


**Mindin: a novel marker for podocyte injury in diabetic nephropathy**

Maki Murakoshi, Mitsuo Tanimoto, Tomohito Gohda, Shinji Hagiwara, Miyuki Takagi, Satoshi Horikoshi and Yasuhiro Tomino

Division of Nephrology, Department of Internal Medicine, Juntendo University Faculty of Medicine, Tokyo, Japan

*Correspondence and offprint requests to: Yasuhiro Tomino; E-mail: yasu@juntendo.ac.jp*

**Abstract**

**Background.** GeneChip Expression Analysis was employed to survey the glomerular gene expression profile in a type 2 diabetes (T2D) model of KK/Ta mice fed with a high-calorie diet (HC), and we focused on the role of mindin (also called spondin 2), whose expression is upregulated by HC.

**Methods.** Isolated glomeruli from three 20-week-old KK/Ta mice fed with HC or a standard diet (SD) were dissected. Total RNA was extracted and labelled for hybridization using the Affymetrix GeneChip Mouse Genome 430 2.0 Array. The gene expression profile was compared between the HC and SD groups using GeneSpring 7.3.1 software. Mindin expression was examined using real-time PCR, western blot analysis and immunohistochemical staining in the glomeruli, cultured podocytes and urine samples of both mice and humans.

**Results.** Podocyte foot process effacement was observed in mice fed with HC. The mindin protein expression levels in mice were localized in the podocytes, and their levels in the glomeruli were increased in the HC group compared with the SD group. The levels of urinary mindin in the HC group at 16 weeks of age were also significantly higher than those in the SD group although albumin/creatinine ratio (ACR) did not differ between the groups. Furthermore, the levels in patients with T2D were higher than those in healthy individuals and increased gradually with increases in ACR.

**Conclusions.** Mindin could be related to podocyte injury and appears to be an early biomarker of the progression of diabetic nephropathy.

**Keywords:** biomarker; high-calorie diet; KK/Ta mouse; spondin 2

**Introduction**

Diabetic nephropathy (DN) is a major cause of end-stage kidney disease (ESKD) in the USA, Japan and most of Europe [1]. Almost 30% of diabetic patients develop DN despite strict blood glucose and/or blood pressure control [2]. Since DN occurs in familial clusters [3–5] and not all patients with poor metabolic control develop nephropathy, genetic factors may contribute to susceptibility to this disease. Genetic analysis of the whole kidney is difficult for studying diabetic glomerulopathy because glomeruli occupy only a small part of the kidney. Recent studies have shown that podocyte injury plays a role in the pathogenesis of various glomerular diseases, including DN [6–9]. However, the precise molecular mechanisms underlying DN remain unclear.
Increasing evidence indicates that inflammatory and immune response mechanisms may contribute significantly to the development and progression of DN [10,11]. These include infiltration of renal compartments by lymphocytes and monocytes/macrophages as well as the local production of cytokines and chemokines in the kidney. Specific inflammatory molecular pathways have recently been linked through the analysis of human glomerular gene expression profiles to DN progression.

Mindin (also called spondin 2, SPON2) is a member of the mindin-/F-spondin family of secreted extracellular matrix (ECM) proteins. This protein was initially identified in zebrafish and is selectively expressed in the basal lamina [12]. Mindin is a secreted protein that promotes adhesion and outgrowth of hippocampal embryonic neurons in vitro [13]. A recent study showed that mindin is essential for the initiation of innate immune response and represents a unique pattern-recognition molecule in the ECM [14]. Furthermore, mindin reportedly functions as an integrin ligand [15]. Integrin is also a key molecule for podocyte injury [16]. Therefore, we focused on mindin expression in the glomeruli and attempted to determine whether an increase in urinary mindin was associated with the development of DN.

The inbred mouse strain KK/Ta, established as a diabetic strain in Japan, spontaneously exhibits characteristics of type 2 diabetes (T2D). Renal lesions in KK/Ta mice closely resemble those in human DN. The albumin/creatinine ratio (ACR) in male KK/Ta mice at 16 weeks of age is 150–200 mg/gCr. KK/Ta mice glomeruli show diffuse hyperplasia in mesangial areas with mild mesangial cell proliferation [17]. This is associated with mild obesity and albuminuria. However, they are not severe when the mice are fed with a standard diet (SD).

In this study, we performed a microarray analysis using isolated glomeruli from diabetic KK/Ta mice fed with a high calorie diet (HC) and investigated mindin, the expression of which is upregulated by HC.

