.

24. Mao Y, Ootaka T, Saito T, Sato H, Sato T, Ito S: **The involvement of advanced glycation endproducts (AGEs) in renal injury of diabetic glomerulosclerosis: association with phenotypic change in renal cells and infiltration of immune cells**. *Clin Exp Nephrol* 2003, **7:**201-9.

25. Benigni A, Zoja C, Corna D, Zatelli C, Conti S, Campana M, Gagliardini E, Rottoli D, Zanchi C, Abbate M, Ledbetter S, Remuzzi G: **Add-on anti-TGF-beta antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat**. *J Am Soc Nephrol* 2003, **14:**1816-24.

26. Atchley DH, Lopes-Virella MF, Zheng D, Kenny D, Virella G: Oxidized LDL-anti-oxidized LDL immune complexes and diabetic nephropathy: **Oxidized LDL-anti-oxidized LDL immune complexes and diabetic nephropathy**. *Diabetologia* 2002, **45:**1562-71.

27. **GeneMath** [http://www.applied-maths.com/ge/ge.htm]

28. **Unigene** [http://www.ncbi.nlm.nih.gov/]

29. Hojlund K, Poulsen M, Staehr P, Brusgaard K, Beck-Nielsen H: **Effect of insulin on protein phosphatase 2A expression in muscle in type 2 diabetes**. *Eur J Clin Invest* 2002, **32:**918-23.

30. Yousif MH, Benter IF, Akhtar S: **Inhibition of calcium/calmodulin-dependent protein kinase II normalizes diabetes-induced abnormal vascular reactivity in the rat perfused mesenteric vascular bed**. *Auton Autacoid Pharmacol* 2003, **23:**27-33.

31. Romero AL, Reddy KS, Johnston TD, Waid TW, Wade MeKeown J, Khan T, Karounos D, Doukas M, Samayoa L, Ranjan D: **Simultaneous kidney-pancreas transplantation in a patient with small lymphocytic lymphoma**. *Transplantation* 2003, **75:**414-6.

32. Shi Y, Ullrich SJ, Zhang J, Connolly K, Grzegorzewski KJ, Barber MC, Wang W, Wathen K, Hodge V, Fisher CL, Olsen H, Ruben SM, Knyazev I, Cho YH, Kao V, Wilkinson KA, Carrell JA, Ebner R: **A novel cytokine receptor-ligand pair.**

Identification, molecular characterization, and in vivo immunomodulatory activity. *J Biol Chem* 2000, **275:**19167-76.

33. Matsumoto, K, Kanmatsuse, K,: **Increased urinary excretion of interleukin-17 in nephrotic patients**. *Nephron* 2002, **91:**243-9.

34. Chow F, Ozols E, Nikolic-Paterson DJ, Atkins RC, Tesch GH: **Macrophages in mouse type 2 diabetic nephropathy: Correlation with diabetic state and progressive renal injury**. *Kidney Int* 2004, **65:**116-28.

35. Moriwaki Y, Yamamoto T, Shibutani Y, Aoki E, Tsutsumi Z, Takahashi S, Okamura H, Koga M, Fukuchi M, Hada T: **Elevated levels of interleukin-18 and tumor necrosis factor-alpha in serum of patients with type 2 diabetes mellitus: relationship with diabetic nephropathy**. *Metabolism* 2003, **52:**605-8.

36. Cummings EA, Sochett EB, Dekker MG, Lawson ML, Daneman D41-6: **Contribution of growth hormone and IGF-I to early diabetic nephropathy in type 1 diabetes**. *Diabetes* 1998, **47:**1341-6.

37. van den Beld AW, Blum WF, Pols HA, Grobbee DE, Lamberts SW: **Serum insulin-like growth factor binding protein-2 levels as an indicator of functional ability in elderly men**. *Eur J Endocrinol* 2003, **148:**627-34.

38. Pugliese G, Pricci F, Iacobini C, Leto G, Amadio L, Barsotti P, Frigeri L, Hsu DK, Vlassara H, Liu FT, Di Mario U: **Accelerated diabetic glomerulopathy in galectin-3/AGE receptor 3 knockout mice**. *FASEB J* 2001, **15:**2471-9.

39. Bluher M, Unger R, Rassoul F, Richter V, Paschke R: **Relation between glycaemic control, hyperinsulinaemia and plasma concentrations of soluble adhesion molecules in patients with impaired glucose tolerance or Type II diabetes**. *Diabetologia* 2002, **45:**210-6.

40. Blaschke S, Mueller CA, Markovic-Lipkovski J, Puch S, Miosge N, Becker V, Mueller GA, Klein G: **Expression of cadherin-8 in renal cell carcinoma and fetal kidney**. *Int J Cancer* 2002, **101:**327-34.

41. Rondinone CM, Kramer D: **Proteasome inhibitors regulate tyrosine phosphorylation of IRS-1 and insulin signaling in adipocytes**. *Biochem Biophys Res Commun* 2002, **296:**1257-63.

42. Rui L, Fisher TL, Thomas J, White MF: **Regulation of insulin/insulin-like growth factor-1 signaling by proteasome-mediated degradation of insulin receptor substrate-2**. *J Biol Chem* 2001, **276:**40362-7.

43. Giorgino F, de Robertis O, Laviola L, Montrone C, Perrini S, McCowen KC, Smith RJ: **The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells**. *Proc Natl Acad Sci U S A* 2000, **97:**1125-30.

