

## ACKNOWLEDGMENTS

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**Sponsor's Role:** None.

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### INCIDENCE AND CHARACTERISTICS OF METABOLIC DISORDERS AND VASCULAR COMPLICATIONS IN INDIVIDUALS WITH WERNER SYNDROME IN JAPAN

*To the Editor:* Werner syndrome (WS) is an autosomal-recessive disorder caused by a mutation of the WNR gene and is considered to be a representative type of progeroid syndrome,<sup>1</sup> which is highly prevalent in Japan. Because individuals with WS often have metabolic disorders and vascular complications, a nationwide epidemiological survey was initiated in Japan to clarify the current relationship between the prevalence of metabolic disorders and vascular complications in these individuals.

The primary survey involved sending 6,000 survey sheets to hospitals with more than 200 beds. This survey

confirmed 336 new patients. The secondary survey in 2011 involved sending questionnaires to hospitals that had responded to the primary survey. Detailed clinical data were obtained for 185 cases. Complication rates of metabolic disorder and morbidity from complications in individuals with WS were compared with those in the average Japanese population.

Of the 185 patients, 86 were men, 98 were women, and the sex of one was unknown. The proportions of patients were 62.7% aged 50 to 59, 22.7% aged 40 to 49, 10.8% aged 30 to 39, 1.1% aged 20 to 29, and 0.5% aged 60 to 69, respectively. Mean height and body weight were  $158.3 \pm 8.6$  cm and  $45.3 \pm 8.3$  kg for 44 male patients and  $148.5 \pm 8.6$  cm and  $37.7 \pm 8.3$  kg for 94 female patients. The prevalence of diabetes mellitus and abnormal glucose tolerance were 55.7% and 6.5%, respectively, with a total combined rate of 62.2% (Table 1). Drugs used for diabetes mellitus included pioglitazone (10.3%), sulfonylurea (7.6%), insulin (7.0%), alpha-glucosidase inhibitor (5.9%), and metformin (4.9%). The morbidity of hyperlipidemia was 51.6%. Treatments for hyperlipidemia included statins (18.4%), fibrates (5.4%), and others (3.8%). The morbidity of hypertension was 25.9%, lower than that of the average Japanese population (Table 1). Therapeutic agents used were angiotensin II receptor antagonists (4.9%) and calcium blockers (4.3%).

Morbidities of vascular diseases in WS were 1.1% for brain hemorrhage, 2.7% for cerebral infarction, 10.3% for angina pectoris or myocardial infarction, and 17.3% for arteriosclerosis obliterans. Individuals with WS were divided into two groups (with (n = 45) and without vascular disease (n = 140)), and correlations with diabetes mellitus ( $\chi^2 = 4.24$ ,  $P = .04$ ), hyperlipidemia ( $\chi^2 = 7.90$ ,  $P = .005$ ), and hypertension ( $\chi^2 = 11.16$ ,  $P < .001$ ) were examined, with a critical value of 3.84, confirming that metabolic disorders are closely related to vascular disease.

This study confirmed a considerably higher prevalence of metabolic disorders and cardiovascular diseases in Japanese with WS than in the average Japanese population (Table 1). Because of the high prevalence of metabolic disorders, the accumulation of visceral fat tissue in WS has been attributed to the development of the metabolic syn-

**Table 1. Morbidity from Metabolic and Atherosclerotic Diseases in Individuals with Werner Syndrome and the General Japanese Population**

Complication	Individuals with Werner Syndrome, n (%)			General Japanese Population Aged 50-59 (%)
	Total (n = 185)	Male (n = 86)	Female (n = 98)	
Diabetes mellitus	115 (62.2)	45 (69.2)	73 (61.4)	10.2*
Hypertension	48 (25.9)	17 (38.6)	31 (33.0)	47.2*
Dyslipidemia	94 (51.6)	27 (41.5)	61 (51.7)	16.4*
Low-density lipoprotein cholesterol $\geq$ 140 mg/dL	42 (22.7)	13 (29.5)	28 (28.6)	
High-density lipoprotein cholesterol $<$ 40 mg/dL	18 (9.7)	7 (15.9)	10 (10.2)	
Triglycerides $\geq$ 150 mg/dL	58 (31.4)	16 (36.4)	41 (41.8)	
<b>Atherosclerotic diseases</b>				
Cerebral vascular diseases	7 (3.8)	5 (5.8)	2 (2.0)	2.04†
Cardiovascular diseases	19 (10.3)	9 (10.5)	10 (10.2)	0.73†

Data from Ministry of Health, Labor, and Welfare in \*2006 and †2008.

drome,<sup>2</sup> but the mechanisms underlying the accumulation of visceral fat tissue frequently observed in WS remains largely unknown.

With regard to the characteristics of vascular disease in WS, the morbidity rate of stroke in individuals with WS was similar to that in the general Japanese population of the same age, although individuals with WS have a considerably greater prevalence of metabolic disorders. Stroke is more commonly caused by arteriolosclerosis than by atherosclerosis. Furthermore, arteriolosclerosis in the brain is associated with changes characterized by hyalinization of the tunica media or fibrinoid necrosis, which are closely associated with hypertension. The present survey demonstrated that the occurrence of hypertension as a complication of WS was lower than in the general Japanese population of the same ages; this has been a contributing factor to the smaller number of cerebral vascular disease in individuals with WS. In accordance with this lower incidence of cerebral vascular disturbances in individuals with WS, the function of the central nervous system is known to be maintained at a normal level, together with a lower incidence of dementia. Although the cause has not been clarified, the difference between the distribution of RecQ-type helicase (a protein that is mutated in WS) in vascular and cerebral blood vessels may be responsible. Furthermore, rapid cell division is associated with telomere stability, which is also associated with the WS protein.<sup>3</sup> Therefore, central nerves undergoing fewer cell divisions may be associated with a small number of disorders.

In conclusion, the frequency of stroke was lower in WS despite these individuals having numerous risk factors. A mutation in the WNR gene has been suggested as a possible protective process against the development of stroke. This finding may be significant for understanding the mechanism of the pathogenesis and progression of stroke, as well as for developing new therapeutic methods.

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**Sponsor's Role:** None.

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#### COMMENTS/RESPONSES

##### REHABILITATION OF ELDERLY ADULTS WITH SEVERE COGNITIVE IMPAIRMENT: IT IS TIME FOR EVIDENCE

*To the Editor:* The article by Poynter and colleagues<sup>1</sup> adds important information to the growing body of literature on the rehabilitation of older adults with dementia. This topic has several important clinical, organizational, and economic implications. In recent years, an increasing number of reports have shown that the rehabilitation of this group of individuals is not only possible and feasible, but is also clinically relevant. People with dementia and hip fracture<sup>2</sup> and other nonspecific medical conditions have been successfully rehabilitated in various studies, despite the severity of their cognitive impairment.<sup>1,3</sup> Furthermore, unconventional and technology-based techniques are now promising strategies to overcome the gap of cognitive impairment in these individuals.<sup>4,5</sup>

Despite these positive remarks, motor rehabilitation of older adults with dementia is far from being an evidence-based discipline. A crucial question is the lack of randomized clinical trials, which are the only way to draw definite conclusions about the effectiveness of rehabilitation in individuals with dementia. For instance, in the field of hip fracture rehabilitation—a topic expected to become prominent in the coming years given the progressive aging of the population<sup>6</sup>—there are only two randomized clinical trials including individuals with dementia.<sup>7,8</sup> Of these, only one,<sup>8</sup> a small subgroup analysis of a previous multicompartment clinical trial to reduce postoperative delirium in elderly adults with hip fractures,<sup>9</sup> used a definition of dementia according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*, criteria.