**Materials and methods**

**Animals**

The Animal Care and Use Committee of Juntendo University reviewed and approved all protocols described in this study. Male KK/Ta Jcl mice (4 weeks of age) were purchased from CLEA Japan Inc. (Tokyo, Japan). The mice were individually housed in plastic cages with free access to food and water throughout the experiment. The mice were randomly divided into the following two groups at 4 weeks of age: 8 mice were fed with either HC (HC group, 5.2 kcal/g, 60% of calories from fat, D12492; Research Diets) for 16 weeks, and 11 mice were fed an SD (SD group, 3.8 kcal/g, 10% of calories from fat, D12450B; Research Diets) for 16 weeks. All mice were maintained in the same room under conventional conditions with a regular 12-h light/dark cycle with the temperature controlled at 24°C ± 1°C. Mice were sacrificed under pentobarbital anaesthesia at 20 weeks of age to obtain kidney samples for isolation of glomeruli and whole kidneys.

**Patients**

This investigation was approved by the Ethical Review Board of the Juntendo University School of Medicine. All patients with T2D and IgA nephropathy (IgAN) gave written informed consent before enrolment. The onset of proteinuria in the DN patients was more than 5 years from the documented onset of T2D, and their urinary sediments did not contain red cells or cellular casts. Urine samples were obtained early in the morning. Control urine samples were taken from healthy adult volunteers and disease control urine samples were also obtained from patients with biopsy-proven IgAN.

**Biochemical characterizations**

The ACR, blood haemoglobin A1c (HbA1c) and fasting body weight were measured at 4, 8, 12, 16 and 20 weeks of age. The levels of fasting serum insulin, triglyceride (TG) and total cholesterol (T-Chol) were measured at 20 weeks of age. Urine samples were collected for a period of 24 h using a mouse metabolic cage (CLEA Japan). HbA1c, urinary albumin and creatinine were measured by immunoassay (DCA 2000 system, Siemens AG, Munich, Germany). Fasting serum insulin was measured using a mouse insulin enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Inc., Tokyo, Japan). Plasma lipoproteins were analysed using an on-line dual enzymatic method for the simultaneous quantification of cholesterol and triglycerides, for which high-performance liquid chromatography (HPLC) by Skylight Biotech (Akita, Japan) was employed as described previously [18].

**Isolation of glomeruli**

Isolated glomerular samples were obtained from mouse kidneys at 20 weeks of age using Dynabeads (Dynal A.S., Oslo, Norway) perfusion as described previously [19]. Briefly, mice were perfused through the heart using magnetic 4.5-μm diameter Dynabeads. Kidneys were excised, minced into small pieces, digested by collagenase and filtered, after which the glomeruli were collected using a magnet. The purity of glomerular samples was confirmed by microscopy, which was shown to be more than 97% on average.

**Microarray gene expression in isolated glomeruli of KK/Ta mice**

Using the Affymetrix GeneChip Mouse Genome 430 2.0 Array, 45 037 genes were simultaneously screened, which enabled us to delineate various biosynthetic or signalling pathways involved in KK/Ta mice nephropathy (GEO accession number: GSE19251). The procedures described in detail in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA, USA) were essentially followed. Briefly, isolated glomeruli from three 20-week-old KK/Ta mice were dissected. Total RNA was extracted from glomerular samples using the RNeasy Mini Kit (Qiagen). Approximately 100 ng of RNA was used for the Two-Cycle cDNA Synthesis Kit (Affymetrix). Biotin-labelled cRNA was produced through in vitro cDNA transcription (IVT Labeling Kit, Affymetrix). Fragmented cRNA (20 μg) was hybridized to the GeneChip Mouse Genome 430 2.0 Array using the Affymetrix Fluidics Station 450 (Affymetrix). The arrays were washed and stained according to the supplied protocols. The GeneChip was scanned with a GeneChip Scanner 3000 (Affymetrix) and the data were analysed using GeneSpring 7.3.1 software (Agilent Technologies, Santa Clara, CA, USA). The GeneSpring 7.3.1 software normalized and scaled the data for each array prepared between KK/Ta mice fed with HC and SD. To identify potential biomarker candidates, Ingenuity Pathways Analysis (IPA) software was employed (Ingenuity Systems, www.ingenuity.com). The biomarker analysis solution allowed us to identify and prioritize the most relevant and promising molecular biomarker candidates from datasets at nearly every step of the disease research.