44. **GenMAPP** [http://www.genmapp.org/MAPPSet-Human/GenMAPP.org_MAPPs/Other_MAPPs/Hs_Proteasome_Degradation.htm]

45. Glickman MH, Ciechanover A: **The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction**. *Physiol Rev* 2002, **82:**373-428.

46. **Locuslink** [http://www.ncbi.nlm.nih.gov/LocusLink/]

47. Bailey JL, Price SR, England BK, Jurkovitz C, Wang X, Ding X, Mitch WE: **Signals regulating accelerated muscle protein catabolism in uremia**. *Miner Electrolyte Metab* 1997, **23:**198-200.

48. Heron, L., Virsolvy A., Peyrollier K., Gribble F. M., Le Cam A., Ashcroft F. M., Bataille D.: **Human alpha-endosulfine, a possible regulator of sulfonylurea-sensitive KATP channel: molecular cloning, expression and biological properties**. *Proc Natl Acad Sci U S A* 1998, **95:**8387-91.

49. Gault VA, O'Harte FP, Harriott P, Flatt PR: **Degradation, cyclic adenosine monophosphate production, insulin secretion, and glycemic effects of two novel N-terminal Ala2-substituted analogs of glucose-dependent insulinotropic polypeptide with preserved biological activity in vivo**. *Metabolism* 2003, **52:**679-87.

50. Nishio Y, Warren CE, Buczek-Thomas JA, Rulfs J, Koya D, Aiello LP, Feener EP, Miller TB Jr, Dennis JW, King GL: **Identification and characterization of a gene regulating enzymatic glycosylation which is induced by diabetes and hyperglycemia specifically in rat cardiac tissue**. *J Clin Invest* 1995, **96:**1759-67.

51. Chibber R, Ben-Mahmud BM, Mann GE, Zhang JJ, Kohner EM: **Protein kinase C beta2-dependent phosphorylation of core 2 GlcNAc-T promotes leukocyte-endothelial cell adhesion: a mechanism underlying capillary occlusion in diabetic retinopathy.** *Diabetes* 2003, **52:**1519-27.

52. Chhabra S, Narang R, Krishnan LR, Vasisht S, Agarwal DP, Srivastava LM, Manchanda SC, Das N: **Apolipoprotein C3 SstI polymorphism and triglyceride levels in Asian Indians**. *BMC Genet* 2002, **3:**9.

53. Hsieh MC, Lin SR, Yang YC, Chen HC, Lin JN, Shin SJ: **Higher frequency of apolipoprotein E2 allele in type 2 diabetic patients with nephropathy in Taiwan**. *J Nephrol* 2002, **15:**368-73.

54. Poland DC, Schalkwijk CG, Stehouwer CD, Koeleman CA, van het Hof B, van Dijk W: Increased alpha3-fucosylation of alpha1-acid glycoprotein in Type I diabetic patients is related to vascular function.: **Increased alpha3-fucosylation of alpha1-acid glycoprotein in Type I diabetic patients is related to vascular function**. *Glycoconj J* 2001, **18:**261-8.

55. Baker FJ, Lee M, Chien YH, Davis MM: **Restricted islet-cell reactive T cell repertoire of early pancreatic islet infiltrates in NOD mice**. *Proc Natl Acad Sci U S A* 2002, **99:**9374-9.

56. Jenkins RL, McDaniel HG, Digerness S, Parrish SW, Ong RL: **Adenine nucleotide metabolism in hearts of diabetic rats. Comparison to diaphragm, liver, and kidney**. *Diabetes* 1988, **37:**629-36.

57. Glover DD, McRobie DJ, Tracy TS: **Effects of gestational and overt diabetes on placental cytochromes P450 and glutathione S-transferase**. *Prim Care Update Ob Gyns* 1998, **5:**189.

58. Dowell P, Ishmael JE, Avram D, Peterson VJ, Nevrivy DJ, Leid M: **Identification of nuclear receptor corepressor as a peroxisome proliferator-activated receptor alpha interacting protein**. *J Biol Chem* 1999, **274:**15901-7.

59. Solban, N., Dumas P., Gossard F., Sun Y., Pravenec M., Kren V., Lewanczuk R., Hamet P., Tremblay J.: **Chromosomal mapping of HCaRG, a novel hypertension-related, calcium-regulated gene**. *Folia Biol (Praha)* 2002, **48:**9-14.

60. Stern MP: **The search for type 2 diabetes susceptibility genes using whole-genome scans: an epidemiologist's perspective**. *Diabetes Metab Res Rev* 2002, **18:**106-13.

61. Scearce LM, Brestelli JE, McWeeney SK, Lee CS, Mazzarelli J, Pinney DF, Pizarro A, Stoeckert CJ Jr, Clifton SW, Permutt MA, Brown J, Melton DA, Kaestner KH: **Functional genomics of the endocrine pancreas: the pancreas clone set and PancChip, new resources for diabetes research**. *Diabetes* 2002, **51:**1997-2004.

62. Chugh SS, Whitesel S, Turner M, Roberts CT Jr, Nagalla SR: **Genetic basis for chamber-specific ventricular phenotypes in the rat infarct model**. *Cardiovasc Res* 2003, **57:**477-85.

63. **Supplementary website** [http://medir.ohsu.edu/~geneview/]

64. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP: **Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation.** *Nucleic Acids Res* 2002, **30:**e15.

65. Reiner A, Yekutieli D, Benjamini Y: **Identifying differentially expressed genes using false discovery rate controlling procedures**. *Bioinformatics* 2003, **19:**368-75.