The results of randomized controlled studies in individuals with dementia will provide important information to physicians and policy-makers to dedicate adequate

## Research Article

# An Angiotensin II Type 1 Receptor Blocker Prevents Renal Injury via Inhibition of the Notch Pathway in Ins2 Akita Diabetic Mice

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Recently, it has been reported that the Notch pathway is involved in the pathogenesis of diabetic nephropathy. In this study, we investigated the activation of the Notch pathway in Ins2 Akita diabetic mouse (Akita mouse) and the effects of telmisartan, an angiotensin II type1 receptor blocker, on the Notch pathway. The intracellular domain of Notch1 (ICN1) is proteolytically cleaved from the cell plasma membrane in the course of Notch activation. The expression of ICN1 and its ligand, Jagged1, were increased in the glomeruli of Akita mice, especially in the podocytes. Administration of telmisartan significantly ameliorated the expression of ICN1 and Jagged1. Telmisartan inhibited the angiotensin II-induced increased expression of transforming growth factor  $\beta$  and vascular endothelial growth factor A which could directly activate the Notch signaling pathway in cultured podocytes. Our results indicate that the telmisartan prevents diabetic nephropathy through the inhibition of the Notch pathway.

## 1. Introduction

The worldwide prevalence of diabetes in all age groups was 2.8% in 2000 and is estimated to be 4.4% in 2030 [1]. The total number of people with diabetes mellitus (DM) is expected to rise from 171 million in 2000 to 366 million in 2030. Diabetic nephropathy, a major microvascular complication of DM, is the most common cause of end-stage renal disease (ESRD) [2]. The number of ESRD cases is expected to increase mainly as a result of the increasing incidence of obesity and type 2 DM.

A number of pathways such as the protein kinase C pathway [3] and the polyol pathway [4] as well as advanced glycation end products [5] have been reported to play important

roles in the development of diabetic nephropathy. It has also been reported that the renin-angiotensin system (RAS) plays a potent role in the initiation and progression of diabetic nephropathy [6].

A number of clinical evidences have suggested that the blockade of the RAS by angiotensin-converting enzyme (ACE) inhibitors (ACEIs) and/or angiotensin II type1 receptor (AT1R) antagonists (ARBs) could improve renal function or slow down disease progression in diabetic nephropathy [7]. Furthermore, it has been reported that ACEIs and/or ARBs inhibit the RAS and have pleiotropic effects, which improve renal prognosis.

Recently, Niranjana et al. reported that the Notch pathway was activated in diabetic nephropathy and in focal segmental

glomerulosclerosis (FSGS) [8]. The activation of the Notch pathway in podocytes has been studied in genetically engineered mice. These mice developed glomerulosclerosis due to the activation of p53, which induced apoptosis in podocytes. The same group also showed that pharmaceutical and genetic blockade of the Notch pathway prevented mice from developing diabetic and puromycin-aminonucleoside-(PAN-) induced glomerulosclerosis.

The Notch signaling pathway is a signaling pathway that determines cell fate [9]. Further, it is regulated by cell-cell communication during the formation of various internal components such as the nerves, blood, blood vessels, heart, and hormonal glands. Notch is a transmembrane receptor protein that interacts with ligands of the Jagged and Delta families [10].

The aim of this study was to examine the activation of the Notch pathway in Akita mice as well as the effects of telmisartan on the Notch pathway both *in vivo* and *in vitro*.

## 2. Materials and Methods

**2.1. Reagents.** Telmisartan was obtained from Nippon Boehringer Ingelheim Co., Ltd. (Tokyo, Japan). Candesartan was purchased from Tronto Research Chemicals (North York, Canada). Angiotensin II was obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human TGF- $\beta$ 1 (#240-B) and recombinant human VEGF-A (#293-VE) were purchased from R&D systems (Minneapolis, MN). GSI was purchased from Calbiochem (San Diego, CA). Hoechst 33342 was from Dojindo laboratories (Kumamoto, Japan).

**2.2. Animals.** Male heterozygous Ins2 Akita diabetic mice (C57BL/6) and C57BL/6 controls were obtained from Japan SLC Inc. (Shizuoka, Japan). Eight-week-old Akita mice and control mice received telmisartan ( $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) or no treatment for 15 weeks ( $n = 8$  in each group). The blood glucose level, body weight, blood pressure, and urinary albumin excretion were measured every two weeks. The blood glucose level was examined using Medisafe-Mini (TERUMO Corporation, Tokyo, Japan), and the blood pressure was determined by the tail cuff method using Softron BP-98A (Softron, Tokyo, Japan). In order to estimate albuminuria, mice were individually housed in metabolic cages for 24 h. Urine was collected, and urinary albumin concentrations were measured with a Lebis Albumin assay kit (Shibayagi, Gunma, Japan). The blood creatinine levels, BUN, fasting blood glucose levels, and HbA1c were measured at the time of sacrifice. All experiments in this study were performed in accordance with the Guidelines of the Animal Care and Use Committee of Chiba University, Japan, which follows the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985). The ethics committee for animal research at Chiba University approved all animal experiments.

**2.3. Immunohistochemistry.** The following commercially available antibodies were used: rabbit anti-Jagged1 (1:200

dilution, sc-11376) and rabbit antihuman TGF- $\beta$ 1 (1:50, sc-146) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-cleaved Notch1 antibody (1:100, Val1744, no. 2421S) was purchased from Cell Signaling (Danvers, MA). Rat anti-podocalyxin monoclonal antibody ( $0.5 \mu\text{g}/\text{mL}$ , MAB1556) was from R&D systems. Mice kidneys were embedded in OCT compound and frozen, and  $10 \mu\text{m}$  sections were made. The sections were air dried, fixed in methanol (10 min on ice), rinsed in phosphate-buffered Tween (PBT), and blocked for 30 min with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Primary antibodies were diluted in PBS containing 1% BSA and were incubated with the sections overnight at  $4^\circ\text{C}$ . The slides were rinsed with PBT for several times. The fluorophore-conjugated secondary antibodies were applied for 2 h. The sections were again rinsed with PBT for several times, mounted (Vectashield Mounting Medium with DAPI; Vector Laboratories, Inc., Burlingame, CA), and viewed under a fluorescence microscope (Axio Observer; Leica) or a confocal laser scanning microscope (Leica LSM5 PASCAL). The images were processed using Adobe Photoshop.

**2.4. Cell Culture.** Mouse podocytes, conditionally immortalized with a temperature-sensitive variant of the SV40 large T-antigen, were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, NY, USA). The preparation and characterization of these cells have been described elsewhere [11]. Podocytes were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco/Life Technologies, Grand Islands, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma Aldrich). To propagate podocytes, cells were cultivated at  $33^\circ\text{C}$  and incubated with 10 U/mL of murine recombinant  $\gamma$ -interferon (Pepro Tech EC Ltd, London, UK) to enhance the expression of the T-antigen (permissive conditions). To induce differentiation, podocytes were cultured at  $37^\circ\text{C}$  without  $\gamma$ -interferon in RPMI 1640. Cells were cultured under nonpermissive conditions for at least 11 d before they were used in the experiments. The medium was changed every 3 d to induce full differentiation. Cells at passages 12 to 18 were used for the experiments in this study.

**2.5. Reverse Transcriptase-Polymerase Chain Reaction.** The expression of mRNA in podocytes was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After treatment with DNase,  $1 \mu\text{g}$  of total RNA was reversely transcribed using oligo dT primer, pd(T)12-18 (Invitrogen, Carlsbad, CA), to avoid genomic contamination. The cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Gene-specific oligonucleotides for the PCR analyses were designed according to the predicted cDNA sequences (<http://www.ensembl.org/>). The PCR was performed in a  $25 \mu\text{L}$  PCR reaction containing  $1 \mu\text{L}$  of complementary DNA (cDNA), Taq reaction buffer

TABLE 1: Characteristics of the experimental groups of mice.

	Wild control	Wild telmisartan	Akita control	Akita telmisartan
Blood glucose (mg/dL)	250 ± 34	284 ± 58	1216 ± 130*	955 ± 137*. <sup>†</sup>
HbA1c (%)	4.3 ± 0.3	4.2 ± 0.3	10.8 ± 1.4*	11.8 ± 0.5*
Body weight (g)	36.4 ± 3.4	40.7 ± 9.0	20.8 ± 0.8*	23.2 ± 1.4*. <sup>†</sup>
Systolic blood pressure (mmHg)	109.3 ± 4.7	96.1 ± 7.3	126.4 ± 5.9*	110 ± 5.1*. <sup>†</sup>
Urinary albumin (mg/day)	21.2 ± 9.4	10.9 ± 2.51	51.4 ± 11.6*	33.8 ± 8.5*. <sup>†</sup>

Data are expressed as the mean ± standard deviation (SD). \* $P < 0.01$  versus wild-type control, <sup>†</sup> $P < 0.01$  versus Akita control.