**Real-time PCR**

Real-time PCR was also used to evaluate mindin mRNA expression in the glomeruli and whole kidneys of mice at 20 weeks of age. The RNA was reverse transcribed using the random decamer primer (Ambion, Austin, TX, USA) and MMLV Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). TaqMan real-time PCR was performed and analysed according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed to determine mindin expression in each tissue fraction using primers supplied with the commercially available assays from Applied Biosystems (Mm00513596_m1). Each data point was repeated three times. The relative mRNA level in the sample was normalized for GAPDH content.
Western blot analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis were performed according to standard protocols and were visualized using enhanced chemiluminescence immunoblot detection kits (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The primary antibodies used were as follows: monoclonal mouse anti-SPON2 antibody (Abnova, Taiwan), monoclonal mouse anti-β1 integrin antibodies (BD Transduction Laboratories, BD Biosciences, San Diego, CA, USA) and monoclonal mouse anti-β-actin antibody (SIGMA). HRP-conjugated second antibodies (Jackson-Immunoresearch Laboratories, Inc., West Grove, PA, USA) were used in this study. The OD was measured by a LAS 3000 image system.

Light microscopy and immunohistochemical staining

Sections were cut to 3 μm, embedded in paraffin and then stained with periodic acid-Schiff (PAS) reagent. Immunohistochemistry was performed as described previously [20]. Immunohistochemical studies were performed using commercially available antibodies as follows: monoclonal mouse anti-SPON2 antibody (Abnova) and polyclonal rabbit anti-WT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Electron microscopy

Tissues were fixed with 2% glutaraldehyde in 0.1 mol/L phosphate buffer at 4°C for 120 min. They were then cut using a regular diamond knife into semi- and ultrathin sections. Semithin sections (thickness 1 μm) stained with 1% toluidine blue were used for light microscopy. Ultrathin sections were collected on 100-mesh copper grids and double stained with 4% uranyl acetate and lead citrate. The sections were examined with a Hitachi 7100 transmission electron microscope (Hitachi High Technologies, Tokyo, Japan) operated at 75 kV.

Podocyte culture

Podocytes were cultured as described previously [21]. To induce differentiation, podocytes were seeded sparsely (5 × 10^4 cells) on type I collagen-coated dishes (Iwaki glass, Tokyo, Japan) at 37°C without γ-interferon (non-permissive conditions). Differentiated podocytes were starved for 24 h in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% fetal calf serum. The cells were then divided into two groups: [1] a normal glucose (NG) group incubated in DMEM containing 5.5 mM glucose as controls, and [2] a high glucose (HG) group incubated in DMEM containing 25 mM glucose. All experiments were repeated at least three times for each indicated condition. Podocytes between passages 15 and 20 were used in all experiments.

Measurement of mindin in urine samples

Both human and mice urine samples were stored at −80°C and centrifuged to remove any urates before use. Urine was diluted, as urinary creatinine for urinary mindin concentrations, and was compensated and analysed by western blotting using antibody to mindin.

Statistical analysis

All data are expressed as mean ± standard error (SE). Comparisons between two parameters were analysed by Student’s unpaired t-test. Comparisons among three or more parameters were analysed by one-way analysis of variance. P-values of <0.05 were defined as statistically significant.

Results

Phenotypic characterization of KK/Ta mice fed with HC

The phenotypic values of KK/Ta mice fed with either HC or normal SD at different ages are shown in Figure 1. The body weight and ACR at 8, 12, 16 and 20 weeks of age in the HC group were significantly higher than those in the SD group (P < 0.01). The HbA1c levels at 12, 16 and 20 weeks of age in the HC group were also significantly higher than those in the SD group (P < 0.01). There was no significant difference in the levels of serum TG and T-Chol between the two groups at 20 weeks of age (Table 1). However, the levels of serum low-density lipoprotein cho-
Lipids in the HC group were significantly higher than those in the SD group (P < 0.05), while the levels of serum high-density lipoprotein cholesterol in the HC group were lower than those in the SD group (P < 0.05). Fasting serum insulin levels in the HC group increased significantly compared with those in the SD group (P < 0.01).

Glomerular injury in the HC group

Diffuse expansion of mesangial matrices was observed by light microscopy in the HC group. The expansion was slightly increased in the HC group compared to the SD group (Figure 2A). Podocyte foot processes were fused in the HC group at 20 weeks of age by electron microscopy (Figure 2B).