Table1

Global view of regulated genes in diabetes in peripheral blood cells

T2D vs. C	T2DN vs. C	T2DN vs. T2D
73↑	86↑	143↑
69↓	72↓	119↓
142 (1.03%)	158 (1.15%)	262 (1.90%)

T2D: type 2 diabetes mellitus without nephropathy
T2DN: type 2 diabetes mellitus with nephropathy
C: controls without diabetes and nephropathy
Up-regulated(↑): Gene expression levels were significantly higher.
Down-regulated(↓): Gene expression levels were significantly lower.

Significant regulation of gene expression levels was defined as a fold change between groups (T2D, T2DN, C) of greater than 2 or less than −2 (using a common error model and a modified z test, all 2-fold changes were statistically significant at $p<0.05$ after FDR correction for multiple comparisons).

Table 2

Diabetes regulated genes in peripheral blood cells

Accession ID	Gene Name (Annotated January 14, 2004)	Cytogenetic Position	Fold change T2D vs. C	Fold change T2DN vs. C	Fold change T2DN vs. T2D	Summary function
Highest responsive genes						
AA454153	*hypothetical protein LOC149837*	20p13	-3.24	1.89	5.54	
N52315	*chromosome 13 open reading frame 10*	13q22.2	-1.20	3.74	4.47	
T63031	*nuclear receptor co-repressor 2*	12q24	-1.19	3.46	4.10	
W95480	*similar to RIKEN cDNA 2310038H17*	13q33.1	4.58	1.07	-4.29	
AA424575	*hematopoietic cell-specific Lyn substrate 1*	3q13	4.93	1.10	-4.47	nucleic acid binding
H29513	*hypothetical protein FLJ10193*	17p11.2	4.11	-1.59	-6.52	
Genes related to Enzyme/Metabolism						
AA464979	*hypothetical protein FLJ21820*	2p24.2	-1.82	3.67	6.70	catalytic activity
W70234	*dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)*	2q24.3	-1.55	2.57	4.00	hydrolase activity
W69379	*hypothetical protein FLJ25059*	11p14.1	-1.41	1.92	2.70	serine-type peptidase activity
AA398218	*non-metastatic cells 3, protein expressed in*	16q13	-1.31	2.01	2.63	GTP biosynthesis
AA427688	*protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform*	19q13.41	-2.19	1.20	2.62	protein phosphatase
AA406081	*eukaryotic translation initiation factor 4A, isoform 2*	3q28	-1.34	1.90	2.54	helicase activity
AA480995	*methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofol*	2p13.1	-1.50	1.64	2.45	hydrolase activity
AA443688	*GTP cyclohydrolase 1 (dopa-responsive dystonia)*	14q22.1-q22.2	-1.77	1.37	2.42	hydrolase activity
N57872	*alanine-glyoxylate aminotransferase (oxalosis I; hyperoxaluria I; glycolicacidur*	2q36-q37	-1.18	2.00	2.36	enzyme
AA143436	*ras homolog gene family, member D*	11q14.3	-1.18	1.94	2.30	GTP binding
H46487	*mannosyl (beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase*	22q13.1	-1.70	1.33	2.25	transferase activity
W94106	*casein kinase 1, epsilon*	22q13.1	1.11	2.48	2.24	protein kinase activity
AA457330	*calpain 6*	Xq23	-1.13	1.98	2.24	calpain activity
AA402855	*polymerase (DNA directed), beta*	8p11.2	-1.40	1.60	2.24	DNA repair
R44822	*phosphoribosyl pyrophosphate synthetase-associated protein 1*	17q24-q25	-1.33	1.63	2.17	enzyme
T62865	*aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)*	1p35.1-p36.23	-1.36	1.59	2.16	carbohydrate metabolism
AA456931	*cytochrome c oxidase subunit VIc*	8q22-q23	-1.49	1.45	2.15	electron transport
N76587	*CDC42 binding protein kinase beta (DMPK-like)*	14q32.3	-1.84	1.15	2.12	protein kinase activity
AA465366	*leukotriene A4 hydrolase*	12q22	-2.03	1.03	2.09	epoxide hydrolase activity
AA454207	*abhydrolase domain containing 2*	15q26.1	-1.23	1.67	2.06	catalytic activity
AA026631	*Ran GTPase activating protein 1*	22q13	-1.33	1.55	2.05	enzyme activator
AA629904	*ADP-ribosylation factor related protein 1*	20q13.3	1.22	2.49	2.04	GTP binding
AA456400	*adenylosuccinate lyase*	22q13.1	1.08	2.16	2.00	hydrolase activity
T49530	*lipase, endothelial*	18q21.1	1.06	2.07	1.95	lipid catabolism
AA401111	*glucose phosphate isomerase*	19q13.1	1.35	2.32	1.72	carbohydrate metabolism
AA453859	*alcohol dehydrogenase 5 (class III), chi polypeptide*	4q21-q25	1.22	2.09	1.71	fatty acid binding
N21546	*topoisomerase (DNA) III alpha*	17p12-17p11.2	1.30	2.18	1.67	DNA binding
AA169724	*protein arginine N-methyltransferase 6*	1p13.3	2.00	3.24	1.62	transferase activity
AA013260	*tribbles homolog 2*	2p25.1	1.51	2.19	1.45	protein kinase activity
R06321	*hydroxymethylbilane synthase*	11q23.3	1.94	2.39	1.24	heme biosynthesis
AA455146	*acetyl-Coenzyme A synthetase 2 (ADP forming)*	20q11.23	-2.21	-2.50	-1.13	lipid biosynthesis

