DNA microarray analysis of the gene expression profile of kidney tissue in a type 2 diabetic rat model

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Abstract. The aim of this study was to determine differences in the gene expression profile of kidney tissue from type 2 diabetes mellitus (T2D) and control rats using DNA microarray analysis. Total RNA was extracted from the kidney tissue of the T2D and control rats using the original single step method. cDNA retro-transcribed from an equal quantity of mRNA was labeled with Cy5 and Cy3 fluorescence and served as the probes. The mixed probes were hybridized to a DNA microarray. Fluorescence signals were scanned by an ScanArray 4000 laser scanner and further analyzed by QuantArray software. Apoptotic cells were detected *in situ* using the Roche TUNEL assay. Serum glucose, ApoAI, ApoB, ApoA1/ApoB, cholesterol and triglyceride levels were significantly higher in the T2D rats than in the controls, but there were no significant differences in serum insulin. When the kidney tissue was screened using the DNA microarray, differential expression was found for 41 genes. Five genes in the T2D rats were upregulated by 2-fold compared to the control rats, while 36 genes were down-regulated by 0.5-fold. Moreover, in the renal tubular epithelial cells, there was a significantly greater number of TUNEL-positive cells in the T2D group than in the control group. A total of 41 genes are associated with the occurrence and development of T2D and diabetic nephropathy. The present study suggests that examining differences in gene expression profiles is of benefit to the diagnosis, treatment and prevention of T2D, diabetic nephropathy and other T2D complications.

Introduction

Type 2 diabetes mellitus (T2D) is a multi-gene disease caused by a combination of genetic and environmental factors, and has a strong heterogeneity. Diabetic nephropathy (DN), a microvascular complication of T2D, is the most serious and most common chronic complication. Five to ten percent of T2D patients develop DN, and it is a leading cause of mortality in patients with T2D. In Europe, the US and other developed countries, DN has become the main cause of the occurrence of end-stage renal disease (1,2). In China, diabetic kidney disease-induced renal failure accounts for 15% of all renal failure cases. The molecular mechanisms of the causes of DN remain unclear due to their complexity.

In recent years, the application of molecular biology technology has contributed to significant progress in T2D research. An increasing number of DNA microarray methods are currently being applied in the study of the gene expression of T2D. Parallel analysis using a DNA microarray to detect differentially expressed genes in different specimens is a great improvement over traditional methods, in which the expression of only a single or several genes was observable with each test.

In the present study, differences between the gene expression patterns in the kidney tissues of T2D and control rats were investigated in order to explore T2D-associated gene clusters and their role in the occurrence and development of T2DN. This may aid in a comprehesive understanding of the molecular mechanisms of T2DN, and in the identification of molecular markers and target genes for the clinical diagnosis, prevention, prediction of susceptibility and treatment of T2DN and T2D.

Materials and methods

Animals. The Licensing Committee of Zhejiang University approved the experiments undertaken. Two-week-old pathogen-free inbred female SD rats (n=15, ~160 g) were obtained from the Animal Research Center of Zhejiang University. All rats were maintained at the Zhejiang University Animal Care Facility under a 12-h light/dark cycle. The rats were randomly divided into two groups: the T2D model group and the control group. The T2D rats (n=10) were fed a high-lipid and high-glucose diet (10% lard, 20% sucrose, 5% cholesterol and 65% conventional components) for 4 months, and were intraperitoneally injected with 25 mg/kg streptozotocin (STZ; Sigma, USA) (0.25% concentration, pH 4.2, 0.1 mol/l citrate buffer). Only 5 rats developed T2D (fasting plasma glucose >7.8 mmol/l) after 2 weeks of STZ injection. The control

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group rats (n=5) were fed conventional chow and were intraperitoneally injected with citrate buffer.

At 22 weeks of age, the rats were sacrificed by cervical dislocation after being fasted for 4 h. All experiments were carried out without the use of an anesthetic, as diethylether affects serum glucose concentrations. Whole blood and kidney tissues were collected after sacrifice. The kidney tissue was harvested and rapidly frozen in liquid N_2, and all samples were stored at -80˚C until use.