(Go Taq, Promega, Madison, WI), and 10  $\mu$ M of dNTPs. The primer sequences and sizes of the expected PCR products are as follows: Hes1, 5'-CCCTGTCTACCTCTCTCCTT-3', 5'-AGGTGCTTACAGTCATTTTC-3', 472 bp; TGF- $\beta$ , 5'-TCC-AAGAAAAAGAAAATGGA-3', 5'-CTCTGAATCAGTTGTGGAT-3', 452 bp; VEGF-A, 5'-GTGGACATCTTCCAGGA-GTA-3', 5'-ATCTGCAAGTACGTTTCGTTT-3', 382 bp;  $\beta$ -actin, 5'-TCGTGCGTGACACATCAACATCAAAGAG-3', 5'-TGGACAGTGAGGCCAGGATG-3', 411 bp. PCR was performed for 25–30 cycles. Each cycle consisted of denaturation at 94°C for 2 min, annealing at 50°C for 30 s, and extension at 72°C for 30 s. PCR amplification was followed by a final extension step at 72°C for 7 min. An aliquot of 10  $\mu$ L of each PCR product was subjected to electrophoresis on a 2% agarose gel (Ronza), followed by staining with an ethidium bromide solution (Sigma). The signals were photographed with a charge-coupled device (CCD) camera system (Printograph, ATTO). Densitometric analyses of the fluorograms were performed using an image scanner (EPSON GT-X900) with ImageJ software (<http://rsbweb.nih.gov/ij/download.html>).

**2.6. Morphometric Analysis.** Five glomeruli ( $n = 3$ , in each) were randomly selected from each specimen. The extent of extracellular mesangial matrix was determined by quantification of the periodic-acid-Schiff-staining- (PAS-) positive area in the mesangium and divided by the glomerular tuft area. The extracellular mesangial matrix area and glomerular tuft area were quantified by ImageJ.

**2.7. Detection of Apoptosis by Hoechst Staining and Flow Cytometric Assays.** Podocytes were treated with AII in the presence or absence of telmisartan for 72 h. After the treatment, apoptosis was defined as the presence of nuclear condensation on Hoechst staining. Alternatively, the cells were collected, washed twice with cold phosphate-buffered saline (PBS), and centrifuged at 1,000 g for 5 minutes. Subsequently, the Annexin V/propidium iodide assay was carried out to determine apoptosis according to the manufacturer's instructions (BD Pharmingen) and analyzed by flow cytometry (FACSCalibur; BD Immunocytometry Systems, San Jose, CA).

**2.8. Statistical Analysis.** Results are expressed as the mean ± standard error of the mean (SEM). Experimental points were performed in triplicates with a minimum of three independent experiments. An unpaired Student's  $t$ -test was

used for comparison of two groups.  $P < 0.05$  was considered significant.

### 3. Results

**3.1. Telmisartan Reduces the Urinary Albumin Excretion in Akita Mice.** First, we evaluated the effect of telmisartan on blood pressure in mice. Table 1 shows that Akita mice had a higher blood pressure than the controls. As expected, administration of telmisartan significantly lowered the blood pressure. Compared to the controls, Akita mice also had considerably higher levels of blood glucose and HbA1c, which eventually led to loss of body weight. Telmisartan decreased the blood glucose level and led to an increase in body weight in Akita mice (Table 1). The urinary albumin excretions were significantly increased in untreated Akita mice compared to wild-type controls, and administration of telmisartan significantly reduced urinary albumin excretion (Table 1).

Next, we investigated the effect of telmisartan on the glomerular morphology. Expansion of the mesangial areas was observed in Akita mice; however, telmisartan had no profound effect on the glomerular morphology as determined by light microscopy (Figure 1).

**3.2. Telmisartan Inhibits the Notch Pathway and the Expression of TGF- $\beta$ , Which Are Activated in the Glomeruli of Akita Mice.** Recently, it has been reported that the Notch pathway is activated in podocytes in DM. Therefore, we examined the Notch pathway in Akita mice. ICN1 staining in kidneys revealed that the number of ICN1-positive cells in the glomeruli was significantly higher in Akita mice (Figures 2(a) and 2(b)). We could not observe ICN1-positive cells other than in the glomeruli. This indicated that the Notch pathway was activated in Akita mice, and the activation of the Notch pathway seemed to be restricted to the glomeruli. In order to identify cell types that were activated by the Notch pathway within the glomeruli, we also carried out coimmunostaining with an anti-ICN1 antibody and an anti-podocalyxin antibody (a marker for podocytes). We localized ICN1 proteins to the nuclei of the cells which were positive for podocalyxin within the cytoplasm (Figure 2(c)). Therefore, Notch pathway was activated in podocytes in diabetic conditions. Administration of telmisartan significantly reduced the number of ICN1-positive cells in the glomeruli (Figures 1(a) and 1(b)). Next, we investigated the expression of Jagged1, which is a ligand for the Notch

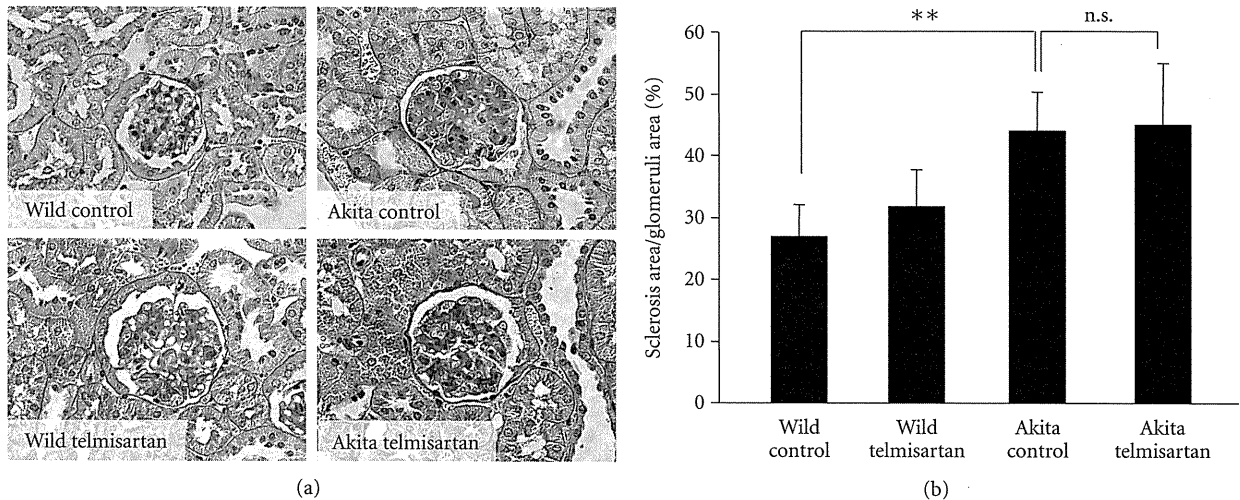


FIGURE 1: Morphometric analyses of the glomeruli of Akita mice. (a) Eight-week-old Akita mice and control mice received telmisartan ( $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ , in their drinking water) or no treatment, respectively, for 15 weeks ( $n = 8$  in each group). After 15 weeks, the mice were sacrificed, the kidneys were harvested, and periodic acid-Schiff staining was performed. (b) Quantification of sclerosis per glomerular area was performed with the ImageJ software.  $**P < 0.01$ , n.s.: not significant.

receptor. The expression pattern of Jagged1 was quite similar to that of ICN1 (Figure 2(d)). These results indicated that telmisartan inhibited the Notch pathway *in vivo* either directly or indirectly. It has been reported that the Notch pathway in podocytes was activated by TGF- $\beta$  signaling [8]. Therefore, we investigated the expression of TGF- $\beta$  by immunohistochemistry. We observed upregulated TGF- $\beta$  expression in the glomeruli of Akita mice (Figure 2(e)), especially in podocytes (Figure 2(f)). Administration of telmisartan also suppressed the expression of TGF- $\beta$  in the glomeruli (Figure 2(e)).

