Gene set enrichment analysis of pathways and transcription factors associated with diabetic retinopathy using a microarray dataset

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Abstract. Diabetic retinopathy (DR) is a serious microvascular complication of diabetes, which causes visual disability and blindness. Several studies have used gene expression profiling of DR to identify the key genes involved in this process; however, few studies have focused on the associated pathways and transcription factors (TFs), or on the co-expression patterns at the multiple pathways level. In this study, we employed a microarray dataset from the public database library of the Gene Expression Omnibus (GEO) associated with DR and applied gene set enrichment analysis (GSEA) to this dataset and performed candidate TF selection. As a result, 10 upregulated pathways, including the type I diabetes mellitus and peroxisome proliferator-activated receptor (PPAR) signaling pathways, as well as 59 downregulated pathways, including the ErbB signaling pathway and the mammalian target of rapamycin (mTOR) signaling pathway, were identified as DR-related pathways. The majority of these pathways have been previously identified, but some were novel. Finally, co-expression networks of related pathways were constructed using the significant core genes and TFs, such as PPARγ and SMAD4. The results of our study may enhance our understanding of the molecular mechanisms associated DR at the genome-wide level.

Introduction

Diabetic retinopathy (DR), a specific microvascular complication of diabetes, is the most common cause of visual disability and blindness. The prevalence of DR increases with the duration of diabetes (1), and nearly 99% of patients with type 1 diabetes and 60% with type 2 have some degree of DR after 20 years (1,2). DR can be classified into 2 stages: non-proliferative and proliferative. The earliest visible signs of non-proliferative DR are microaneurysms, hemorrhages, hard exudates, cotton wool spots, intraretinal microvascular abnormalities and venous beading. The more severe state of proliferative DR (PDR) is characterized by the growth of new blood vessels on the surface of the retina or the optic disc, which are prone to hemorrhaging. Finally, visual impairment results in vitreous hemorrhage, subsequent fibrosis and tractional retinal detachment (3,4). Although the pathogenesis of DR has not yet been fully elucidated, the pathogenesis of diabetes is believed to be multifactorial, with genetic risk factors playing a fundamental role. However, several factors, including hyperglycemia, aldose reductase, advanced glycation end products (AGEs) and cytokines, such as vascular endothelial growth factor (VEGF) have been implicated in the disease pathogenesis (5).

Despite DR being a common complication of diabetes, little is known about the underlying molecular mechanisms. In recent years, the complex association that exists between the most relevant contributors to the onset and progression of DR, such as AGEs, oxidative stress, inflammation and angiogenesis have been elucidated and analyzed, particularly via whole-genome expression analyses using cells and animal models (6,7). Based on published studies, several systems, pathways and processes have been strongly implicated in DR; these include the renin-angiotensin system, the polyol pathway, non-enzymatic glycation, endothelial dysfunction, the maintenance of vascular tone, extracellular matrix remodeling and angiogenesis, which is dysregulated in diabetes and leads to the proliferation of new, fragile retinal capillaries and culminates in PDR (8,9). A host of genes involved in these pathways/processes have been treated as potential candidate genes. These genes include angiotensin I-converting enzyme (ACE), angiotensin II type 1 receptor (AGTR1), angiotensinogen (AGT), VEGF, aldose reductase (AR2), receptor for advanced glycation end products (RAGE), glucose trans-

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porter 1 (GLUT1), inducible nitric oxide synthase (NOS2A), constitutive nitric oxide synthase (NOS3), transforming growth factor-β (TGF-β), endothelin isoforms and their cellular receptors, amongst others (10-19). However, to the best of our knowledge, few studies to date have focused on the associated pathways and transcription factors (TFs), or on the co-expression patterns at the multiple pathways level.