Detection of glucose, insulin, total cholesterol, ApoA1, ApoB and triglyceride levels. Serum insulin was determined using a radioimmunoassay kit (Shanghai Institute of Biological Products). Serum glucose, total cholesterol and triglyceride levels were measured using an automatic biochemical analyzer (Hitachi 7060). ApoA1 and ApoB were detected using immune turbidimetry.

Microarray analysis

Isolation of total RNA. Total RNA from stocked kidney samples was extracted using TRIzol reagent (Invitrogen, USA) and purified using an RNeasy mini kit combined with an RNase-free DNase Digest set (Qiagen, Germany) for the degradation of genomic DNA in the total RNA samples. Total RNA extracted from 5 rats (5 μg each) was then pooled into one sample in order to normalize individual differences, followed by gene expression analysis of the tissue by cDNA microarray (3). Total RNA extracted from the kidney tissue was stored at -80˚C until use.

Microarray preparation. A cDNA microarray with 4096 target cDNA clones was provided by United Gene Ltd. (Shanghai, China). These genes were amplified with PCR using universal primers and purified using a standard method (4). The obtained genes were dissolved in spotting buffer and then spotted on silylated slides (Telechem Inc., USA) by Cartesian 7500 Spotting Robotics (Cartesian Inc., USA). Each target gene was spotted twice, then the slides were hydrated for 2 h and dried for 0.5 h at room temperature (RT). The samples were cross-linked with UV light and treated with 0.2% SDS, H_2O and 0.2% $NaNBH_4$ for 10 min, respectively. The slides were lastly dried under cold conditions.

Probe preparation. The fluorescent-labeled cDNA probes were prepared through retro-transcription according to the method of Wang *et al* (5). The cDNA probes from the normal kidney tissue were labeled with Cy3-dCTP, while those from the T2D rat kidney tissue were labeled with Cy5-dCTP. Two groups of cDNA probes were mixed (Cy3-dCTP control + Cy5-dCTP T2D rats) and precipitated by ethanol, then dissolved in 13 μl hybridization solution.

Hybridization and washing. Probes and the cDNA microarray were denatured in a 95˚C bath for 2 min and 30 sec, respectively, then the probes were added to the cDNA microarray. Hybridization was performed in a sealed chamber at 42˚C for 16-18 h. Subsequently, the probes were washed in turn with solutions of 2X SSC + 0.2% SDS, 0.1X SSC + 0.2% SDS and 0.1X SSC for 10 min each, and then dried at RT for 10 min.

Fluorescent scanning and analysis. The cDNA microarray was read by the Scan Array 4000 laser scanner (Packard Biochip Technologies Inc., USA). The overall intensities of Cy3 and Cy5 were normalized and corrected by a coefficient according to the normalization factor. The acquired image was further analyzed by QuantArray Software with a digital computer to obtain the intensities of the fluorescent signals and the Cy3/Cy5 ratio. Data were gathered for an average of the two repeated spots. A gene was defined as being differentially expressed when i) the PCR results were satisfactory, ii) either the Cy3 or Cy5 signal value was >600, and iii) the absolute value of the Cy5/Cy3 natural logarithm was >0.69 (with a variation in gene expression >2-fold).

TdT-mediated dUTP nick end labeling (TUNEL). The apoptotic cells were detected *in situ* using the Roche TUNEL kit. The TUNEL method was performed to visualize the 3'-OH ends of DNA fragments in apoptotic cells according to the manufacturer's protocol. After xylene dewaxing, the sections were rinsed three times in DW for 5 min and then dipped in methanol containing 0.3% H_2O_2 at RT for 30 min to inhibit endogenous peroxidase activity. After rinsing in PBS three times at RT for 5 min, the sections were treated with proteinase K (Sigma, Germany) at 37˚C for 8 min. After rinsing in PBS three times at RT for 5 min, the sections were soaked in the TdT buffer for 10 min and then incubated at 37˚C for 60 min in a moist chamber with 50 μl of the TdT buffer containing TdT (Roche, Germany). After rinsing in PBS three times at RT for 5 min, the sections were placed in 50 μl FITC (Roche) and then incubated at 37˚C for 40 min. After further rinsing in PBS three times for 5 min, the sections were dipped in DAB (Roche) at RT for 3 min, and the reaction was observed under a microscope. The reaction was terminated with DW, then the nuclei were counterstained with hematoxylin buffer.