**3.3. Angiotensin II Activates the Notch Signaling Pathway through Increased Expression of TGF- $\beta$  and VEGF-A in Cultured Podocytes.** Telmisartan lowered the blood pressure and improved the blood glucose level in Akita mice. From these findings, we were not able to completely exclude the possibility that the inhibitory effect of telmisartan on the Notch pathway *in vivo* was due to a systemic effect. Therefore, we used cultured mouse podocytes that were conditionally immortalized in order to not only rule out the influence of blood pressure and glucose levels but also elucidate the mechanism by which telmisartan inhibits the Notch pathway. Telmisartan is an AT1R blocker. For this reason, we studied the effect of angiotensin II (AII), a ligand for AT1R, on the activation of the Notch pathway. As shown in Figure 3(a), the mRNA expression of hairy enhancer of split homolog-1 (Hes1), which was a target gene of the Notch signaling pathway, increased considerably in the presence of  $10^{-6} \text{ M}$  AII. In addition, telmisartan inhibited the AII-induced mRNA expression of Hes1 (Figure 3(a)). The expression of Jagged1 mRNA was also increased in the presence of AII, and telmisartan inhibited AII-induced mRNA expression of Jagged1 (data not shown). We also examined the effect of candesartan, another type of AT1R blocker, and found that

candesartan inhibited the AII-induced mRNA expression of Hes1 same as telmisartan (Figure 3(b)). It has been reported that TGF- $\beta$  and VEGF-A activate the Notch pathway [12]; therefore, the effect of AII on the expression of TGF- $\beta$  and VEGF-A was investigated. As shown in Figures 3(c) and 3(d), incubation with AII significantly increased the expression of both TGF- $\beta$  and VEGF-A. Telmisartan reversed this effect.

Finally, we observed the effects of TGF- $\beta$  and VEGF-A on the activation of the Notch pathway and found that these growth factors could activate the Notch pathway. However, telmisartan had no effect on the Notch pathway in the presence of TGF- $\beta$  or VEGF-A (Figure 4).

**3.4. Telmisartan Suppresses the Podocyte Apoptosis Induced by Angiotensin II.** It has been reported that the activated Notch pathway induces apoptosis to the glomerular podocytes which eventually causes glomerulosclerosis. Therefore, we investigated whether telmisartan could prevent podocyte apoptosis. As shown in Figures 5(a) and 5(b), flow cytometer studies using annexin V and propidium iodide showed that apoptotic cells were increased in the podocytes treated with AII ( $12.56 \pm 1.9\%$  versus  $7.09 \pm 1.4\%$  in the control group,  $P < 0.01$ ), and telmisartan treatment significantly decreased the AII-induced apoptotic cells ( $8.51 \pm 2.0\%$  versus  $12.56 \pm 1.9\%$  in the AII group,  $P < 0.01$ ). We also examined the apoptosis by the use of Hoechst 33342 staining as shown in Figures 5(c) and 5(d). Nuclear condensation was observed in the podocytes in the presence of AII, and those changes were significantly decreased when the podocytes were treated with telmisartan. We also examined the effects of  $\gamma$ -secretase inhibitor (GSI) on the AII-induced apoptosis and found that GSI, an inhibitor of Notch signaling, was able to inhibit the AII-induced apoptosis (Figure 4). Collectively, these results indicated that the AII induced podocytes apoptosis via the activating Notch signaling pathway, and telmisartan



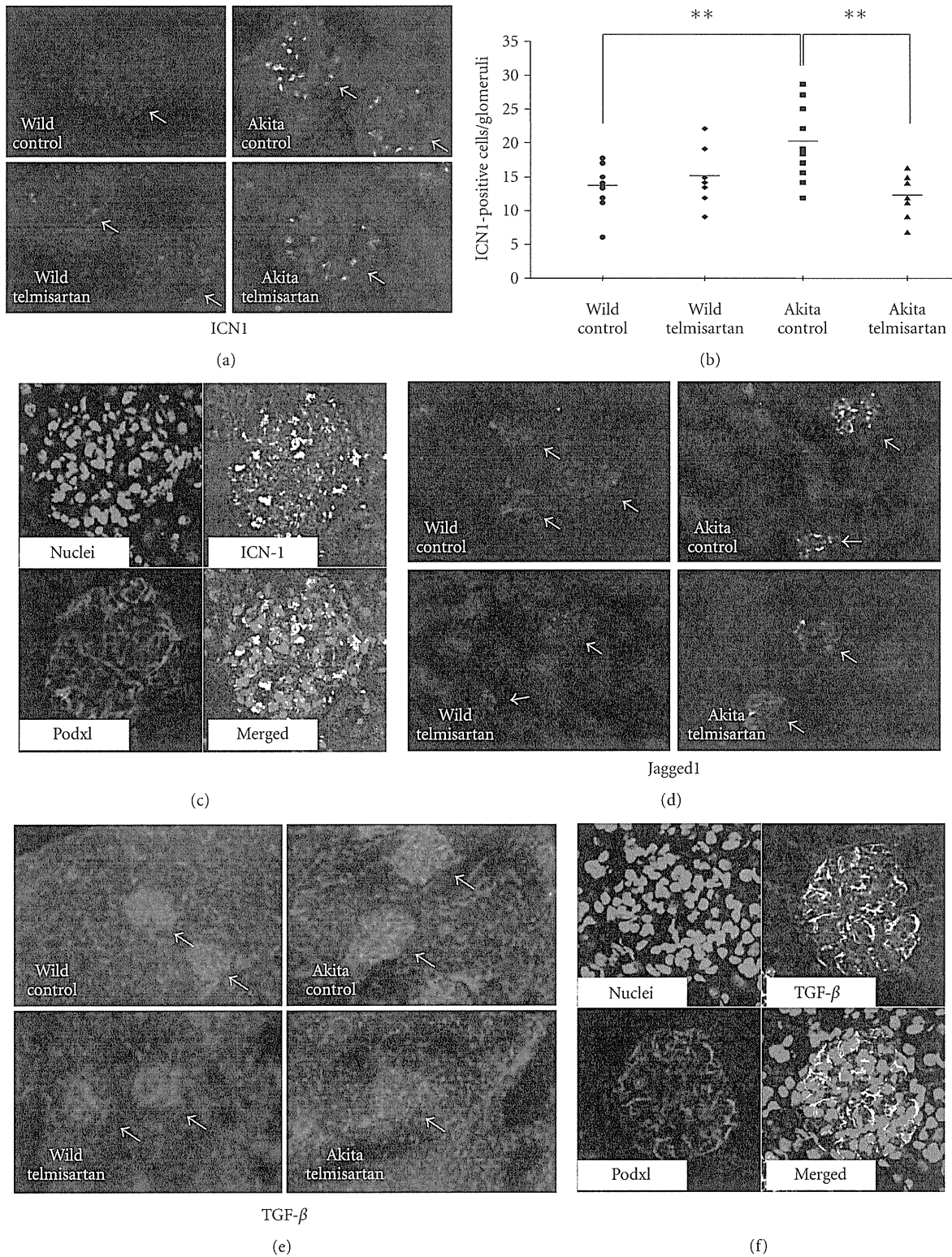
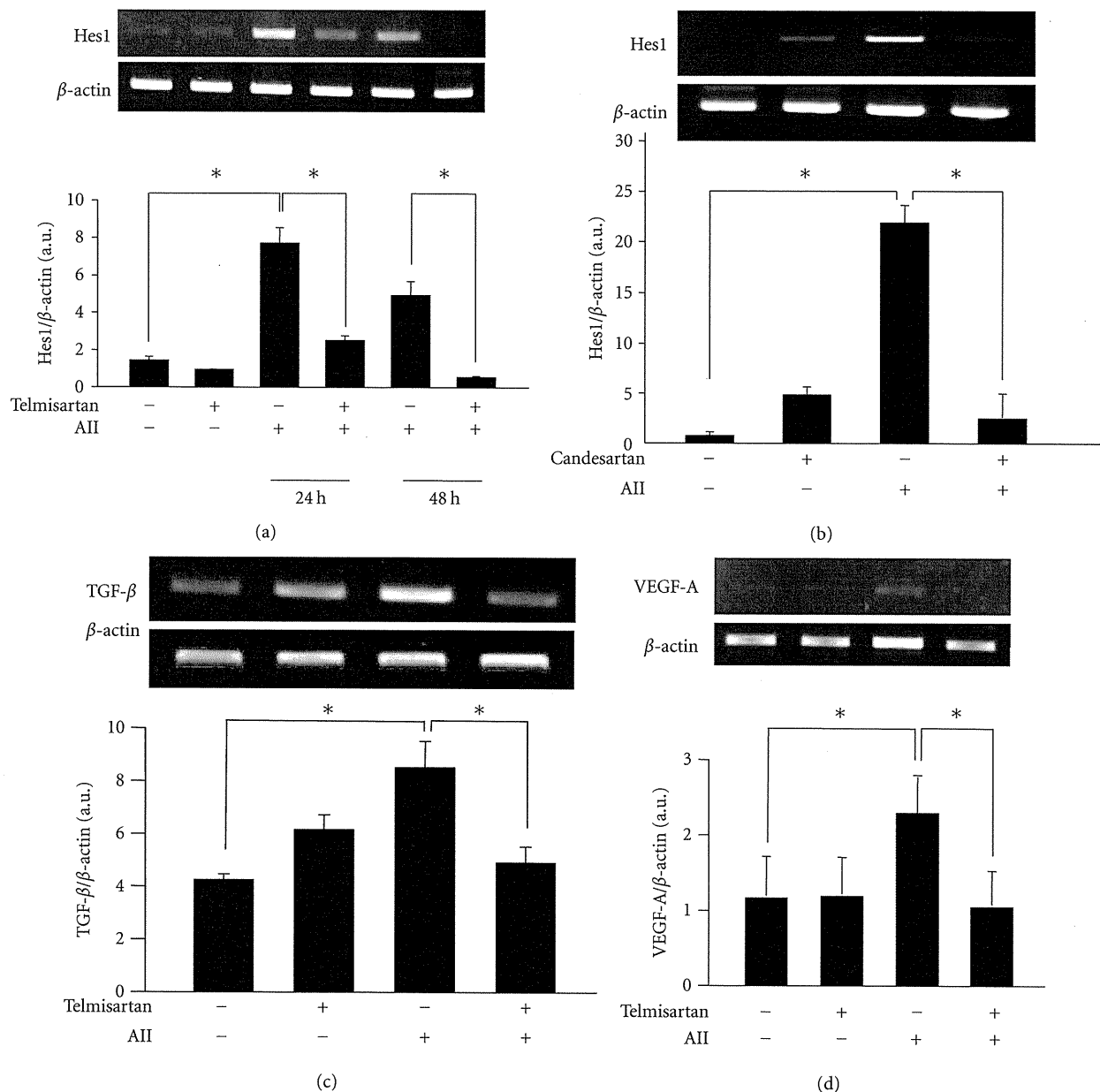


