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Gene Expression Profile in Leucocytes of Type 2 Diabetic Subjects

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Abstract

Communication between insulin target tissues and beta-cells initiate compensatory responses which increase insulin production. Correlated changes, in gene expression between tissues, can provide evidence for such intercellular communication. We profiled gene expression studies in Type 2 diabetic subjects to unveil the mechanistic factors involved. Our results showed that genes involved in carbohydrate, lipid and amino acid metabolism pathways, glycan of biosynthesis, metabolism of cofactors and vitamin pathways, ubiquitin-mediated proteolysis, signal transduction pathways, neuroactive ligand receptor interaction were upregulated in diabetes compared to healthy subjects. In contrast, genes involved in cell adhesion, cytokine-cytokine receptor interaction, insulin signaling, PPAR signaling pathways were downregulated in subjects with type 2 diabetes mellitus (T2DM). β_2-microglobulin, a MHC class I molecule was strongly downregulated in diabetic subjects. Further, genes involved in inflammatory pathway are differentially expressed in subjects with T2DM. Hence, it was evident that genes concerned with pathways of carbohydrate, lipid and amino acid metabolism, neuronal function and inflammation play a significant role in the pathobiology of T2DM.

Key words: Gene expression profile, leucocytes, PCR array, Type 2 diabetes mellitus

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Introduction

Type 2 diabetes mellitus (T2DM) is a disorder that involves an increased demand for insulin brought about by insulin resistance, together with a failure to compensate with sufficient insulin production. Although insulin resistance occurs in most obese individuals, diabetes is generally forestalled through compensation with increased insulin. This increase in insulin occurs through an expansion of beta-cell mass and/or increased insulin secretion by individual beta-cells. Failure to compensate for insulin resistance leads to T2DM.

T2DM is one of the most challenging health problems in many developing and industrialized countries [1]. The exact cause of T2DM, which affects millions of people all over the world, is not known. One of the foremost challenges we face is to account mechanistically for not only the definition of hyperglycemia, but also for the myriad of other biochemical and physiological abnormalities, which are characteristics of this disease. The abnormalities include central obesity, hypertension, accelerated atherosclerosis, hypertriglyceridemia and low serum concentrations of high density lipoproteins [2,3].

One way to understand the pathophysiology of diabetes is to examine the coordinated changes in gene expression in T2DM. In each case, there are groups of genes that undergo changes in expression in a highly correlated fashion. Genomic approaches to determine differential expression profiles utilizing serial analysis of gene expression (SAGE) [4] and DNA microarrays [5] 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 [5].

Systemically circulating peripheral blood mononuclear cells (PBMCs) are considered unique tissue affected by

the host condition and may reflect oxidative stress caused by high levels of glucose, insulin, free fatty acids, and tissue-derived circulating bioactive mediators. To verify the hypothesis that the gene expression of PBMCs changes in response to diabetic circumstances, we comprehensively compared global gene expression profiles of PBMCs between patients with and without T2DM by using Reverse Transcription Quantitative Real Time PCR array technology. Even though there is extensive literature relating peripheral blood cells to diabetic complications, the association of gene expression changes in PBMCs in T2DM is largely unknown. In this study, we examined gene expression profiles of peripheral blood cells in T2DM to identify potential gene signatures.

Material and Methods

Study subjects

The study population was composed of six type 2 diabetics and three normal subjects from the mixed races of Malaysian population. Patients were in the age group 30-65 years. .The clinical characteristics of the study subjects were recorded.

Anthropometric measurements

Anthropometric measurements, like weight and height, were obtained using standardized techniques as detailed elsewhere [6]. Height was measured with a tape to the nearest cm. Weight was measured with traditional spring balance that was kept on a firm horizontal surface. The body mass index (BMI) was calculated using the formula

$$\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height (m}^2\text{)}}$$

Laboratory studies

After an overnight fast, venous blood samples were withdrawn from each patient in sterile EDTA tubes. Serum samples were assayed for plasma glucose, HbA1C, total cholesterol, triglycerides and HDL cholesterol.