H94944	*v-ral simian leukemia viral oncogene homolog A (ras related)*	7p15-p13	-1.96	-2.30	-1.18	GTP binding
AA464568	*proteasome (prosome, macropain) 26S subunit, ATPase, 4*	19q13.11-q13.13	-1.71	-2.58	-1.51	hydrolase activity
AA459572	*protein phosphatase 1, regulatory subunit 7*	2q37.3	1.86	-1.09	-2.02	intrinsic regulator activity
R16838	*cytochrome P450, family 17, subfamily A, polypeptide 1*	10q24.3	1.72	-1.18	-2.03	electron transport
AA490902	*guanylate kinase 1*	1q32-q41	2.44	1.18	-2.07	GTP biosynthesis
AA451781	*dynactin 6*	8p12-p11	1.41	-1.47	-2.07	lipid biosynthesis
AA676749	*dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A*	21q22.13	1.56	-1.41	-2.20	protein kinase activity
R25825	*N-acetylgalactosaminidase, alpha-*	22q13-qter	1.66	-1.36	-2.25	carbohydrate metabolism
H68845	*peroxiredoxin 2*	19p13.2	2.40	-1.08	-2.58	peroxidase activity
N23112	*protein kinase, AMP-activated, alpha 1 catalytic subunit*	5p12	2.36	-1.39	-3.28	protein kinase activity
AA192419	*biliverdin reductase A*	7p14-cen	3.14	-1.08	-3.40	electron transport
AA487681	*ornithine decarboxylase antizyme 1*	19p13.3	3.30	-1.11	-3.68	enzyme inhibitor
W76331	*leukotriene B4 12-hydroxydehydrogenase*	9q32	4.70	1.02	-4.60	oxidoreductase activity
AA456156	*uroporphyrinogen III synthase (congenital erythropoietic porphyria)*	10q25.2-q26.3	4.09	-1.20	-4.91	lyase activity
AA043551	*UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5*	3q28	2.05	-4.29	-8.77	transferase activity

Genes related to cell cycle/regulator

AA425755	*deleted in lymphocytic leukemia, 1*	13q14.3	-1.94	1.42	2.74	regulation of cell cycle
W21482	*chromosome 1 open reading frame 38*	1p35.3	-1.10	2.19	2.42	cell adhesion
R13558	*activated leukocyte cell adhesion molecule*	3q13.1	-1.17	1.72	2.02	cell adhesion
AA083032	*cyclin G1*	5q32-q34	1.16	2.21	1.91	regulation of cell cycle
AA598637	*chaperonin containing TCP1, subunit 4 (delta)*	2p15	1.13	2.05	1.81	regulation of cell cycle
AA676590	*telomeric repeat binding factor 2*	16q22.1	1.64	-1.22	-2.00	regulation of cell cycle
T90767	*cyclin T1*	12pter-qter	1.57	-1.28	-2.00	regulation of cell cycle
AA448641	*E2F transcription factor 4, p107/p130-binding*	16q21-q22	1.22	-1.67	-2.04	regulation of cell cycle
R56219	*cadherin 8, type 2*	16q22.1	1.92	-1.09	-2.09	cell adhesion
H16637	*vascular cell adhesion molecule 1*	1p32-p31	1.77	-1.24	-2.20	cell adhesion

Genes related to signal transducer

T48312	*endosulfine alpha*	1q21.3	-2.08	2.33	4.85	Signal transducer
AA427667	*T cell receptor alpha locus*	14q11.2	-1.08	2.26	2.45	Signal transducer
AA262988	*brain-derived neurotrophic factor*	11p13	-1.43	1.70	2.42	Signal transducer
N75745	*interleukin 2 receptor, gamma (severe combined immunodeficiency)*	Xq13.1	-1.14	1.97	2.24	Signal transducer
AA427595	*SHB (Src homology 2 domain containing) adaptor protein B*	9p12-p11	-1.62	1.33	2.17	Signal transducer
AA461424	*ephrin-B2*	13q33	-1.12	1.79	2.00	Signal transducer
H64601	*interleukin 17 receptor B*	3p21.1	-1.62	-2.27	-1.40	Signal transducer
N48080	*G-protein coupled receptor 88*	1p21.3	1.49	-1.57	-2.34	Signal transducer
H79353	*Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide*	1q23	3.37	1.22	-2.75	Signal transducer
AA410383	*chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)*	4q21	1.95	-1.49	-2.90	Signal transducer
H54629	*tumor necrosis factor (ligand) superfamily, member 10*	3q26	4.28	-1.06	-4.54	Signal transducer

Genes related to Transporter/ligand binding or carrier

AA450037	*KIAA0033 protein*	11p15.3	1.05	3.50	3.32	transporter
AA464121	*myosin, light polypeptide 5, regulatory*	4p16.3	-1.87	1.57	2.95	ligand binding or carrier
N53169	*apolipoprotein C-III*	11q23.1-q23.2	1.09	2.71	2.49	transporter
W55997	*F-box and leucine-rich repeat protein 11*	11q13.1	-1.26	1.86	2.34	ligand binding or carrier
H79047	*insulin-like growth factor binding protein 2, 36kDa*	2q33-q34	-1.46	1.53	2.24	ligand binding or carrier
AA172096	*endoplasmic reticulum thioredoxin superfamily member, 18 kDa*	1p32.3	-1.66	1.33	2.22	ligand binding or carrier