FIGURE 2: Notch pathway was activated in the glomeruli of Akita diabetic mice and telmisartan inhibited its expression. The expression of the intracellular domain of Notch1 (ICN1) (a and c), Jagged1 (d), and transforming growth factor  $\beta$  (TGF- $\beta$ ) (e and f) were examined by immunohistochemistry. Anti-podocalyxin (Podxl) antibody was used as a marker for podocyte. ICN-1 was localized to podocyte nuclei (c), while TGF- $\beta$  was localized to podocyte cytoplasm, respectively (f). Quantification of ICN1-positive cells per glomeruli was performed (b). Ten glomeruli of each specimen were randomly selected. The ICN1-positive cells within the glomeruli were counted under a fluorescence microscope. Statistical significance was analyzed using Student's *t*-test. Arrows indicated the glomerulus. Bars indicated the mean value. **\*\**P* < 0.01.**



**FIGURE 3:** Telmisartan suppressed the activation of the Notch signaling pathway through inhibition of the angiotensin II type 1 receptor. The mRNA expression of Hes1, one of the Notch target genes; transforming growth factor  $\beta$  (TGF- $\beta$ ); vascular endothelial growth factor-A (VEGF-A) were examined by reverse transcriptase-polymerase chain reaction. (a) The podocytes were stimulated with  $10^{-6}$  M Angiotensin II (AII) for 24 to 48 h. The mRNA expression of Hes1 increased in the presence of AII and peaked at 24 h. On the other hand,  $10^{-6}$  M telmisartan suppressed the AII-induced mRNA expression of Hes1 (upper panel). Quantification of the Hes1 mRNA expression compared to the internal control ( $\beta$ -actin) (lower panel). (b) The podocytes were treated with  $10^{-6}$  M AII in the presence or absence of  $10^{-8}$  M candesartan for 24 h. Candesartan also suppressed the AII-induced mRNA expression of Hes1. (c) AII increased the TGF- $\beta$  mRNA by 2.5-fold within 12 h. Telmisartan ( $10^{-6}$  M) suppressed the expression of TGF- $\beta$  significantly. (d) AII increased the VEGF-A expression by 2.0-fold. Telmisartan suppressed the expression of VEGF-A significantly. \* $P < 0.05$ .

inhibited podocytes apoptosis through the inhibition of Notch signaling pathway (Figure 5(e)).

#### 4. Discussion

In the present study, we investigated the activation of the Notch pathway in the glomeruli (especially in the podocytes)

of Akita mice. Treatment with telmisartan significantly reduced not only the urinary albumin excretion which was usually seen as an early manifestation of diabetic nephropathy but also the activation of the Notch pathway. We also confirmed that AII induced the activation of the Notch pathway in cultured podocytes. Incubation with AII increased the expression of TGF- $\beta$  and VEGF-A, and telmisartan reversed



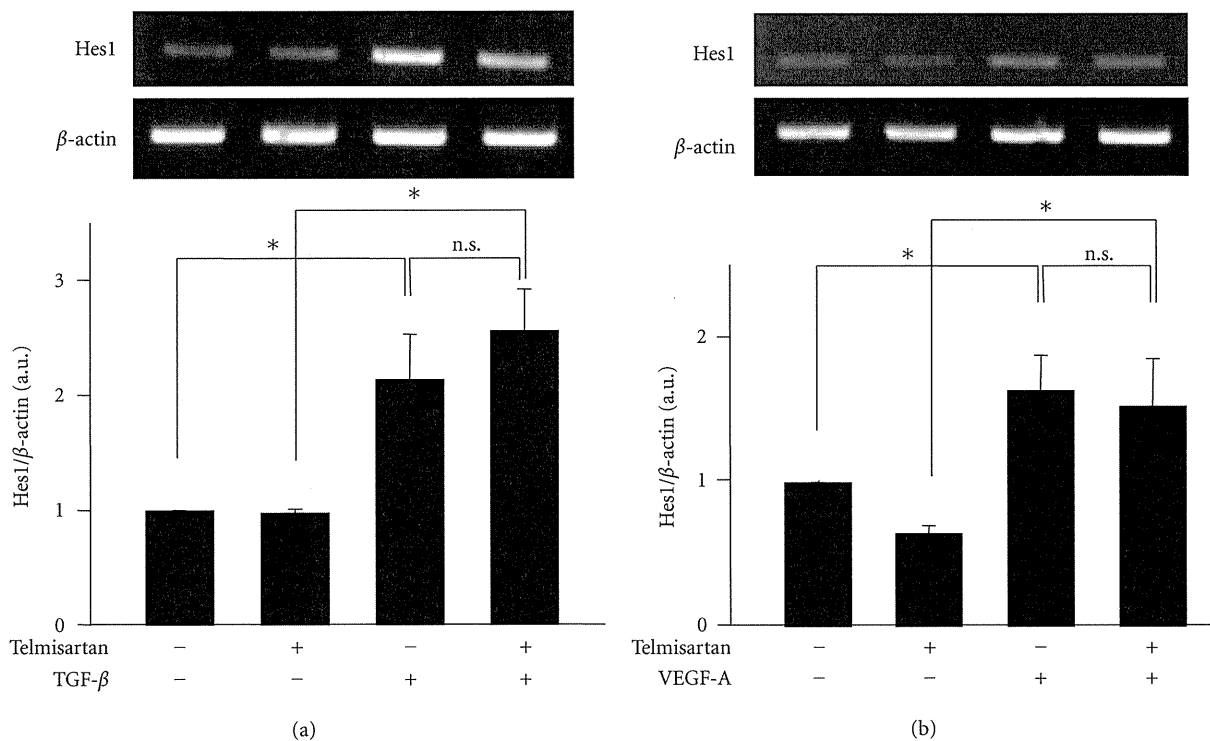
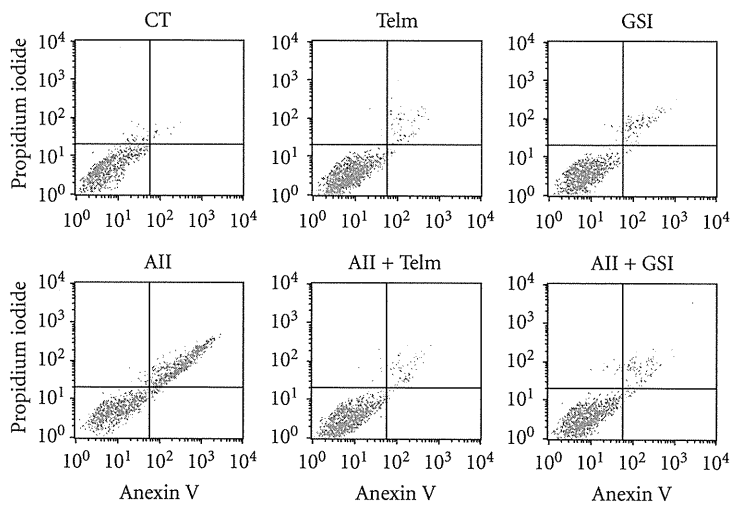