In the present study, we employed a microarray dataset of genome-wide gene expression profiling from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) (20,21), which is associated with DR. The most well-known method of gene set enrichment analysis (GSEA) was used to analyze the genomic data in order to uncover the regulatory mechanisms of retinopathy (damage to the retina) caused by diabetic complications at the multiple pathways level. GSEA is widely used to analyze gene expression profiles, particularly to identify pre-defined gene sets which exhibit significant differences in expression between samples from the control and treatment groups (22-24). The goal of GSEA is to determine other interesting categories (pathways) in which the constituent genes exhibit coordinated changes in expression under the given experimental conditions, other than in the form of sets of differentially expressed genes (DEGs). One of the advantages of GSEA is that it has the ability to highlight genes that are weakly connected to the phenotype through pathway analysis, something which may be difficult to detect using classical univariate statistics (22).

Materials and methods

Microarray data collection and pre-processing. We searched the GEO database (www.ncbi.nlm.nih.gov/geo/) for gene expression profiling studies associated with DR. Data were included in our re-analysis if they met the following conditions: i) the data were genome-wide; ii) a comparison was conducted between DR samples and control (CT) samples; and iii) complete microarray raw or normalized data were available. Finally, we chose the GSE12610 dataset for our re-analysis, which was contributed by Liang et al. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12610). In this dataset, a total of 5 RNA samples extracted from retinas were examined for RNA quality and then hybridized to 2 different GeneChip® Mouse Genome 430 2.0 arrays (technical replicates; Affymetrix, Santa Clara, CA, USA). There were 3 biological replicates for DR (the samples from GSM315892 to GSM315894, designated as DR-1, DR-2 and DR-3) and 2 for CT (the samples GSM315895 and GSM315896, designated as CT-1 and CT-2).

In order to determine the influence of pre-processing on the comparison, data pre-processing was performed using software packages developed in version 2.6.0 of Bioconductor and R version 2.10.1. Each Affymetrix dataset was background adjusted, normalized, and log2 probe-set intensities were calculated using the Robust Multichip Average (RMA) algorithm from the affy package (25).

GSEA. Our GSEA of pathways and genes was performed using the Category package in version 2.6.0 of Bioconductor (26). The goal of GSEA is to determine whether the members of a gene set S are randomly distributed throughout the entire reference gene list L or are primarily found at the top or bottom. One of the advantages of GSEA is its relative robustness in the face of noise and outliers in the data. In our analysis, the gene sets represented by <10 genes were excluded. The t-statistic mean of the genes was computed in each Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Using a permutation test with 1,000 repetitions, the cut-off of significance level P-values was chosen as 0.05 for the significant pathways associated with DR. Accordingly, the significant pathways and genes were then identified by comparing the samples with DR and those with no DR. The following classification of identified pathways was based on the pathway maps br08901 of BRITF Functional Hierarchies in the KEGG database (http://www.genome.jp/kegg-bin/get_htext?br08901.kee). The annotation of significant genes in each pathway was performed using the bioMartT package, BioMart v 0.8 rc3 (version of 0.8 release candidate 3; http://www.biomart.org/). Next, clustering of groups and genes was performed based on the expression of the identified genes in each significant pathway, using the hierarchical clustering method and Pearson’s correlation co-efficient.

Regulatory elements (REs) and TFs of co-regulated genes. We used a web server known as the DiRE (distant regulatory elements of co-expressed genes, http://dire.dcode.org/), which uses the Enhancer Identification (EI) method, to predict common REs for our input genes that have co-function in each identified, significantly related pathway (27). It predicts function-specific REs that consist of clusters of specifically associated transcription factor binding sites (TFBSs), and it also scores the association of individual TFs with the biological function shared by the group of input genes. We selected a random set of 5,000 genes in the genome of Mus musculus 9 (mm9) as the background genes. As a result, our predicted TFs have two major parameters, including TF occurrence (the percentage of candidate REs containing a conserved binding site for a particular TF) and TF importance (the product of TF occurrence and TF weight). From our candidate associated TFs with input gene sets, we selected the cut-off value of TF importance as >0.05.