N26823	*retinoblastoma binding protein 6*	16p12.2	-1.34	1.61	2.16	ligand binding or carrier
AA027230	*exportin 6*	16p12.1	-1.39	1.44	2.00	transporter
AA261796	*multiple endocrine neoplasia I*	11q13	1.41	2.62	1.85	ligand binding or carrier
AA058314	*lectin, galactoside-binding, soluble, 3 (galectin 3)*	14q21-q22	1.35	2.18	1.62	ligand binding or carrier
N72116	*solute carrier family 11 (proton-coupled divalent metal ion transporters), membe*	12q13	-2.77	-2.20	1.26	transporter
N22297	*hypothetical protein FLJ90430*	7q11.21	1.80	2.01	1.11	ligand binding or carrier
N22827	*ferredoxin 1*	11q22	-1.31	-2.02	-1.54	ligand binding or carrier
AA186686	*prostaglandin E synthase 2*	9q34.13	-1.20	-2.14	-1.79	ligand binding or carrier
AA455126	*ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9),*	12q13.13	1.14	-1.75	-2.00	ligand binding or carrier
T83098	*adducin 2 (beta)*	2p14-p13	2.11	1.02	-2.07	ligand binding or carrier
N66591	*retinoblastoma binding protein 6*	16p12.2	1.36	-1.53	-2.08	ligand binding or carrier
H85454	*potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1*	20q12	-1.06	-2.25	-2.13	ligand binding or carrier
AA521389	*tumor protein p53 binding protein, 1*	15q15-q21	1.45	-1.59	-2.30	ligand binding or carrier
AA599078	*signal recognition particle 54kDa*	14q13.2	2.06	-1.20	-2.46	ligand binding or carrier
AA436372	*zinc finger protein 151 (pHZ-67)*	1p36.2-p36.1	3.17	1.10	-2.87	ligand binding or carrier

Genes related to ubiquitin-Proteasome

AA411876	*ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)*	7q32	-1.14	2.05	2.34	ubiquitin cycle
AA496541	*KIAA0317 gene product*	14q24.2	-1.25	1.64	2.05	ubiquitin cycle
R97788	*ring-box 1*	22q13.2	-2.78	-2.01	1.39	ubiquitin-dependent protein catabolism
R70174	*ubiquitin specific protease 13 (isopeptidase T-3)*	3q26.2-q26.3	-3.40	-2.76	1.23	ubiquitin-dependent protein catabolism
N66068	*ubiquitin specific protease 42*	7p22.2	1.94	-1.11	-2.16	ubiquitin-dependent protein catabolism
AA182680	*ubiquitin specific protease 9, Y-linked (fat facets-like, Drosophila)*	Yq11.2	1.56	-1.47	-2.28	ubiquitin-dependent protein catabolism
AA401853	*proteasome (prosome, macropain) 26S subunit, non-ATPase, 9*	12q24.31-q24.32	3.55	1.11	-3.20	ligand binding or carrier

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T2D: type 2 diabetes mellitus without nephropathy

T2DN: type 2 diabetes mellitus with nephropathy
C:controls without diabetes and nephropathy

Values indicate differential expression at $p<0.05$. Significant regulation indicated a 2-fold change between groups using a common error model and a modified z test. All 2-fold changes were statistically significant at $p<0.05$ after FDR correction for multiple comparisons.

Table 3

Genes associated with nephropathy in type 2 diabetes mellitus.

32 genes with known functions and preferentially expressed in diabetic nephropathy as compared to controls or diabetics without nephropathy

Accession ID	Gene Name (Annotated January 14, 2004)	Cytogenetic Position	Fold change			Broad Function
			T2D vs. C	T2DN vs. T2D	T2DN vs. C	
AA464979	*hypothetical protein FLJ21820*	2p24.2	-1.82	6.70	3.67	
T48312	*endosulfine alpha*	1q21.2	-2.08	4.85	2.33	signal transducer
N52315	*chromosome 13 open reading frame 10*	13q22.2	-1.20	4.47	3.74	
T63031	*nuclear receptor co-repressor 2*	12q24	-1.19	4.10	3.46	transcription factor binding
W70234	*dipeptidylpeptidase IV (CD26, adenosine deaminase complexing protein 2)*	2q24.3	-1.55	4.00	2.57	Enzyme
AA450037	*KIAA0033 protein*	11p15.3	1.05	3.32	3.50	
H51765	*paternally expressed 10*	7q21	-1.36	3.19	2.35	
R78725	*vitamin A responsive; cytoskeleton related*	3p14	-1.21	2.86	2.36	
AA398218	*non-metastatic cells 3, protein expressed in*	16q13	-1.31	2.63	2.01	
M17886	*ribosomal protein, large, P1*	15q22	-1.29	2.63	2.04	nucleic acid binding
AA598840	*polyhomeotic-like 2 (Drosophila)*	1p34.3	-1.13	2.53	2.25	ligand binding or carrier
N53169	*apolipoprotein C-III*	11q23.1-q23.2	1.09	2.49	2.71	structural protein
AA427667	*T cell receptor alpha locus*	14q11.2	-1.08	2.45	2.26	
AA192527	*fucosyltransferase 8 (alpha (1,6) fucosyltransferase)*	14q24.3	-1.09	2.44	2.24	Enzyme
W21482	*chromosome 1 open reading frame 38*	1p35.2	-1.10	2.42	2.19	
T73468	*glutathione S-transferase A2*	6p12.1	1.38	2.39	3.30	Enzyme
N57872	*alanine-glyoxylate aminotransferase (oxalosis I; hyperoxaluria I; glycolicacidur*	2q36-q37	-1.18	2.36	2.00	Enzyme
AA411876	*ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)*	7q32	-1.14	2.34	2.05	Enzyme
H81199	*hypothetical protein MGC2198*	5q35.3	-1.05	2.25	2.14	
W94106	*casein kinase 1, epsilon*	22q13.1	1.11	2.24	2.48	Enzyme
AA464962	*HT002 protein; hypertension-related calcium-regulated gene*	8q24-qter	1.37	2.19	3.01	
N53616	*melanoma ubiquitous mutated protein*	19p13.3	-1.07	2.16	2.02	
T91080	*zinc finger protein, subfamily 1A, 1 (Ikaros)*	7p13-p11.1	2.19	2.11	4.62	nucleic acid binding
R33335	*hypothetical protein FLJ32069*	7q11.21	1.09	2.10	2.29	
W04996	*periphilin 1*	12q12	1.12	2.05	2.29	
R26960	*peripheral myelin protein 22*	17p12-p11.2	1.28	2.05	2.62	
AA629904	*ADP-ribosylation factor related protein 1*	20q13.3	1.22	2.04	2.49	Enzyme
R99627	*chromosome 6 open reading frame 203*	6q21	1.01	2.02	2.04	
AA456400	*adenylosuccinate lyase*	22q13.2	1.08	2.00	2.16	Enzyme
H85454	*potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1*	20q12	-1.06	-2.13	-2.25	Transporter
AA411900	*Mov10l1, Moloney leukemia virus 10-like 1, homolog (mouse)*	22q13.33	-1.07	-2.62	-2.79	
AA043551	*UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5*	3q28	2.05	-8.77	-4.29	