FIGURE 4: TGF- $\beta$  and VEGF-A directly activated the Notch pathway. The podocytes were stimulated with 5 ng/mL transforming growth factor  $\beta$  (TGF- $\beta$ ) or 10 ng/mL vascular endothelial growth factor-A (VEGF-A) in the presence or absence of  $10^{-6}$  M telmisartan. The mRNA expression of Hes1 was examined by reverse transcriptase-polymerase chain reaction. (a) TGF- $\beta$  increased the expression of Hes1 irrespective of the presence or absence of telmisartan (upper panel). Quantification of Hes1 expression compared to the internal control ( $\beta$ -actin). TGF- $\beta$  significantly increased the Hes1 expression within 2 h by 2.1-fold (lower panel). (b) VEGF-A increased the expression of Hes1 irrespective of the presence or absence of telmisartan (upper panel). Quantification of the Hes1 expression compared to the internal control ( $\beta$ -actin). VEGF-A significantly increased the Hes1 expression within 2 h by 1.6-fold (lower panel). \* $P < 0.05$ , n.s.: not significant.

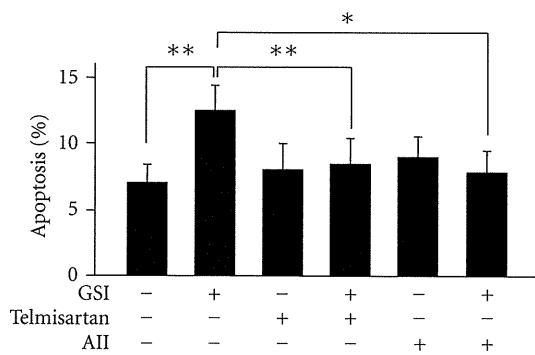
this effect. TGF- $\beta$  and VEGF-A could directly activate the Notch pathway.

Diabetic nephropathy, the leading cause of ESRD in the western world and Asia, is a considerable socioeconomic burden. Investigation of the pathophysiology and establishment of a treatment for diabetic nephropathy is urgently needed. Angiotensin II (Ang II) is a potent vasoconstrictor hormone that is cleaved from angiotensinogen by renin and ACE. In addition to its known vital role in both cardiovascular and blood pressure homeostasis, several lines of evidence implicate a role in diabetic nephropathy. Durvasula and Shankland have reported that high glucose activates the local RAS in podocytes (independent of ACE activity), which led to injury of the podocytes [13]. Therefore, RAS are locally and systemically activated under diabetic conditions. It has also been reported that the injury of podocytes, referred to as podocytopathy, is a hallmark not only in diabetic nephropathy but also in virtually all glomerular diseases [14]. There are not many pharmacological options to treat diabetic nephropathy; ACEIs and/or ARBs are currently the only drugs that effectively slow the progression of diabetic nephropathy [15]. Furthermore, clinical trials demonstrated that ARBs also lower the risk of type 2 DM compared with other antihypertensive therapies. These observations indicate that ARBs can potentially be used to induce effects other than blood pressure lowering effects. Indeed, ARBs have recently

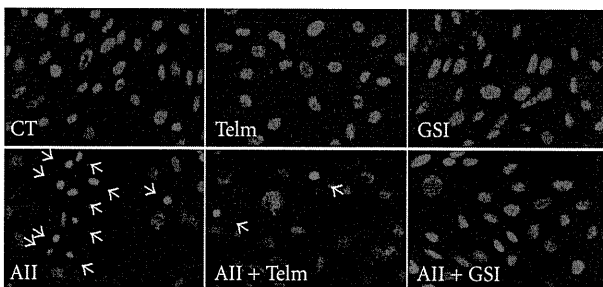
been proven to attenuate inflammation and oxidative stress and inhibit apoptosis [16]. These effects are known as pleiotropic effects. In addition to the previously reported pleiotropic effects, in the present study, we identified that telmisartan inhibited the activation of the Notch pathway. The Notch pathway is known to control a number of cell-fate-specific events in multiple organisms, especially during development, and it also plays a crucial role in diseases such as cancers and autoimmune diseases [17]. It has been recently reported that the Notch pathway is activated in mouse models of DM such as *Lpr<sup>db/db</sup>* mice (which mimics type 2 DM), in streptozotocin-treated mice (which leads to type 1 DM), and in kidney specimens from patients with DM [8]. It has also been reported that high glucose activated Notch pathway and increased the expression of VEGF in cultured podocyte [18]. We confirmed the activation of the Notch pathway in another diabetic animal, the Akita mouse. Our findings support the idea that the Notch pathway is generally activated in podocytes in DM. In recent years, GSIs received significant attention as drug candidates for the treatment of Alzheimer's disease and cancers [19]. Since GSIs are capable of inhibiting the Notch signaling pathway, they can be used in the treatment of diabetic nephropathy in the future. In addition to GSIs, our data also suggest that telmisartan inhibits the Notch pathway. To the best of our knowledge, this is the first report that describes the ARB-induced



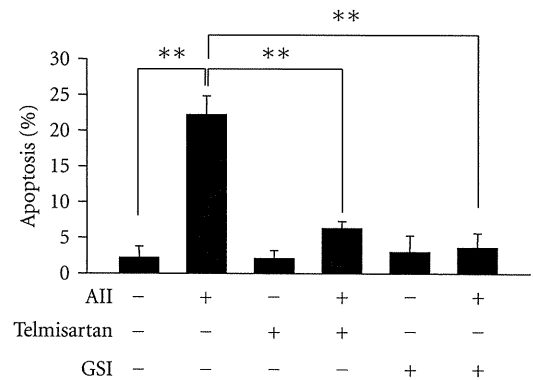
(a)



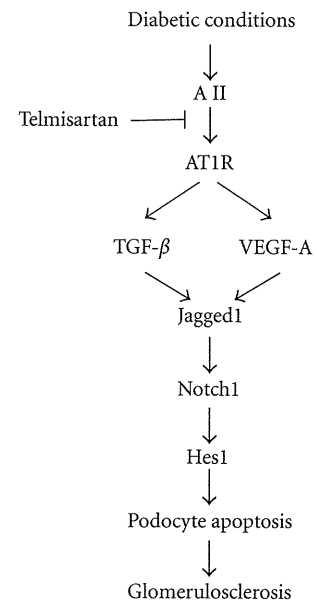
(b)



(c)



(d)



(e)