Results and Discussion

Identification of significant pathways associated with DR. Compared to the approach of DEGs, the strategy of GSEA that we used in this study is likely to be more powerful than conventional single-gene methods in the study of complex diseases, in which many genes make subtle contributions. According to our GSEA of the dataset of 5 samples, achieved by comparing the DR to the CT samples, there were 69 significant pathways associated with DR, whose P-values were <0.05, including 10 upregulated and 59 downregulated pathways. The coregulated pathways network is highlighted in Fig. 1 (red text indicates upregulated pathways, and green text indicates downregulated pathways). Furthermore, the details of significant genes in these 69 pathways related to DR are available upon request, as is the information on probe set ID and gene symbol. Among these 69 pathways associated with DR, the samples were classified and divided into DR and CT groups by clustering. For example, based on the expression of 86 significant genes whose significance level P-value was <0.05 in the downregulated ErbB signaling pathway, which may be clustered into 7 groups of gene
sets (Fig. 2; group A-G), 5 samples were clustered into 2 groups, with DR-1, DR-2 and DR-3 in one group and CT-1 and CT-2 in the other group. Similarly, in the downregulated mammalian target of rapamycin (mTOR) pathway, 5 samples were also grouped, as CT-1 and CT-2 in the CT group and DR-1, DR-2 and DR-3 in the DR group (Fig. 3). The 52 genes involved in the mTOR signaling pathway may also be clustered into 4 groups of gene sets (Fig. 3, group A-D). Moreover, in the upregulated peroxisome proliferator-activated receptor (PPAR) pathway, 5 samples were also clustered into 2 groups, with CT-1 and CT-2 in one group and DR-1, DR-2 and DR-3 in the other group (Fig. 4). Furthermore, 71 genes were involved in the PPAR signaling pathway associated with DR, which may be clustered into 6 groups of gene sets (Fig. 4, group A-F). Moreover, based on the KEGG pathway maps in the KEGG database (http://www.genome.jp/kegg/), the 69 significant pathways could be mapped into 6 functional classes: cellular processes, environmental information processing, genetic information processing, human diseases, metabolism and organ systems. The details of the pathways involved in each class are described in Tables I-V.

In the functional class of cellular processes, there were 6 significantly downregulated pathways related to DR (Table I). These pathways were involved in cell communication, cell motility, and transport and catabolism. Of these pathways, the tight junction one was the most significant, and was classified as belonging to the functional group of cell communication. As is well known, the breakdown of the blood-retinal barrier (BRB) is one of the most important characteristics of DR and is largely attributed to the disruption of endothelial tight junction. X-box binding protein 1 (XBP1), which is a major transcription factor activated by ER stress, plays an important role in maintaining endothelial barrier function (28). The activation of XBP1 protects against ER stress-induced tight junction damage.

There were 7 significantly downregulated pathways in the functional class of environmental information processing, 5 significantly downregulated pathways in the functional class of genetic information processing, and only 2 significantly upregulated pathways in the functional class of genetic information processing related to DR (Table II). The ATP-binding cassette (ABC) transporters pathway was associated with the function of membrane transport, and the extracellular matrix (ECM)-receptor interaction pathway was associated with the function of the signaling of molecules and interaction. The environmental information processing pathways, such as the ErbB, mTOR, VEGF and Wnt signaling pathways, and the phosphatidylinositol signaling system were related to the functions of signal transduction. The genetic information processing pathways, such as soluble N-ethylmaleimidesensitive attachment protein receptor (SNARE) interactions in vesicular transport and proteasome were related to the functions of folding, sorting and degradation. The genetic information processing pathways of homologous recombination and DNA replication were related to the functions of replication and repair, and. The genetic information processing pathways of aminoacyl-tRNA biosynthesis and ribosome were related to the functions of translation, and the RNA polymerase pathway

![Diagram of coregulated pathways network related to diabetic retinopathy (DR)](image-url)
was transcription-related. Of these aforementioned pathways, the ErbB and mTOR signaling pathways were the most significant in this class (the functional class of environmental and genetic information processing). The epidermal growth factor receptor (EGFR), also known as ErbB1/human epidermal growth factor receptor 1 (HER1), is a member of the ErbB family of receptor tyrosine kinases which also includes ErbB2 (Neu, HER2), ErbB3 (HER3) and ErbB4 (HER4). It was recently observed that hyperglycemia perturbs the EGFR-PI3K-AKT and extracellular signal-regulated kinase (ERK) signaling pathways in normal and healing corneas and that increased levels of cellular apoptosis and decreased cell proliferation may be contributing factors in the impairment of corneal epithelial wound healing in diabetic corneas (29,30). Signaling through the mTOR pathway plays a major role in smooth muscle and endothelial cell proliferation in response to hypoxia (31). There have been signs that the inhibition of the PI3K/Akt/mTOR pathway may have beneficial therapeutic effects in the management of PDR, which stems from findings that indicate that growth factors known to play major roles in the induction of angiogenesis depend on