Processing of blood samples

Blood was collected in EDTA tubes. PBMCs were isolated using Ficoll gradients within 4h of each blood draw; if not processed immediately, cells were lysed in RLT buffer containing β-mercaptoethanol and stored at minus 80°C.

Isolation of RNA from PBMCs

Mononuclear cells were isolated by the Ficoll density-gradient method as previously described [7]. Total RNA was extracted using an RNA isolation kit as per the manufacturer's protocol (SuperArray, catalog number PA-001). RNA integrity was assessed using an Agilent 2100 bio-analyzer (Agilent, Palo Alto, Ca).

PCR arrays

From 0.5–1.0 μg of total RNA, double-stranded cDNA was generated using a cDNA synthesis kit (SA Bioscience, Catalog. Number C-03) that eliminated genomic DNA contamination. cDNA was used as a template for SYBR-green based RT^2-qPCR array using the diabetes array plate (SA Biosciences).

Statistical analysis

All data is expressed as means plus/minus SD. Statistical analysis of the results was performed by student't' test. Values were considered significant when *p was less than* 0.0001.

Results and Discussion

In this study, we demonstrate the possibility that gene expression profile in PBMCs reflects the pathophysiology of T2DM. As T2DM is a multifactorial disorder [8], a comprehensive approach identifying biological pathways or co-regulated gene sets associated with the diseases is required to understand the molecular signature of T2DM [9]. Thus, we screened known human pathways and extracted information on the metabolic pathways that were significantly altered in the PBMCs of the diabetic subjects.

Clinical characteristics of control subjects and patients with diabetes are shown in Table 1. Age, BMI, and levels of fasting plasma glucose, HbA1c, total cholesterol, triglyceride and LDL-cholesterol were significantly increased while HDL-cholesterol was found to be decreased in patients with T2DM.

Table 1: Clinical characteristics of patients with T2DM (n = 6) and normal subjects (n=3)

	Healthy controls	T2DM
M:F	1:2	3:3
Age, Yr	34.7 ± 9.07	53.5 ± 7.4*
BMI, Kg/m2	22.07 ± 1.78	26.7 ± 1.93*
Fasting plasma glucose (mmol/L)	4.5 ± 0.45	11.5 ± 1.36**
HbA1c (%)	5.49 ± 0.13	11.1 ± 0.68**
Total cholesterol (mmol/L)	4.73 ± 0.25	7.03 ± 0.34**
Triglyceride (mmol/L)	1.70 ± 0.26	4.83 ± 0.44**
HDL cholesterol (mmol/L)	2.14 ± 0.17	1.04 ± 0.04**
LDL cholesterol (mmol/L)	2.25 ± 0.15	4.87 ± 0.18**

*Data are expressed as Mean ± SD. *P < 0.01, **P < 0.0001 as compared to control.*

Gene expression profiling is a powerful alternative strategy to test for differences in expression of pre-defined clusters or networks of genes rather than individual genes, thus reducing the number of tested hypotheses. Gene expression analysis using RNA extracted from peripheral blood cells of subjects with T2DM showed significant alterations in the expression of 84 candidate genes (Table 2). For the purpose of analysis, genes were clustered functionally into

1. Receptors, transporters and channels
2. Nuclear receptors
3. Metabolic enzymes
4. Secreted factors
5. Signal Transduction molecules
6. Transcription factors.

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Table 2: Fold change in gene expression in subjects with T2DM over normal subjects