FIGURE 5: Telmisartan suppressed the podocyte apoptosis which was induced by angiotensin II. The effects of AII as well as telmisartan on the podocytes apoptosis were examined by the flow cytometry or by the Hoechst staining. (a, b) The podocytes were treated with  $10^{-6}$  M AII in the presence or absence of  $10^{-6}$  M telmisartan or 5 mM  $\gamma$ -secretase inhibitor (GSI) for 72 h. Apoptosis in podocytes was determined by low propidium iodide staining and prominent annexin V labeling using the flow cytometry. AII significantly induced podocytes apoptosis compared to the controls ( $12.56 \pm 1.9\%$  versus  $7.09 \pm 1.4\%$ ). Telmisartan significantly suppressed AII-induced apoptosis in podocytes ( $8.51 \pm 2.0\%$  versus  $12.56 \pm 1.9\%$ ). GSI also significantly suppressed that ( $7.89 \pm 1.6\%$  versus  $12.56 \pm 1.9\%$ ). Representative results of three independent experiments were presented.  $*P < 0.05$ ,  $**P < 0.01$ . (c) The apoptosis in podocytes was examined by Hoechst staining. The podocytes were treated with  $10^{-6}$  M AII,  $10^{-6}$  M telmisartan, and 5 mM GSI as indicated in the figures for 72 h. Apoptosis was determined by nuclear condensation pattern and expressed as the percentage of apoptotic cells per high-power field. A total of 5 high-power fields in a pericentric distribution were quantitated per well. (d) Telmisartan and GSIs suppressed the podocyte apoptosis (CT  $2.3 \pm 1.5\%$ , AII  $22.3 \pm 2.54\%$ , Telm + AII  $6.3 \pm 0.9\%$ , and GSI + AII  $3.6 \pm 2.0$ , resp.). *Telm*: telmisartan,  $**P < 0.01$ . (e) Schematic illustration of the effects of telmisartan on the Notch pathway in podocytes.

inhibition of the Notch pathway both *in vivo* and *in vitro*. Telmisartan is a potent and highly selective AT1R antagonist. Furthermore, telmisartan exerted effects other than the blockade of AT1R, such as PPAR $\gamma$  activation [20]. Our data showed that telmisartan improved the levels of blood glucose, which might indicate that telmisartan functioned as a PPAR $\gamma$  agonist and improved insulin resistance in Akita mice. Although telmisartan significantly reduced urinary albumin excretion, we were not able to detect profound histological improvement. There might be some time difference between the improvement in urinary albumin excretion and the improvement histologically. Telmisartan lowered the blood pressure and improved the blood glucose level in Akita mice. From these findings, we were not able to completely exclude the possibility that the inhibitory effect of telmisartan on the Notch pathway *in vivo* was due to a systemic effect. However, we also used cultured podocytes in order to rule out the influence of blood pressure and glucose levels. Therefore, we argue that telmisartan could directly affect podocytes in order to inhibit the Notch pathway. We also investigated whether candesartan, another ARB, could suppress the Notch pathway and found that candesartan also inhibited Notch signaling pathway. Therefore, the inhibitory effect of Notch pathway by telmisartan seems to be a class effect of ARB.

It has been reported that the genetically activated Notch pathway in podocytes in mice activated p53 and induced apoptosis, which led to decreased expression of the slit diaphragm-related protein such as nephrin, causing proteinuria and renal dysfunction [8]. We tried to detect apoptosis by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and by staining for activated caspase 3. However, we could not observe apoptosis in the glomeruli of Akita mice, and this could be attributed to technical reasons.

There are some limitations to this present study. First, we were not able to completely exclude the possibility systemic effects of telmisartan for reducing Notch signal *in vivo*. Second, we are not able to explain the reason why telmisartan did not improve the glomerulosclerosis which was seen in Akita mice. Third, we still do not completely understand the biological significance of activated Notch pathway in diabetic condition.

In summary, we showed that the Notch pathway was activated in podocytes of Akita mice and that administration of telmisartan inhibited the Notch pathway. Our data might indicate that telmisartan inhibits the Notch pathway. In addition to its blood pressure lowering effect, which leads to reduced cardiovascular morbidity and mortality, telmisartan might improve the renal prognosis, especially in diabetic subpopulations. Further investigations are needed to prove this hypothesis in the future.

## Acknowledgments

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## WRN遺伝子に複合型ヘテロ接合体変異を同定した Werner症候群の1例

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大野 恭太<sup>1)</sup> 竹本 稔<sup>2)</sup> 横手幸太郎<sup>2)</sup> 喜多 哲也<sup>1)</sup>  
筒泉 正春<sup>1)</sup>

### 要 旨

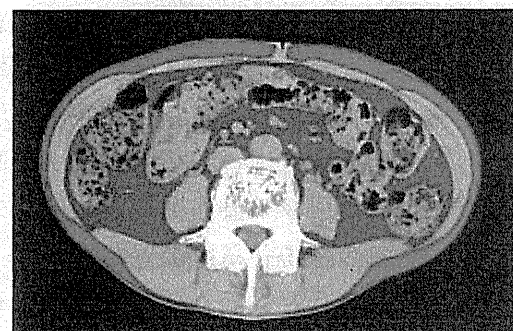
Werner症候群はRecQ型DNAヘリカーゼであるWRN遺伝子の異常に起因する遺伝性の早老症で、日本における報告例が多い。今回、我々はWRN遺伝子にMutation4 およびMutation6 の複合型ヘテロ接合体変異を有するWerner症候群を見出した。合併する糖尿病に対しては、チアゾリジン薬を投与することで、血糖コントロールが改善するとともに、インスリン抵抗性や炎症状態を示す指標が軽快した。

[日内会誌 100:1642~1644, 2011]

**Key words** Werner症候群, 早老症, 糖尿病

### 症 例

患者：51歳，女性。主訴：血糖のコントロール。既往歴：特記すべきものなし。家族歴：糖尿病なし。血族結婚なし。現病歴：高校生の頃から頭髪が白髪になるとともに、同時期から足底の鶏眼や胼胝が出現しだした。30歳代には白内障で手術を受けた。月経は12歳から40歳頃まであり、女兒を1人出産している。40歳頃から肘頭部に難治性の皮膚潰瘍が出現し、皮膚移植術を受けた。また40歳頃から尿糖を指摘され、現在、経口血糖降下薬の処方を受けている。現症：身長155 cm，体重29.5 kg，BMI 12.3。顔貌



内臓脂肪面積 (V) 皮下脂肪面積 (S)

44 cm<sup>2</sup>

24 cm<sup>2</sup>

V/S=1.83

図. 腹部CT (臍高) による内臓脂肪と皮下脂肪の面積

はやや鼻梁が突出した鳥様顔貌で全身の皮下脂

[第191回近畿地方会 (2010/10/05) 推薦][受稿2010/12/03, 採用2010/12/17]

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Case Report; A case of Werner syndrome with compound heterozygous mutations of WRN gene.

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表. 抗糖尿病薬による治療経過での各種代謝指標の変化

	グリメピリド 1 mg/day		ピオグリタゾン 30 mg/day ×16週間
空腹時血糖 (mg/dL)	102	→	98
HbA1c (JDS値) (%)	7.1	→	6.8
(HbA1c (国際標準値) (%)	7.5	→	7.2)
空腹時IRI (μU/mL)	12.7	→	4.5
空腹時CPR (ng/mL)	3.74	→	1.48
HOMA-R	3.2	→	1.1
高感度CRP (ng/mL)	1,280	→	486
IL-6 (pg/mL)	5.1	→	1.7
Adiponectin (μg/mL)	2	→	6.4
レプチン (ng/mL)	3.8	→	3.3

肪は極めて少なく四肢が非常に細い。両足底は皮膚の硬化があり、胼胝や鶏眼が多発し、右踵底部には潰瘍とピンホール瘻孔を認める。神経学的所見に異常を認めず。検査所見：尿所見、末梢血液所見、一般生化学所見に異常なし。糖尿病はグリメピリド 1 mg/dayを投与され、空腹時血糖値 102 mg/dl, HbA1c 7.1% (JDS値)であった。血中CA19-9 88.3 U/ml, CEA 16.9 ng/mlと腫瘍マーカーの上昇を認めた。腹部CT検査では皮下脂肪量に比して、腹腔内脂肪量の相対的な増加が認められた (V/S比 1.83) (図)。足部X線撮影では、アキレス腱の石灰化を認めた。