Glomerular mindin mRNA in the HC group

Table 2 shows the top 20 genes with increased fold change in glomeruli of the HC group on a GeneChip filtered using the IPA-Biomarker. Mindin expression showed the highest fold change in the list of HC-induced genes, and this result was confirmed using real-time PCR (Figure 3A). Glomerular expression of mindin mRNA was significantly upregulated under HC at 20 weeks of age (P < 0.005). However, there was no significant change in whole-kidney mindin mRNA expression between the HC group and the SD group (Figure 3B).

Mindin protein expression in glomeruli

In the immunohistochemical staining, mindin protein was mainly localized in the podocytes because the expression of WT1 is restricted to podocyte in the mature glomeruli (Figure 3C). Western blot analysis showed that mindin protein expression levels in the glomeruli of the HC group at 20 weeks of age were also significantly higher than those in the SD group (Figure 3D).

Production of mindin protein by podocytes

To determine whether HG stimulation increases mindin protein production, we examined podocyte cultures incubated in HG. Mindin was detected in podocyte culture supernatants because it is a secreted protein. Mindin protein expression levels in podocyte culture supernatants
under HG stimulation were significantly higher than those under NG stimulation (P < 0.05, Figure 4A).

β1-integrin expression in cultured podocytes

β1-integrin expression in the podocyte cell lysate under HG conditions was increased compared to that under NG conditions (P < 0.05, Figure 4B).

Mindin in urine samples from mouse and human DN

After confirming that podocytes secreted mindin under HG conditions, we examined the urinary secretion of mindin in mice and humans. The urinary mindin level in the HC group at 20 weeks of age was significantly higher than that in the SD group (Figure 5A).

As shown in Figure 5B, the urinary levels of mindin were higher in patients with T2D than in healthy individuals and increased gradually with the progression of DN. However, those levels in diabetic patients with low normoalbuminuria (<10 mg/g Cr) did not differ when compared with healthy individuals with low normoalbuminuria (Figure 5B). The levels of urinary mindin in patients with T2D were significantly higher than those in patients with IgAN despite a similar degree of albuminuria (P ≤ 0.05, Figure 5B).

Discussion

The KK/Ta mice in the HC group showed increased body weight, ACR, HbA1c and fasting serum insulin than those in the SD group. We used GeneChip Expression Analysis to screen the genes expressed differentially in isolated glomeruli of KK/Ta mice fed with HC and SD. Mindin expression showed the highest fold change in HC-induced genes.

This study is the first to report the association of high glomerular mindin expression with DN with HC. Mindin functions as a pattern-recognition molecule for microbial pathogens and as an integrin ligand for inflammatory cell recruitment [14]. Mindin-deficient mice were more resistant to bacteria-induced shock when they were systemically

**Fig. 4.** Mean mindin and β1-integrin protein levels in podocyte cell culture. (A) Mindin protein expression in podocyte cell culture supernatants, (B) β1-integrin protein expression in podocyte cell lysate. NG, normal glucose group used as controls incubated in DMEM containing 5.5 mM glucose; HG, high glucose group incubated in DMEM containing 25 mM glucose.

**Earlier increase in urinary mindin secretion**

At 20 weeks of age, the levels of ACR in the HC group were significantly higher than those in the SD group. However, the levels at 16 weeks of age did not differ between the groups (Figure 6A). On the other hand, urinary mindin expression levels in the HC group were already significantly higher than those in the SD group at 16 weeks of age (Figure 6B).
infected with bacteria. The bacteria were unable to induce inflammatory cytokine production in mindin-deficient cells. Therefore, mindin is essential for the initiation of innate immune response [14]. T2D and its complications are associated with an abnormal inflammatory state. Inflammatory signalling pathways such as those coupled with Toll-like receptors trigger immune responses associated with a decline in renal function [22]. Activation of systemic and local immune responses is thought to contribute to renal dysfunction [23]. Thus, mindin may also play a role in the pathogenesis of DN.