Unigene	Genbank	Position	Symbol	Description	Fold change
				T2D vs C	
Hs.54470	NM_000352	A01	ABCC8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	-1.35
Hs.298469	NM_152831	A02	ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	2.10
Hs.387567	NM_001096	A03	ACLY	ATP citrate lyase	1.45
Hs.2549	NM_000025	A04	ADRB3	Adrenergic, beta-3-, receptor	-1.20
Hs.19383	NM_000029	A05	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	1.48
Hs.631535	NM_001626	A06	AKT2	V-akt murine thymoma viral oncogene homolog 2	-1.35
Hs.130730	NM_000486	A07	AQP2	Aquaporin 2 (collecting duct)	-1.36
Hs.514821	NM_002985	A08	CCL5	Chemokine (C-C motif) ligand 5	1.48
Hs.644637	NM_000648	A09	CCR2	Chemokine (C-C motif) receptor 2	1.66
Hs.591629	NM_006139	A10	CD28	CD28 molecule	-1.70
Hs.512682	NM_001712	A11	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	-1.37
Hs.76171	NM_004364	A12	CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	1.86
Hs.247824	NM_005214	B01	CTLA4	Cytotoxic T-lymphocyte-associated protein 4	-1.28
Hs.417962	NM_057158	B02	DUSP4	Dual specificity phosphatase 4	1.68
Hs.527295	NM_006208	B03	ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	2.11
Hs.494496	NM_000507	B04	FBP1	Fructose-1,6-bisphosphatase 1	1.33
Hs.436448	NM_005251	B05	FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)	-1.50
Hs.632336	NM_005249	B06	FOXG1B	Forkhead box G1B	-1.19
Hs.247700	NM_014009	B07	FOXP3	Forkhead box P3	-1.51
Hs.212293	NM_000151	B08	G6PC	Glucose-6-phosphatase, catalytic subunit	2.11
Hs.461047	NM_000402	B09	G6PD	Glucose-6-phosphate dehydrogenase	-1.34
Hs.516494	NM_002054	B10	GCG	Glucagon	1.66
Hs.208	NM_000160	B11	GCGR	Glucagon receptor	1.36
Hs.1270	NM_000162	B12	GCK	Glucokinase (hexokinase 4, maturity onset diabetes of the young 2)	-1.61
Hs.389103	NM_002062	C01	GLP1R	Glucagon-like peptide 1 receptor	-1.34
Hs.524418	NM_005276	C02	GPD1	Glycerol-3-phosphate dehydrogenase 1 (soluble)	-1.19
Hs.445733	NM_002093	C03	GSK3B	Glycogen synthase kinase 3 beta	1.80
Hs.517581	NM_002133	C04	HMOX1	Heme oxygenase (decycling) 1	1.49
Hs.116462	NM_178849	C05	HNF4A	Hepatocyte nuclear factor 4, alpha	-1.54
Hs.643447	NM_000201	C06	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	1.49
Hs.500546	NM_004969	C07	IDE	Insulin-degrading enzyme	-1.07
Hs.856	NM_000619	C08	IFNG	Interferon, gamma	1.67
Hs.635441	NM_000599	C09	IGFBP5	Insulin-like growth factor binding protein 5	-1.42
Hs.413513	NM_001556	C10	IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	-1.20
Hs.193717	NM_000572	C11	IL10	Interleukin 10	-1.53
Hs.674	NM_002187	C12	IL12B	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation	1.69