Statistical analysis. Statistical analysis was carried out with SPSS 11.0 statistical software. Data are expressed as the mean ± SD. Analysis of differences between the two groups was carried out using the Satterthwaite t-test.

Results

Body weight, serum glucose and insulin levels. The body weight of the T2D model rats administered a high-lipid and high-glucose diet for 4 months was significantly higher than that of the control rats (P=0.0084). There was no difference in serum glucose levels (P=0.8576). After the injection of 25 mg/kg STZ for 2 weeks, hyperglycemia was noted in 5 of the T2D rats. The serum glucose of these hyperglycemic rats was significantly increased (P=0.0023), while their weight was significantly decreased (P=0.018). The 5 hyperglycemic rats ingested more food and water than the control group rats. No similar changes in weight and serum glucose levels were found among the control group rats (P=0.6574 and 0.2056). No significant difference (P=0.994) was found between the serum insulin levels of the T2D and control groups (Table I). The levels of ApoAI, ApoB, ApoA1/ApoB, cholesterol and triglyceride in the T2D rats were significantly higher than those of the control group (Table II).

Purity of total RNA. The total RNA absorbance of A260/A280 was 1.8-2.0, demonstrating that the purity of the total RNA was satisfactory. Total RNA was not significantly degraded,

Table I. Comparison of weight, serum glucose and insulin (mean ± SD) in the T2D model and control groups.

	Weight before injection (g)	Weight after injection (g)	Glucose before injection (mmol/l)	Glucose after injection (mmol/l)	Insulin after injection (μU/ml)
T2D	337.80±21.83	297.20±21.44	4.94±0.56	18.19±7.23	15.36±4.53
Control	285.40±25.40	293.80±31.92	4.38±0.70	5.17±1.08	15.34±4.37
P-value	0.0084	0.8489	0.8576	0.015	0.994

Table II. Comparison of total cholesterol (CHO), ApoA1, ApoB and triglyceride (TG) levels in the T2D model and control groups.

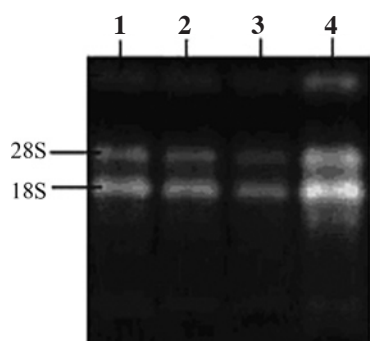|	ApoA1 (mg/dl)	ApoB (mg/dl)	ApoA1/ApoB	CHO (mmol/l)	TG (mmol/l)
T2D	121.46±49.62	265.24±16.21	0.46±0.18	21.12±9.43	1.96±0.82
Control	22.52±2.48	35.80±4.27	0.63±0.02	2.17±0.08	1.34±0.75
P-value	0.0111	0.0115	0.03562	0.0109	0.04253

Figure 1. Gelose electrophoresis of the total RNA extracted from the kidney tissues. Lanes 1 and 2, total RNA of the kidney tissue of the control rats; 3 and 4, total RNA of the kidney tissue of the T2D rats.

as indicated by the presence of 28S and 18S RNA bands in the results of gelose electrophoresis (Fig. 1).

Hybridization signals on the gene chip. A scatter plot was plotted with the Cy3 and Cy5 fluorescent signal values, and displayed a quite disperse distribution pattern. Most of the spots were gathered around the 45° diagonal line, with red spots representing the area where the signal intensities varied between 0.5- and 2-fold compared to the control. Some yellow spots distributed beyond or far from the 45° diagonal line indicated the existence of abnormal gene expression in the diabetic kidney. The signal intensities for the T2D group were 2 times stronger than those of the control.

Scanning analysis and gene expression patterns. cDNA probes labeled with Cy3 for the control and Cy5 for the diabetic kidney tissue groups were hybridized to a DNA microarray. Hybridization results were obtained in parallel by comparison of the sample gene expression patterns demonstrated. In the diabetic kidney tissue, 41 genes were found to have variations in expression >2-fold compared to the control. Among these 41 genes, 15 were not recorded in GenBank. The function of 29 genes remained undetermined. Five genes were up-regulated and 36 genes were down-regulated, as shown in Tables III and IV, respectively.