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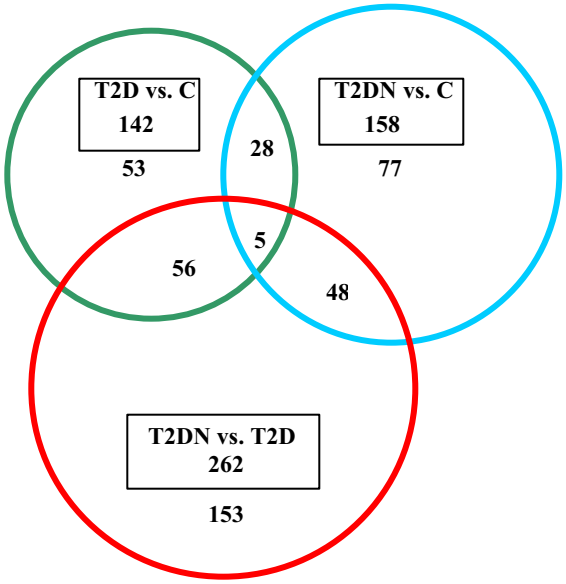
Figure 1a. **Venn Diagram of overlap in regulated genes in type 2 diabetes mellitus with and without nephropathy.**

T2D: type 2 diabetes mellitus without nephropathy
T2DN: type 2 diabetes mellitus with nephropathy
C: controls without diabetes and nephropathy



Acc.No	Gene Name	Gene Symbol	Broad Function	Cytogenetic Posit...
AA035347	glucosamine (N-acetyl)-6-sulfatase...	GNS	enzyme	12q14
AA431181	"thyroid hormone receptor-associa...	TRAP95	transcription factor binding	19p13.3
T83098	adducin 2 (beta)	ADD2	ligand binding or carrier	2p14-p13
AA443557	protocadherin 1 (cadherin-like 1)	PCDH1		5q32-q33
AA490902	guanylate kinase 1	GUK1	enzyme | ligand binding or ...	1q32-q41
H21520	rap2 interacting protein x	RIPX		4q21.1
R01619	Homo sapiens transcribed sequen...			
AA485974	"golgi autoantigen, golgin subfamily...	GOLGB1		3q13
N47443	KIAA0390 gene product	KIAA0390		19q13.11
AA130193	acheron	FLJ11196	nucleic acid binding	15q22.32
N63864	Homo sapiens transcribed sequen...			
H91216	"Homo sapiens histone 1, H2ac, m...			
R93875	nucleosome assembly protein 1-lik...	NAP1L1		12q21.1
AA490771	UV radiation resistance associated...	UVRAG		11q13.5
H48677	caldesmon 1	CALD1		7q33
H01039	Homo sapiens transcribed sequen...			
AA132094	"down-regulator of transcription 1, ...	DR1	nucleic acid binding | trans...	1p22.1
AA401853	"proteasome (prosome, macropain)...	PSMD9	ligand binding or carrier | m...	12q24.31-q24.32
H55907	"GrpE-like 1, mitochondrial (E. coli)"	GRPEL1	chaperone	4p16
AA436372	zinc finger protein 151 (pHZ-67)	ZNF151	ligand binding or carrier | n...	1p36.2-p36.1
H79353	"Fc fragment of IgE, high affinity I, r...	FCER1G	signal transducer	1q23
W76331	leukotriene B4 12-hydroxydehydro...	LTB4DH	enzyme | ligand binding or ...	9q32
AA424575	hematopoietic cell-specific Lyn sub...	HCLS1	nucleic acid binding	3q13
W95480	similar to RIKEN cDNA 2310038H17	na-unofficial		13q33.1
AA463458	glutathione synthetase	GSS	enzyme | ligand binding or ...	20q11.2

Figure 1b. **Global Gene Expression changes in type 2 diabetes mellitus with and without nephropathy**

Genes (n=420) with significant expression were normalized by the absolute value of the maximum fold change for the gene and grouped by hierarchical clustering using Euclidean distances. Genes included were statistically significant by z test using a pooled error model, cut-offs were adjusted for multiple comparisons using a false detection rate (FDR) of 0.05.