Hs.513457	NM_000418	D01	IL4R	Interleukin 4 receptor	1.17
Hs.512234	NM_000600	D02	IL6	Interleukin 6 (interferon, beta 2)	1.17
Hs.523875	NM_001567	D03	INPPL1	Inositol polyphosphate phosphatase-like 1	1.48
Hs.89832	NM_000207	D04	INS	Insulin	-1.56
Hs.465744	NM_000208	D05	INSR	Insulin receptor	-1.69
Hs.32938	NM_000209	D06	PDX1	Pancreatic and duodenal homeobox 1	-1.68
Hs.471508	NM_005544	D07	IRS1	Insulin receptor substrate 1	-1.53
Hs.442344	NM_003749	D08	IRS2	Insulin receptor substrate 2	-1.34
Hs.588289	NM_001315	D09	MAPK14	Mitogen-activated protein kinase 14	1.20
Hs.138211	NM_002750	D10	MAPK8	Mitogen-activated protein kinase 8	1.66
Hs.21160	NM_002395	D11	ME1	Malic enzyme 1, NADP(+)-dependent, cytosolic	-1.82
Hs.72981	NM_002500	D12	NEUROD1	Neurogenic differentiation 1	-1.60
Hs.431926	NM_003998	E01	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	1.48
Hs.653170	NM_000603	E02	NOS3	Nitric oxide synthase 3 (endothelial cell)	-1.51
Hs.298069	NM_005011	E03	NRF1	Nuclear respiratory factor 1	-1.35
Hs.431279	NM_006178	E04	NSF	N-ethylmaleimide-sensitive factor	-1.34
Hs.177766	NM_001618	E05	PARP1	Poly (ADP-ribose) polymerase family, member 1	1.79
Hs.497487	NM_002646	E06	PIK3C2B	Phosphoinositide-3-kinase, class 2, beta polypeptide	-1.20
Hs.518451	NM_005026	E07	PIK3CD	Phosphoinositide-3-kinase, catalytic, delta polypeptide	-1.19
Hs.132225	NM_181504	E08	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	-1.36
Hs.103110	NM_005036	E09	PPARA	Peroxisome proliferative activated receptor, alpha	1.67
Hs.162646	NM_015869	E10	PPARG	Peroxisome proliferator-activated receptor gamma	-1.20
Hs.527078	NM_013261	E11	PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	-1.27
Hs.591261	NM_133263	E12	PPARGC1B	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta	-1.06
Hs.43322	NM_006251	F01	PRKAA1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	-1.27
Hs.549162	NM_016203	F02	PRKAG2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-1.20
Hs.460355	NM_002738	F03	PRKCB1	Protein kinase C, beta 1	1.47
Hs.417549	NM_002827	F04	PTPN1	Protein tyrosine phosphatase, non-receptor type 1	1.78
Hs.282417	NM_002863	F05	PYGL	Phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	1.27
Hs.296169	NM_004578	F06	RAB4A	RAB4A, member RAS oncogene family	-1.51
Hs.283091	NM_020415	F07	RETN	Resistin	1.87
Hs.82848	NM_000655	F08	SELL	Selectin L (lymphocyte adhesion molecule 1)	-1.20
Hs.380691	NM_001042	F09	SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4	-1.21
Hs.511149	NM_003825	F10	SNAP23	Synaptosomal-associated protein, 23kDa	-1.35
Hs.167317	NM_003081	F11	SNAP25	Synaptosomal-associated protein, 25kDa	-1.51
Hs.592123	NM_004176	F12	SREBF1	Sterol regulatory element binding transcription factor 1	-1.44
Hs.83734	NM_004604	G01	STX4	Syntaxin 4	-1.07
Hs.288229	NM_003165	G02	STXBP1	Syntaxin binding protein 1	-1.34
Hs.534352	NM_006949	G03	STXBP2	Syntaxin binding protein 2	-1.51
Hs.191144	NM_006481	G04	TCF2	Transcription factor 2, hepatic; LF-B3; variant hepatic nuclear factor	-1.21
.Hs.645227	NM_000660	G05	TGFB1	Transforming growth factor, beta 1	1.48
Hs.94367	NM_003317	G06	TITF1	Thyroid transcription factor 1	-1.52
Hs.241570	NM_000594	G07	TNF	Tumor necrosis factor (TNF superfamily, member 2)	1.32
Hs.279594	NM_001065	G08	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	1.66
Hs.516826	NM_021158	G09	TRIB3	Tribbles homolog 3 (Drosophila)	1.67
Hs.66708	NM_004781	G10	VAMP3	Vesicle-associated membrane protein 3 (cellubrevin)	-1.32
Hs.653207	NM_194434	G11	VAPA	(vesicle-associated membrane protein)-associated protein A, 33kDa	-1.35
Hs.73793	NM_003376	G12	VEGFA	Vascular endothelial growth factor A	1.39

conditions by studying gene expression in blood cells [10,11]. Hence, we used such an approach to delineate similarities and differences in gene expression between normal and type 2 diabetic patients.

Hierarchical clustering and heat map of the genes significantly regulated in diabetes showed distinct gene clusters that are unique to T2DM (Figure 1a and b). This demonstrates that gene expression signatures in PBMCs could potentially provide a mechanism to distinguish diabetic state from the normal state or to explain the pathophysiology of diabetes. Sixty per cent of significantly altered genes in the diabetic group were down regulated while 40% were up regulated when compared with the non-diabetic groups (Figure 2a and b). This relationship emphasizes how strikingly different the pattern of expression is between these two groups.