TUNEL-positive cells. Few TUNEL-positive cells were noted in each visual field in the renal tubules of the control group. In the T2D group, TUNEL-positive cells were scattered or clumped, were mostly located in epithelial cell tissues, and were significantly more numerous as compared to the control group ($P<0.01$) (Table V; Fig. 2).

Discussion

Non-insulin-dependent diabetes mellitus (T2D) is a decompensatory endocrinopathy with a high incidence of 7%. T2D results from a series of molecular changes and is caused by the abnormal expression of diabetes-associated genes (6). Therefore, it is necessary to investigate the kinetic changes in thousands of genes which make up the normal genome, rather than the study of one or several genes. Investigating gene expression may help understand the interrelation of genotype and phenotype. DNA microarray methods may be used to detect differences in gene expression in various specimens by parallel analysis on a large scale using just one procedure (7-16). Therefore, DNA microarray techniques have increasingly been applied to the study of gene expression. Detection of changes in gene expression in diabetes may reveal the cause of pathological changes and may also provide new targets for diagnosis and prevention.

In the present study, the DNA microarray technique was employed to analyze changes in gene expression patterns in a T2DN model. The expression levels of 41 genes in the diabetic kidney were were found to be increased or decreased. The functions of many of the genes were undetermined, or the genes were not yet registered in GenBank. Most of these genes were associated with glucose metabolism, fat metabolism and apoptosis-related enzymes.

Table III. Genes with a differential expression >2 between the diabetic kidney and normal control tissues.

Genbank-ID	UniGene	Definition	Average ratio
X86561	Rn.5500	Rat gene for α-fibrinogen	2.138
AW915104	Rn.6725	EST346395 *Rattus norvegicus* cDNA, 5' end	2.364
BF282390	Rn.9278	EST446893 *Rattus norvegicus* cDNA, 3' end	2.376
BI292167	Rn.19056	UI-R-DN0-civ-c-07-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	2.108
NM_053819	Rn.25754	*Rattus norvegicus* tissue inhibitor of metalloproteinase 1 (Timp-1), mRNA	2.160

Table IV. Genes with a differential expression <0.5 between the diabetic kidney and normal control tissues.

Genbank-ID	UniGene	Definition	Average ratio
AW915650	Rn.17901	EST346954 *Rattus norvegicus* cDNA, 5' end	0.389
BE107033	Rn.20198	UI-R-BS1-ayp-a-04-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.399
J02585	Rn.1023	Rat liver stearyl-CoA desaturase mRNA, complete cds	0.399
AA963985	Rn.73529	UI-R-C0-gs-e-04-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.446
NM_012533	Rn.12780	*Rattus norvegicus* carboxypeptidase B (Cpb), mRNA	0.472
AA997888	Rn.33114	UI-R-C0-hu-h-12-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.488
NM_012898	Rn.32083	*Rattus norvegicus* α 2 HS-glycoprotein α 2 (fetuin) (Ahsg), mRNA	0.492
NM_012552	Rn.6044	*Rattus norvegicus* elastase 1 (Ela1), mRNA	0.264
BI296422	Rn.49412	UI-R-CV2-cgr-a-06-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.324
AW916433	Rn.11526	EST347737 *Rattus norvegicus* cDNA, 5' end	0.348
AI639370	Rn.40539	rx00135s *Rattus norvegicus* cDNA, 3' end	0.364
BF415649	Rn.62771	UI-R-CA1-bkb-h-10-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.377
AI548311	Rn.43990	UI-R-C3-tg-b-08-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.388
AA997888	Rn.33114	UI-R-C0-hu-h-12-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.395
BI296773	Rn.16849	UI-R-DK0-cfz-b-06-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.402
AW918368	Rn.8749	EST349672 *Rattus norvegicus* cDNA, 5' end	0.407
BF555986	Rn.15320	UI-R-A1-dy-d-02-0-UI.r1 *Rattus norvegicus* cDNA, 5' end	0.407
AA858817	Rn.22047	UI-R-A0-bn-h-04-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.411
NM_031537	Rn.48721	*Rattus norvegicus* solute carrier family 11 member 1 (natural resistance-associated macrophage protein 1), see also D9Arb3 (Slc11a1), mRNA	0.427
BF567520	Rn.45035	UI-R-BO0-agp-b-06-0-UI.r1 *Rattus norvegicus* cDNA, 5' end	0.431
NM_022183	Rn.55119	*Rattus norvegicus* topoisomerase (DNA) II α (Top2a), mRNA	0.432
BF398042	Rn.12550	UI-R-BS2-beg-f-04-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.433
BI299197	Rn.3433	UI-R-CV2-chs-d-03-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.435
BG378288	Rn.22202	UI-R-CV1-bvq-c-10-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.436
AW917734	Rn.17053	EST349038 *Rattus norvegicus* cDNA, 5' end	0.443
NM_021752	Rn.43867	*Rattus norvegicus* apoptosis inhibitor 2 (Api2), mRNA	0.446
NM_022297	Rn.4241	*Rattus norvegicus* NG, NG dimethylarginine dimethylaminohydrolase (Ddah1), mRNA	0.447
BF566725	Rn.54198	UI-R-BJ0p-aff-a-06-0-UI.r1 *Rattus norvegicus* cDNA, 5' end	0.448
BI295067	Rn.25242	UI-R-DK0-cef-d-10-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.453
NM_012503	Rn.44300	*Rattus norvegicus* asialoglycoprotein receptor 1 (hepatic lectin) (Asgr1), mRNA	0.453
AI172457	Rn.36857	EST218468 *Rattus norvegicus* cDNA, 3' end	0.460
AF016387	Rn.8808	*Rattus norvegicus* retinoid X receptor γ (RXRγ) mRNA, partial cds	0.480
AA818602	Rn.40378	UI-R-A0-bc-g-12-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.480
BI282211	Rn.28004	UI-R-CU0s-cbx-h-09-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.483
BG372437	Rn.12364	UI-R-CV1-bsc-g-03-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.492
BI285816	Rn.11799	UI-R-CX0s-ccv-b-12-0-UI.s1 *Rattus norvegicus* cDNA, 3' end	0.494