### 臨床経過

特徴的な徴候と検査成績から臨床的にWerner症候群と診断した。遺伝子検査を行ったところ、WRN遺伝子にMutation6 (エクソン9内で1336番目の塩基がCからTへ変異することによる切断型変異蛋白の形成)とMutation4 (エクソン26の直前の塩基がGからCへ変異することによる切断型変異蛋白の形成)の複合型ヘテロ接合体変異を見だし、各種の病態がこの遺伝子異常に由来することを確認した。腫瘍マーカーが増加していたことから、悪性腫瘍の発症を疑い、全

身の検索を行ったが、現在のところ、明らかな悪性腫瘍の合併は認めていない。Werner症候群は全身の脂肪が減少し、やせ型を呈するが、インスリン抵抗性が認められる。本症例でもHOMA-Rは3.2とインスリン抵抗性があり、SU薬(グリメピリド 1 mg/day)よりもインスリン抵抗性改善薬(ピオグリタゾン 30 mg/day)のほうが病態に適合すると考え、変更を行ったところ、血中アディポネクチン濃度が増加するとともにインスリン抵抗性が軽減し、血糖コントロールも改善傾向にある(表)。

### 考 察

Werner症候群は第8染色体短腕に位置するRecQ型DNAヘリカーゼ遺伝子異常のため、DNAの複製、修復、組み換え、テロメアの維持などに障害を来す結果、遺伝子の不安定化が起こり、成人期になって様々な早老化が出現する常染色体劣性遺伝疾患である<sup>1)</sup>。患者はやせ型で皮下脂肪組織が少なく、鳥様顔貌、白髪、白内障、皮膚硬化、足底の鶏眼や胼胝、難治性皮膚潰瘍、糖尿病を高頻度に合併し、悪性腫瘍や心血管疾患によって50歳代で死亡することが多い。日本人ではMutation4あるいはMutation6のホモ接合



体変異による発症が多く、本症例に認められる Mutation4/6 の複合型ヘテロ接合体変異による発症は約 5% 程度である<sup>2)</sup>。Werner 症候群では全身の炎症病態の亢進が認められる。また皮下脂肪組織は萎縮傾向にあるものの、内臓脂肪量は保たれ相対的な内臓脂肪の増加を来すことが多い<sup>3)</sup>。こういった特徴的な病態が Werner 症候群に認められるインスリン抵抗性と密接に関連するものと考えられる。それ故、Werner 症候群に併発する糖尿病の治療薬としては、インスリン抵抗性改善薬であるビッグアナイド薬やチアゾリジン薬が効果的であると報告されている<sup>4~8)</sup>。全身の慢性炎症がインスリン抵抗性や動脈硬化の発症進展に関連していることや、Werner 症候群の死亡原因として心血管疾患が多いことを考慮すると、抗炎症効果も期待できるチアゾリジン薬は<sup>9)</sup>、Werner 症候群に伴う糖尿病治療に適していると考えられる。本症例においても、チアゾリジン薬を投与することによって血糖値の改善とともに血中高感度 CRP や IL-6 の低下といった炎症病態の改善が確認された (表)。

著者の COI (conflicts of interest) 開示：本論文発表内容に関連して特に申告なし

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## V. 參考資料

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資料 1. 共同通信社取材 新聞掲載記事

H23年度 共同通信社取材 新聞記事

2012年3月12日 東奥日報

2012年3月13日 琉球新聞

2012年3月15日 日本海新聞

2012年3月25日 千葉日報

2012年3月30日 高知新聞

2012年3月31日 岐阜新聞

2012年4月27日 神戸新聞

# 早老症に診療指針 世界初

若くして老化が進んでしまう早老症(ワエルナー症候群)は、患者数は少ないが、世界中で日本人の患者が7割を占める。このほど厚生労働省の研究班による世界初の診療ガイドラインがまとまり、日本からの発表を控え、東京で患者家族と研究班が顔を合わせ、最新の情報を交換した。



- ワエルナー症候群の主要な兆候**  
(10歳以降、40歳までに出現)
- 男性性腺摘除(白髪、はげ頭など)
  - 声のかすれ
  - 白内障(両側)
  - 皮膚の萎縮、硬化、または慢性炎症性潰瘍
  - アキレス腱の痛み(石灰化)など

## ワエルナー症候群で家族会 早期治療に効果

ワエルナー症候群は、自身発症のきっかけは「幸」で発症するが、成人になると病状が進行し、骨密度が低下し、心臓病や糖尿病などの合併症が現れる。このため、早期治療が重要とされている。東京で開かれたワエルナー症候群患者家族会では、最新の診療指針が発表された。研究班の発表によると、ワエルナー症候群は、遺伝性疾患で、10歳以降、40歳までに出現する。主要な兆候として、男性性腺摘除(白髪、はげ頭など)、声のかすれ、白内障(両側)、皮膚の萎縮、硬化、または慢性炎症性潰瘍、アキレス腱の痛み(石灰化)などが挙げられる。研究班は、ワエルナー症候群の患者は、骨密度が低下し、心臓病や糖尿病などの合併症が現れる。このため、早期治療が重要とされている。東京で開かれたワエルナー症候群患者家族会では、最新の診療指針が発表された。

## ワエルナー症候群 初の診療指針まとまる

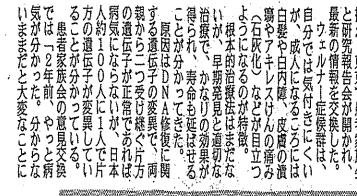
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## 患者や医師 最新情報交換 東京で家族会、研究報告会

東京で開かれたワエルナー症候群患者家族会。患者や医師が最新の診療指針について話し合った。研究班の発表によると、ワエルナー症候群は、遺伝性疾患で、10歳以降、40歳までに出現する。主要な兆候として、男性性腺摘除(白髪、はげ頭など)、声のかすれ、白内障(両側)、皮膚の萎縮、硬化、または慢性炎症性潰瘍、アキレス腱の痛み(石灰化)などが挙げられる。研究班は、ワエルナー症候群の患者は、骨密度が低下し、心臓病や糖尿病などの合併症が現れる。このため、早期治療が重要とされている。東京で開かれたワエルナー症候群患者家族会では、最新の診療指針が発表された。

# 早老症の患者家族会開く

若くして老化が進んでしまう早老症(ワエルナー症候群)は、患者数は少ないが、世界中で日本人の患者が7割を占める。このほど厚生労働省の研究班による世界初の診療ガイドラインがまとまり、日本からの発表を控え、東京で患者家族と研究班が顔を合わせ、最新の情報を交換した。



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## ワエルナー症候群 初の診療指針もまとまる

ワエルナー症候群は、自身発症のきっかけは「幸」で発症するが、成人になると病状が進行し、骨密度が低下し、心臓病や糖尿病などの合併症が現れる。このため、早期治療が重要とされている。東京で開かれたワエルナー症候群患者家族会では、最新の診療指針が発表された。研究班の発表によると、ワエルナー症候群は、遺伝性疾患で、10歳以降、40歳までに出現する。主要な兆候として、男性性腺摘除(白髪、はげ頭など)、声のかすれ、白内障(両側)、皮膚の萎縮、硬化、または慢性炎症性潰瘍、アキレス腱の痛み(石灰化)などが挙げられる。研究班は、ワエルナー症候群の患者は、骨密度が低下し、心臓病や糖尿病などの合併症が現れる。このため、早期治療が重要とされている。東京で開かれたワエルナー症候群患者家族会では、最新の診療指針が発表された。

## 早老症の患者家族会開く 初の診療指針も

若くして老化が進んでしまう早老症(ワエルナー症候群)は、患者数は少ないが、世界中で日本人の患者が7割を占める。このほど厚生労働省の研究班による世界初の診療ガイドラインがまとまり、日本からの発表を控え、東京で患者家族と研究班が顔を合わせ、最新の情報を交換した。ワエルナー症候群も疑はせることが、佐村が説明している。佐村は「成人になる(原因は遺伝子の変異)ことで、骨密度が低下し、心臓病や糖尿病などの合併症が現れる。このため、早期治療が重要とされている。東京で開かれたワエルナー症候群患者家族会では、最新の診療指針が発表された。

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