It is obvious from previous studies that podocyte injury is important in DN. Podocytes cover the outer aspect of the glomerular basement membrane (GBM) and form the final barrier to protein loss. This explains why podocyte injury, i.e. foot process effacement, is typically associated with marked proteinuria [16]. In this study, podocytes in the HC group showed foot process effacement on electron microscopy. Glomerular mindin protein expression in the HC group was higher than that in the SD group. Mindin protein expression was also higher in podocyte culture supernatants incubated in HG medium than in those incubated in NG medium. These results suggested that mindin was produced by damaged podocytes. The foot process is fixed to the GBM via $\alpha_3\beta_1$-integrin and $\alpha$-$\beta$-dystroglycans [24–26], and $\alpha_3\beta_1$-integrin is the major integrin expressed by podocytes. Downstream signalling events are being elucidated, concentrating mainly on integrin-dependent cascades and their consequences for podocyte adhesion and proliferation [16]. Jia et al. reported that mindin serves as an integrin ligand [15]. We also showed that $\beta_1$-integrin protein expression increased in cultured podocytes stimulated by HG conditions. Cytoskeletal changes of podocytes are also critically involved in the pathogenesis of glomerular disease. Small GTPases of the Rho family are key regulators of the cellular cytoskeleton. Signalling through integrins activates Rho GTPases. Mindin was reported as a key regulator of Rho GTPase expression [27]. As mentioned above, mindin protein was expressed strongly in glomeruli of the HC group, which had podocyte foot

Fig. 5. Urinary mindin expression by western blot analysis. (A) Urinary mindin expression in KK/Ta mice at 20 weeks of age, (B) urinary mindin expression in healthy control and in patients with type 2 diabetes and IgA nephropathy. CTRL ($n = 4$), healthy control; DN1 ($n = 3$), diabetic patients with low normoalbuminuria (ACR ≤10 mg/gCr); DN2 ($n = 3$), diabetic patients with high normoalbuminuria (ACR 10–30 mg/gCr); DN3 ($n = 4$), diabetic patients with microalbuminuria (ACR 30–300 mg); DN4 ($n = 3$), diabetic patients with macroalbuminuria (ACR >300 mg/gCr); DN5 ($n = 4$), diabetic patients with renal failure; IgAN ($n = 5$), patients with IgA nephropathy. *P ≤ 0.05 vs CTRL or DN1, †P ≤ 0.01 vs DN2, ‡P ≤ 0.001 vs DN3. ACR, albumin/creatinine ratio; other abbreviations used in this figure are same as in Figure 3.
process effacement, and in cultured podocytes stimulated by HG conditions, suggesting that mindin is related to podocyte injury under diabetic conditions.

In this study, we confirmed that podocytes produce mindin in culture. We assume that urinary mindin was secreted by podocytes because mindin was dominantly detected in podocytes by immunohistochemistry. Urinary expression of mindin was also examined. Mindin was detectable in human and mouse urine. Urinary mindin expression in patients with T2D increased compared with that in healthy individuals, reflecting the stage of DN. Albuminuria has been used as a clinically useful diagnostic marker for predicting future overt nephropathy, but both sensitivity and specificity of albuminuria are not high enough for detecting the initial stage of nephropathy [28–30]. To evaluate the severity of glomerular injury, at least two aspects should be considered: the results of glomerular and urinary expression of mindin was significantly upregulated under HC compared with SD in diabetic KK/Ta mice. Furthermore, urinary mindin was detectable both in healthy individuals and in patients with T2D and DN, and the level increased in proportion to the levels of albuminuria. This study provides the first evidence that mindin is excreted in the urine in patients with T2D and DN. Mindin might be related to podocyte injury in patients with T2D and DN.

**Fig. 6.** Earlier increase in urinary mindin expression than urinary albumin to creatinine ratio. (A) Urinary levels of ACR, (B) urinary mindin expression in KK/Ta mice. HC, high-calorie diet group starting at 12 weeks of age; SD, standard diet group. *P ≤ 0.05 vs SD, †P ≤ 0.01 vs SD. Abbreviations used in this figure are same as in Figure 5.

**Conclusions**

Glomerular and urinary expression of mindin was significantly upregulated under HC compared with SD in diabetic KK/Ta mice. Furthermore, urinary mindin was detectable both in healthy individuals and in patients with T2D and DN, and the level increased in proportion to the levels of albuminuria. This study provides the first evidence that mindin is excreted in the urine in patients with T2D and DN. Mindin might be related to podocyte injury in patients with T2D and DN.

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**Conflict of interest statement.** None declared.

**References**

15. Jia W, Li H, He YW. The extracellular matrix protein mindin serves as an integrin ligand and is critical for inflammatory cell recruitment. *Blood* 2005; 106: 3854–3859

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