Table V. Changes in apoptosis noted in the renal tissues of the T2D and control groups.

Group	Slice no.	No. of apoptotic cells in a single field	P-value
T2D	20	11.18±5.32	<0.01
Control	20	2.56±0.65	

The *α-fibrinogen* gene was one of the genes up-regulated in the kidney tissue of the T2D rats. In conjunction with increased blood glucose in T2D, the glycosylation level of blood fibrinogen increases significantly. This abnormal glycosylation reduces the glycosylation sensitivity of the blood fibrinogen fibrinolysis enzyme, which increases blood fibrinogen and creates erythrocyte aggregation. These changes easily lead to peripheral microvascular disease and induce DN (17).

Matrix metalloproteinase inhibitor gene 1 (*Timp-1*) was also found to be up-regulated in the kidney tissue of the T2D rats, consistent with a report by Suzuki *et al* (18), which showed that the expression of the *Timp-1* gene was up-regulated in renal parenchymal and infiltrating cells in patients with ND. TIMP-1 is thought to play a role in resistance to lymphocyte-mediated apoptosis *in vivo* by decreasing the activity of NF-κB (19). Hence, the overexpression of *Timp-1* in the kidneys of T2D rats may be an emergency response to resistance to tissue damage caused by increased blood glucose.

Wang *et al* (20,21) reported that the *retinoid X receptor* (*Rxr*) gene located on chromosome lq21-lq23 is among the best replicated regions with linkage to T2D in genome-wide scans. RXR is a member of the steroid/thyroid superfamily of nuclear receptors, and is likely to play a role in both glucose and lipid metabolism. RXR forms heterodimers or homodimers with a variety of nuclear receptors, including peroxisome-proliferator-activated receptors (PPARs) α and γ, thyroid hormone receptors and vitamin-D receptors, and subsequently alters insulin secretion and T2D susceptibility. Hence, a decrease in *Rxr* gene expression results in the disruption of glucose and lipid metabolism. In the present study, the RXR protein was also down-regulated in the T2D rats, which may have led to abnormal levels of cholesterol, triglycerides, apolipoprotein A and apolipoprotein B.