Figure 2a. Functional classes of regulated genes in type 2 diabetes mellitus.

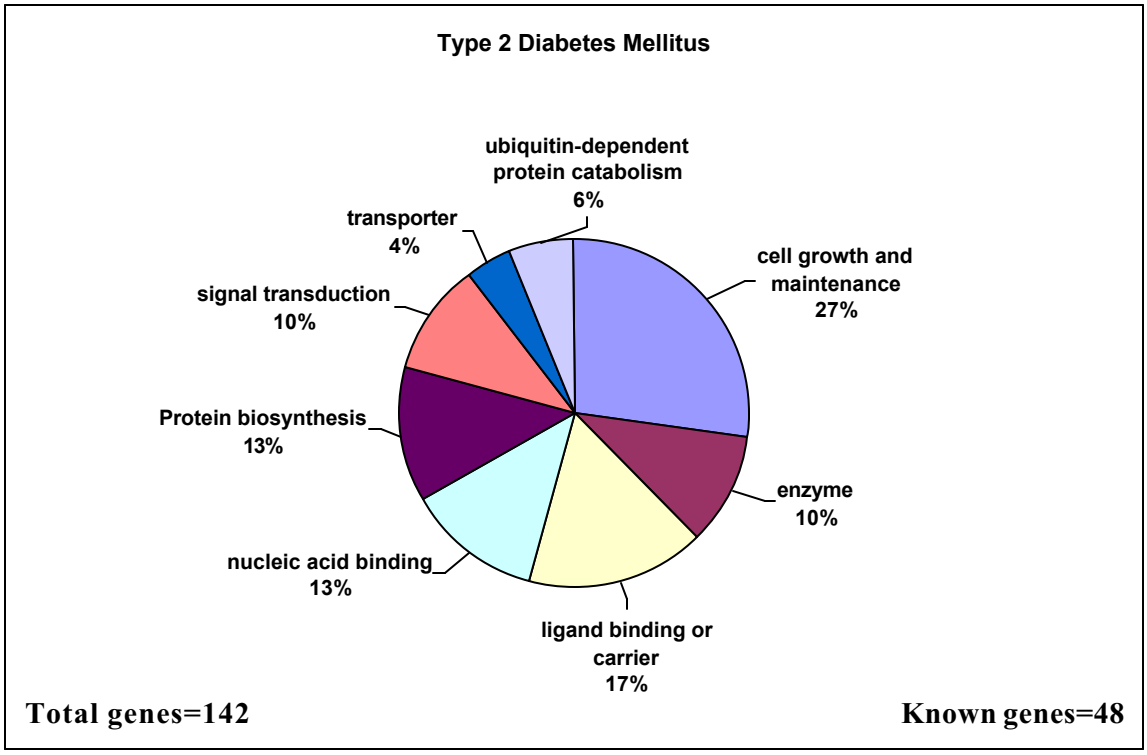
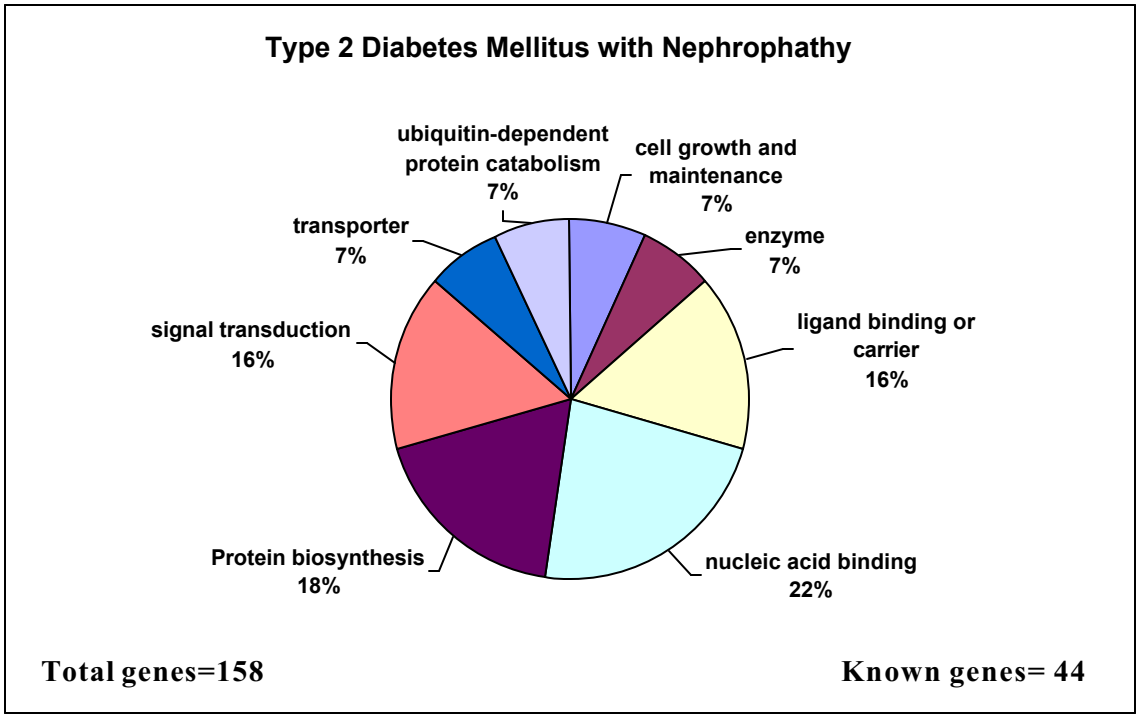