We found that distinct pathophysiology of patients with type 2 diabetes was reflected in coordinate alterations in the gene expression levels of pathways involving carbohydrate, lipid and amino acid metabolism, insulin biosynthesis, secretion and its signaling mechanisms in the liver, pancreas, skeletal muscle and adipocytes and its co-factors.

Metabolic homeostasis has long been considered a major component in the pathophysiology of diabetes. In PBMCs, the genes encoding enzymes regulating carbohydrate and fat metabolism showed significant differential expression in diabetes.

This shows that insulin regulation of energy homeostasis in PBMCs is distinctly different from other target tissue such as skeletal muscle and pancreas [12].

Genes encoding metabolic enzymes, receptors, substrates and signaling molecules (ENPP1, IDE, NEUROD1, INSR, ABCC8, IRS1, IRS2, INS, PXD-1, SNARE protein complex) that showed significant differential expression (Table 2) are known to play a role in insulin signaling and homeostasis. Protein kinases such as mitogen activated protein kinases (MAPK) were found to be up regulated and AMP-activated protein kinases (AMPK) were down regulated in T2DM but their association with the pathogenesis of diabetes is unclear.

The liver is regarded as one of the central metabolic organs in the body, regulating and maintaining homeostasis. It performs most of the reactions involved in the synthesis and utilization of glucose. Diabetes results in a decrease in glucose utilization, an increase in glucose production,