Figure 2. Heatmap of the downregulated ErbB signaling pathway was constructed using the expression of 86 significant genes (probe sets), which may be clustered into 7 groups of gene sets based on hierarchical clustering with Pearson's correlation co-efficient (group A-G). Five samples were clustered into 2 groups, diabetic retinopathy (DR)-1, DR-2 and DR-3 in one group and control (CT)-1 and CT-2 in the other.
PI3K/Akt/mTOR for prolonging the cell survival signals that are operating in pathological angiogenesis (32).

Thirteen significantly associated pathways were classified and assigned to the functional class of human diseases, including 2 upregulated endocrine and metabolic diseases related pathways, 1 upregulated immune diseases related pathways, 3 downregulated cardiovascular diseases related pathways, 1 downregulated infectious diseases: bacterial related pathways, 4 downregulated infectious diseases: parasitic related pathways and 2 downregulated cancer related pathways (Table III). Of these, the pathway of type I diabetes mellitus was one of the most significant endocrine and metabolic diseases-related pathways. The prevalence of DR increases with the duration of diabetes. After 20 years of diabetes, nearly all patients with type I diabetes and >60% of patients with type II diabetes have some degree of retinopathy (33).

In the functional class of metabolism, there were 14 downregulated and 4 upregulated significant pathways associated...
with DR (Table IV). These were involved in 7 different types of metabolism, including carbohydrate metabolism, amino acid metabolism, nucleotide metabolism, lipid metabolism, metabolism of co-factors and vitamins, metabolism of terpenoids and polyketides, and glycan biosynthesis and metabolism. Of these, the glycolysis/gluconeogenesis pathway was one of the most significant pathways, which was classified and assigned to the functional group of carbohydrate metabolism. Protein kinase C (PKC) is a serine/threonine kinase, which is involved in signal transduction events with regard to specific hormonal, neuronal and growth factor stimuli. Hyperglycaemia leads to an increase in glucose flux through the glycolysis pathway, which in turn increases the de novo synthesis of diacylglycerol (DAG), the key activator of PKC in physiology (34). PKC is a molecule which plays an important role in the regulation of numerous physiological processes, whose upregulation contributes to the pathogenesis of DR.

In the last functional class of organismal systems, there was 1 significantly upregulated and 17 significantly downregulated pathways associated with DR (Table V). These were involved in the endocrine system, development, and the circulatory, excretory, digestive, nervous and immune systems. Of these, the PPAR signaling pathway was one of the most associated pathways, and was classified and assigned to the functional group of the endocrine system. PPARs are ligand-activated TFs (members of the nuclear receptor family) which offer promising

Figure 4. Heatmap of the upregulated peroxisome proliferator-activated receptor (PPAR) signaling pathway was constructed using the expression of 71 significant genes (probe sets), which may be clustered into 6 groups of gene sets based on hierarchical clustering with Pearson's correlation coefficient (group A-F). Five samples were clustered into 2 groups, with control (CT)-1 and CT-2 in one group and diabetic retinopathy (DR)-1, DR-2 and DR-3 in the other group.
Table I. Significant pathways associated with DR in the functional class of cellular processes.

| Pathways                                         | Map B                          | No. of genes | No. of TFs |
|--------------------------------------------------|--------------------------------|--------------|------------|
| 04142: Lysosome                                   | Transport and catabolism        | 119          | 28         |
| 04144: Endocytosis                                | Transport and catabolism        | 199          | 22         |
| 04145: Phagosome                                  | Transport and catabolism        | 140          | 22         |
| 04530: Tight junction                             | Cell communication              | 127          | 34         |
| 04810: Regulation of actin cytoskeleton           | Cell motility                   | 206          | 26         |
| 05142: Chagas disease (American trypanosomiasis)  |                                 | 99           | 28         |

DR, diabetic retinopathy; TFs, transcription factors.