Stearyl-CoA desaturase (SCD), the terminal component of the SCD system, is a key enzyme involved in the synthesis of unsaturated fatty acids. It catalyzes the insertion of a double bond between carbons 9 and 10 in a spectrum of fatty stearyl-CoA substrates. When the activity of SCD is decreased, the ratio of saturated fatty acids and unsaturated fatty acids is altered. The abnormal alteration of this ratio has been shown to play a role in several physiological and disease states. In this study, we found that the activity of SCD was down-regulated in T2D rats, similar to reports by Waters and Ntambi (22) and Ntambi (23).

It has been demonstrated that the activity of topoisomerase II (Topo II) is rapidly decreased in T2D rats, which means that the enzyme may be related to islet cell destruction.
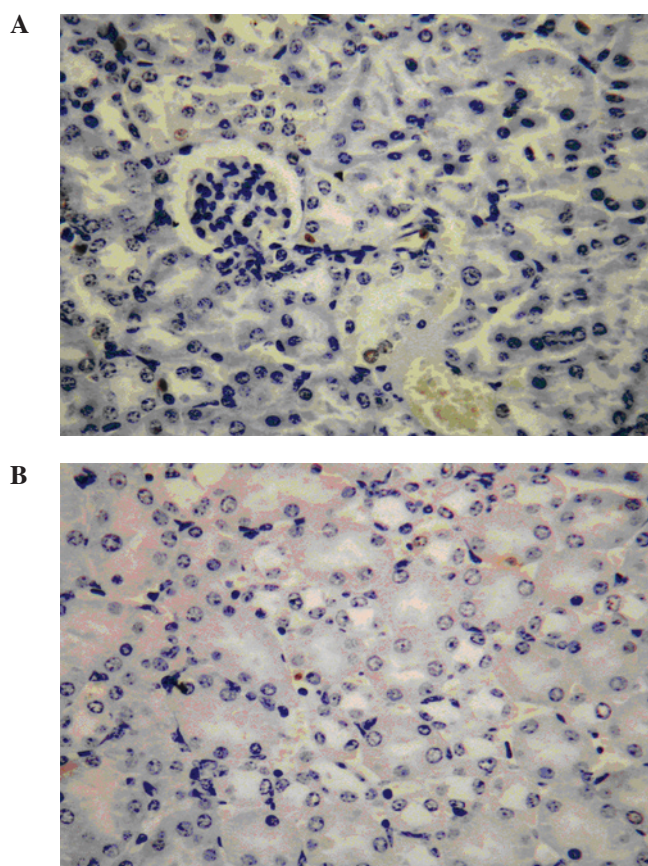
Figure 2. (A) TUNEL markers of apoptosis in renal tissue from T2D rats. (B) TUNEL markers of apoptosis in renal tissue from control rats. Apoptotic cells are stained brown and the nuclei of cells blue; magnification x100.

Janik *et al* (24) reported that *Topo II* expression levels were obviously down-regulated in T2D rats, and produced irreversible DNA strand breakage during DNA replication or RNA transcription. In the present study, *Topo II* expression was similarly down-regulated.

Carboxypeptidase B, which is present in the insulin secretion pathway, is an exocrine peptide protease containing zinc. It is closely related to insulin processing and is an indispensable protease that activates proinsulin to insulin. Hence, the activation and stability of carboxypeptidase B is an important parameter for proinsulin activation. In the present study, *carboxypeptidase B* was down-regulated in the T2D rats, as in studies reported by Fricker *et al* (25) and Sanchez *et al* (26). This may be associated with a decreased proinsulin to insulin conversion.

The *Apoptosis inhibitor 2* gene was also found to be down-regulated. This implies that the occurrence and development of T2D is related to an impaired dynamic balance of cell proliferation and apoptosis. Hence, there is uncontrolled apoptosis in T2D, which is related to islet β cell damage. Moreover, we found that the apoptosis rate of renal tubular epithelial cells in the T2D group was significantly higher compared to the control group, as similarly reported by Oritiz *et al* (27). We suggest that the expression of apoptosis-regulating genes is altered by metabolic disorders resulting from high-blood glucose levels, which increases apoptosis. Thus, the incidence of DN is related to apoptosis.

The application of the DNA microarray technique is a revolutionary research method in the life sciences, and has great potential use for the discovery of novel type 2 diabetes-related genes. The present study demonstrates that the detection of differences in gene expression between diabetic and normal kidney tissue using DNA microarray methods may provide novel prospects for the diagnosis, therapy and prevention of human T2D, DN and other complications.

Acknowledgements

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