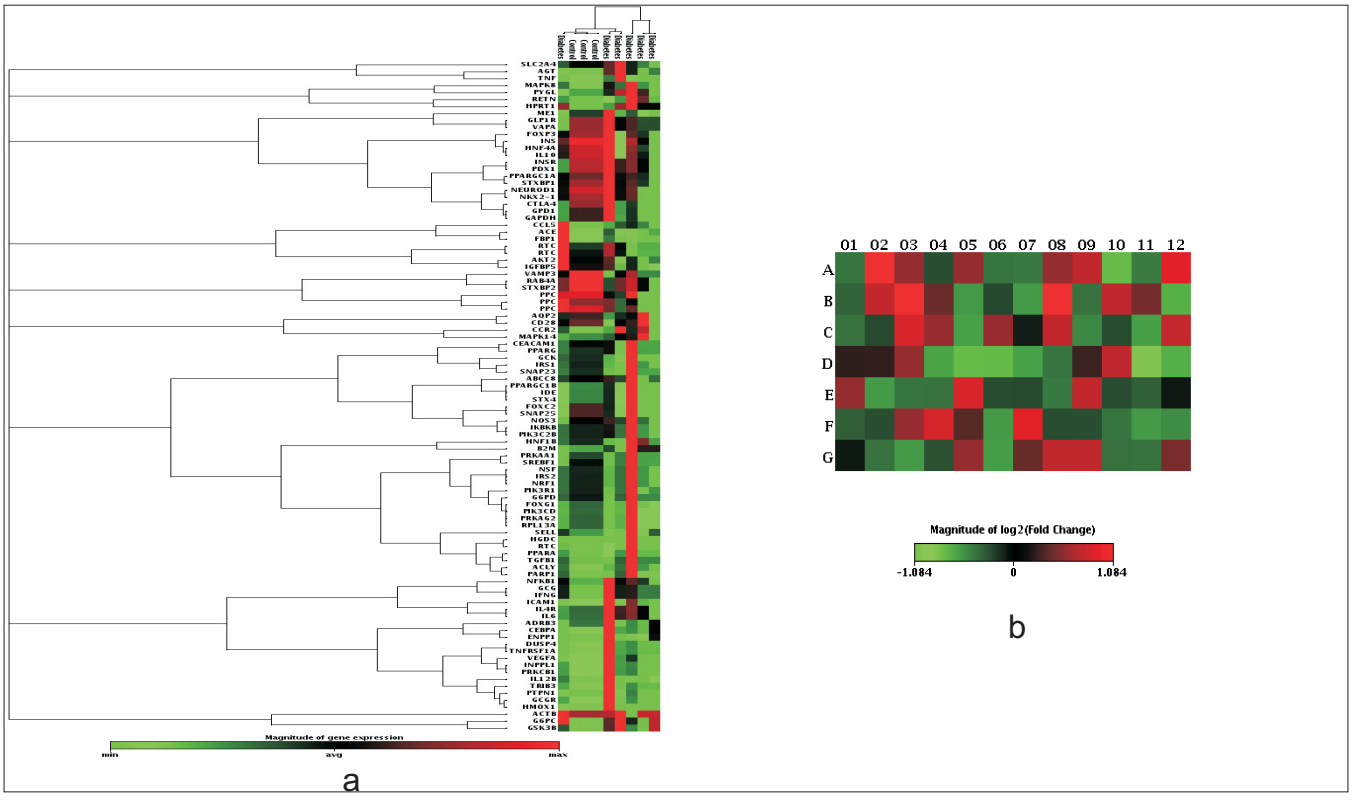

Figure 1: *Hierarchical cluster (a) and Heat map (b) illustration of gene-expression profiles of peripheral-blood mononuclear cell (PBMC) samples from type 2 diabetics (n=6) and healthy controls (n=3). "Heat map," which is the part of the figure containing colors (red, green, and black). The color represents the expression level of the gene. Red represents high expression, while green represents low expression. A01 to G12 refer to positions shown in Table 2*

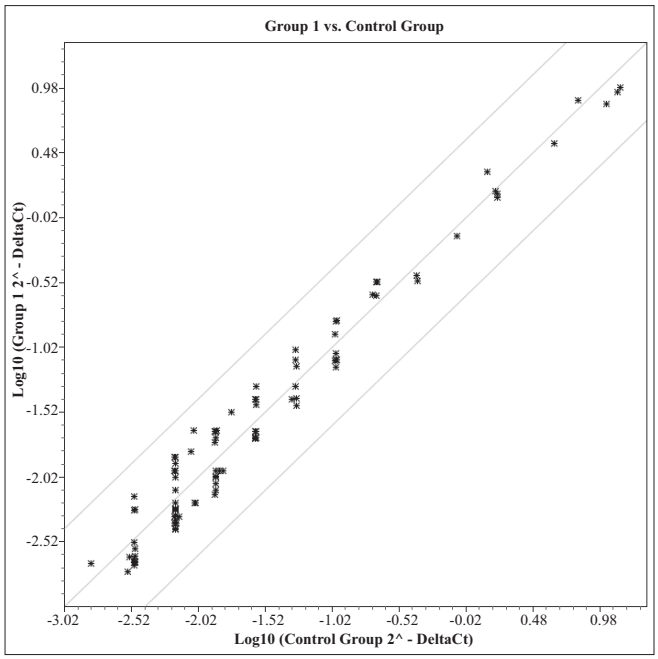

Figure 2 (a)*: Scatter plot analysis of gene expression profiling on T2DM patients demonstrates differential gene expression. The y-axis represents log scores from T2DM patients (group 1) and the x-axis represents log scores from normal controls. Each symbol represents one gene within two-fold cutoff threshold*

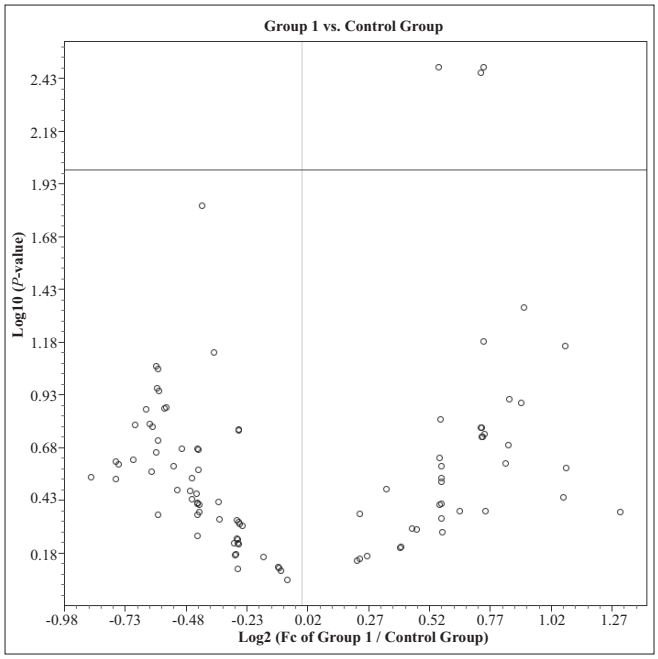

Figure 2 (b): *Volcano plot of log intensities for Control vs.T2DM. Each circle corresponds to one gene represents the average log-ratio (log fold-change) in a two group comparison. The two-fold change method selects as differentially expressed all genes above the line 0.02 (up regulation) and below the line minus 0.02 (down regulation)*