Table II. Significant pathways associated with DR in the functional class of environmental and genetic information processing.

| Pathways                                         | Map B                          | No. of genes | No. of TFs |
|--------------------------------------------------|--------------------------------|--------------|------------|
| 02010: ABC transporters                           | Membrane transport             | 44           | 25         |
| 04512: ECM-receptor interaction                   | Signaling molecules and interaction | 83         | 34         |
| 04102: ErbB signaling pathway                     | Signal transduction             | 86           | 40         |
| 04150: mTOR signaling pathway                     | Signal transduction             | 52           | 30         |
| 04370: VEGF signaling pathway                     | Signal transduction             | 74           | 25         |
| 04310: Wnt signaling pathway                      | Signal transduction             | 145          | 30         |
| 04070: Phosphatidylinositol signaling system      | Signal transduction             | 73           | 26         |
| 04130: SNARE interactions in vesicular transport | Folding, sorting and degradation | 35         | 6          |
| 03050: Proteasome                                 | Folding, sorting and degradation | 44         | 20         |
| 03440: Homologous recombination                   | Replication and repair          | 27           | 17         |
| 03030: DNA replication                            | Replication and repair          | 35           | 23         |
| 00970: Aminoacyl-tRNA biosynthesis                | Translation                     | 41           | 7          |
| 03010: Ribosome                                | Translation                     | 53           | 19         |
| 03020: RNA polymerase                            | Translation                     | 24           | 29         |

aSignificantly upregulated pathways associated with DR. DR, diabetic retinopathy; TFs, transcription factors; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; ABC, ATP-binding cassette; mTOR, mammalian target of rapamycin; SNARE, soluble N-ethylmaleimide-sensitive attachment protein receptor.

Table III. Significant pathways associated with DR in the functional class of human diseases.

| Pathways                                         | Map B                          | No. of genes | No. of TFs |
|--------------------------------------------------|--------------------------------|--------------|------------|
| 04950: Maturity onset diabetes of the young       | Endocrine and metabolic diseases | 25           | 25         |
| 04940: Type I diabetes mellitus                   | Endocrine and metabolic diseases | 40           | 30         |
| 05330: Allograft rejection                        | Immune diseases                | 33           | 22         |
| 05416: Viral myocarditis                          | Cardiovascular diseases        | 65           | 21         |
| 05410: Hypertrophic cardiomyopathy (HCM)          | Cardiovascular diseases        | 81           | 32         |
| 05412: Arrhythmogenic right ventricular cardiomyopathy (ARVC) | Cardiovascular diseases | 73           | 43         |
| 05100: Bacterial invasion of epithelial cells     | Infectious diseases: bacterial | 66           | 29         |
| 05140: Leishmaniasis                              | Infectious diseases: parasitic | 62           | 20         |
| 05143: African trypanosomiasis                    | Infectious diseases: parasitic | 30           | 19         |
| 05146: Amoebiasis                                 | Infectious diseases: parasitic | 106          | 26         |
| 05145: Toxoplasmosis                              | Infectious diseases: parasitic | 123          | 19         |
| 05211: Renal cell carcinoma                       | Cancers                        | 70           | 30         |
| 05212: Pancreatic cancer                          | Cancers                        | 69           | 25         |

DR, diabetic retinopathy; TFs, transcription factors.
targets for the development of novel, efficient treatments for several metabolic disorders. An indication suggesting that antidiabetic thiazolidinediones and adipogenic prostanoids are ligands of one of the PPARs reveals a novel signaling pathway.