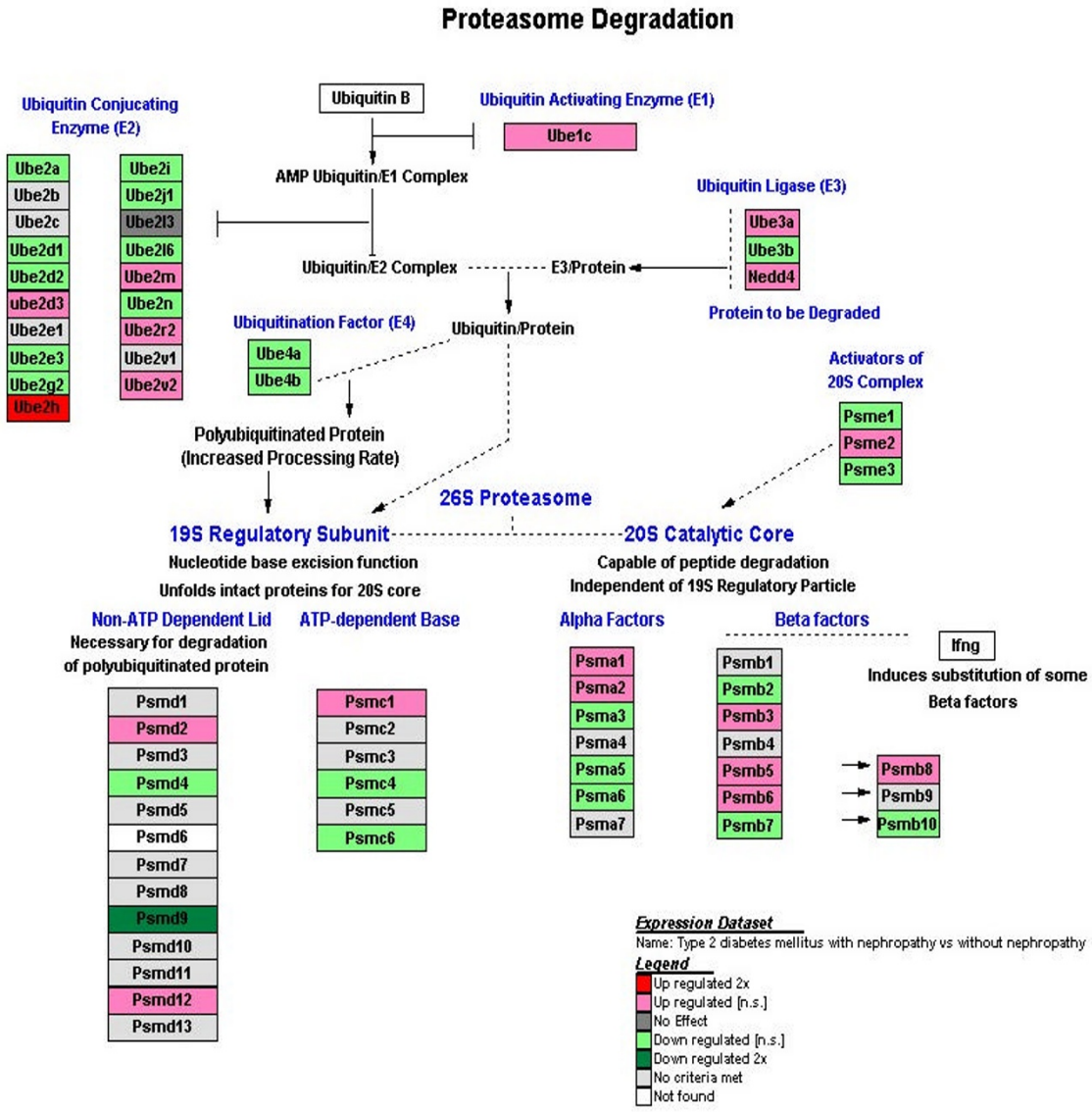
Figure 2b. Functional classes of regulated genes in type 2 diabetes mellitus with nephropathy



All named genes with functional annotations in the Unigene database were categorized by broad functional class and broad process class

Figure 3. Regulation of Proteasome Pathway in type 2 diabetes mellitus with nephropathy or without nephropathy

The gene expression of components of the ubiquitin-proteasome degradation pathway in type 2 diabetes mellitus with nephropathy or without nephropathy. Color scale represents relative fold change of type 2 diabetes mellitus with nephropathy compared to diabetes without nephropathy. Dark red = T2DN/T2D $\geq$ 2; Light red = T2DN/T2D > 1; Dark green = T2DN/T2D $\leq$ -2; Light Green = T2DN/T2D < -1. The GenMAPP-derived proteasome pathway was modified utilizing information from Glickman and Ciechanover and updated with gene symbols from Locuslink.



T2D: type 2 diabetes mellitus without nephropathy
T2DN: type 2 diabetes mellitus with nephropathy