increased angiogenesis, a reduced stress-defence system, and altered mitochondrial oxidative phosphorylation (OXPHOS) in insulin-dependent tissues such as liver [13,9,14]. A marked increase in the hepatic lipid concentration has been observed during diabetes [15-17]. In contrast, the rate of hepatic lipogenesis and related enzymes is decreased. Decreased glycolysis, impeded glycogenesis and increased gluconeogenesis are some of the changes of glucose metabolism in the diabetic liver [18]. Patti *et al*. [19] in their study showed decreased expression of PGC1α coupled with reduced expression of NRF1 and PPARγ eventually result in decreased oxidative phosphorylation and lipid oxidation, accumulation of lipid in skeletal muscle and ultimately diabetes. In our studies on PBMCs, NRF1 and PPARγ were down regulated 1.35- and 1.20-fold, respectively.

Immunoregulation plays a significant role in diabetes and its pathogenesis. Enhanced expression and activation of transcription factor, nuclear factor κB and degradation of IκB triggers activation of genes involved in immune responses such as the pro-inflammaory cytokines (IL-4, TNF-α, IFN-γ). IL-4, TNF-α and IFN-γ expression was up regulated in our experiments, an indication of activation of the pathways of inflammation. An imbalance between Th1 and Th2 cells has been shown to results in decreased activity of CD28 and CTLA4-mediated immunosuppressant and activation of macrophage-mediated inflammation involved in the process of β-cell destruction [20]. In PBMC both CD28 and CTL4 are downregulated and may be indicative of similar processes.

In human eosinophils, intracellular signaling molecules ERK, p38 MAPK and c-Jun N-terminal protein kinase (JNK), together with proinflammatory cytokines IL-6, IL-10, chemokines MCP-1/C-C chemokine receptor-2 (CCR2), CXCL9/monokine induced by interferon-γ (MIG), CCL5/regulated upon activation normal T cell expressed and secreted (RANTES) and CXCL10/IFN-γ inducible protein-10 (IP-10) form a network in orchestrating inflammation in diabetes [21,22]. Our results show upregulation of CCL2, CCL5, IL-6 and MAPKs 8 and 14 in diabetes indicating triggering of proinflammatory pathways.

Regulation of cell adhesion molecules plays an important role in vascular complications in T2DM. Intracellular adhesion molecule-1 (ICAM1) is a member of the immunoglobulin super-family of adhesion molecules. This type I membrane protein mediates leukocyte-endothelial cell adhesion and signal transduction, may play a role in the development of atherosclerosis. In the retina, its increased expression contributes to the microcirculation dysfunction by increasing leucocyte adhesion, aggregation

and migration in retinal vasculature [23]. In PBMC, ICAM1 expression is markedly elevated and this would enhance leucocyte adhesion. Plasma concentrations of ICAM1, in patients with T2DM are more reflective of hyperglycemia than hyperinsulinemia or insulin resistance [24].

In summary, the current study demonstrates that 84 gene transcripts, involved in a variety of functions, were altered in PBMCs of people with T2DM. Results of this study suggest that there are significant differences in the expression of various genes concerned with carbohydrate, lipid, and protein metabolism, ubiquitin-mediated proteolysis, signal transduction pathways, neuroactive ligand-receptor interaction, cell adhesion molecules, cytokine-cytokine receptor interaction, insulin signaling and immune system pathways, oxidative phosphorylation, and PPAR signaling pathways in subjects with T2DM compared to normal.

These alterations in gene transcripts may represent the response to increased circulating insulin levels necessary to maintain normal glucose levels in these patients. Further, studies on important known and novel targets regulated in T2DM 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.

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