Table V. Significant pathways associated with DR in the functional class of organismal systems.

| Pathways | Map B | No. of genes | No. of TFs |
|----------|-------|--------------|------------|
| 03320: PPAR signaling pathway\(^a\) | Endocrine system | 71 | 25 |
| 04910: Insulin signaling pathway | Endocrine system | 128 | 27 |
| 04916: Melanogenesis | Endocrine system | 95 | 40 |
| 04920: Adipocytokine signaling pathway | Endocrine system | 67 | 19 |
| 04320: Dorso-ventral axis formation | Development | 22 | 24 |
| 04360: Axon guidance | Development | 129 | 28 |
| 04380: Osteoclast differentiation | Development | 112 | 34 |
| 04270: Vascular smooth muscle contraction | Circulatory system | 110 | 32 |
| 04960: Aldosterone-regulated sodium reabsorption | Excretory system | 44 | 33 |
| 04962: Vasopressin-regulated water reabsorption | Excretory system | 43 | 27 |
| 04970: Salivary secretion | Digestive system | 70 | 41 |
| 04972: Pancreatic secretion | Digestive system | 99 | 44 |
| 04720: Long-term potentiation | Nervous system | 63 | 27 |
| 04730: Long-term depression | Nervous system | 68 | 22 |
| 04660: T cell receptor signaling pathway | Immune system | 106 | 33 |
| 04664: Fc epsilon RI signaling pathway | Immune system | 77 | 28 |
| 04666: Fc gamma R-mediated phagocytosis | Immune system | 86 | 39 |
| 04670: Leukocyte transendothelial migration | Immune system | 115 | 26 |

\(^a\)Significantly upregulated pathways associated with DR. DR, diabetic retinopathy; TFs, transcription factors; PPAR, peroxisome proliferator-activated receptor.
that directly links these compounds to processes involved in glucose homeostasis and lipid metabolism, including adipocyte differentiation (35).

**Candidate TF selection associated with DR.** To predict TFs potentially involved in the regulation of DR, we performed an analysis of TFBSs and predicted TFs using the significant genes in each identified pathway. Based on the cut-off value of TF importance, we identified the candidate TFs related to DR with potential target genes which are co-regulated in each of the above 69 pathways. The details are available upon request. As a result, 2 protein families, PPARs and SMADs, including members PPARα, PPARγ, PPAR_DR1, SMAD, SMAD3 and SMAD4, were predicted as candidate TFs in the majority of the identified pathways, particularly the downregulated pathways. PPARs are ligand-activated nuclear TFs that control gene expression by binding to specific response elements (PPREs) within promoters. They play a critical physiological role as lipid sensors and regulators of lipid metabolism (36). More potent synthetic PPAR ligands, including the fibrates and thiazolidinediones, have proven effective in the treatment of dyslipidemia and diabetes (35). The powerful therapeutic ability of PPARα and PPARγ agonists to favorably influence systemic lipid levels, glucose homeostasis, and the risk of atherosclerosis (in the case of PPARα activation in humans) have been demonstrated (37). PPARγ plays a vitally important role in the pathogenesis of DR by inhibiting retinal leukostasis and leakage in response to diabetes (38). Fenofibrate, a PPARα agonist, has demonstrated robust protective effects against DR in diabetic patients (39). Our data also support the hypothesis that PPARα and PPARγ may be important therapeutic targets for the management of DR.

The TGF-β signal is predominantly transduced by a family of TFs, the Smad proteins (40). After binding the TGF-β ligand from outside the cell surface, the type II receptor activates the type I receptor kinase, and this is followed by the phosphorylation of receptor-regulated Smads (R-Smads), Smad2 and Smad3. After associating with a common-partner Smad (co-Smad), or Smad4, the Smad complex translocates to the nucleus where they bind to specific response elements (PPREs) for the management of diabetic retinopathy: Aa systematic review. JAMA 298: 902-916, 2007. After binding the TGF-β ligand from outside the cell surface, the type II receptor activates the type I receptor kinase, and this is followed by the phosphorylation of receptor-regulated Smads (R-Smads), Smad2 and Smad3. After associating with a common-partner Smad (co-Smad), or Smad4, the Smad complex translocates to the nucleus where they bind to specific response elements (PPREs) for the management of diabetic retinopathy: Aa systematic review. JAMA 298: 902-916, 2007.

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