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糖尿病性腎症のRemissionをめざして

糖尿病性腎症における代表的な結節性病変（ヒト腎生検例，PAS染色）
和泉洋平，山本直哉，槇野博史（腎と透析 Vol.59, No.1, 2005）

巻頭言

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糖尿病性腎症は慢性腎臓病（chronic kidney disease: CKD）の中で最も主要な位置を占めている。それはCKDの患者数のみならず、病期が進行して透析に陥る患者数も最も多いからである。

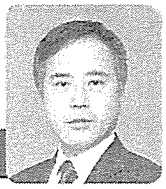
Mauerらのグループにより、1型糖尿病性腎症患者に対して膵単独移植により血糖を正常化すると、糖尿病性腎症に特徴的とされる結節性病変が消失したり、メサンギウム基質の縮小や、糸球体・尿細管基底膜の肥厚の改善が見られるなど、腎病変が病理学的に軽快することが報告された。従来は糖尿病性腎症は組織学的変化がいったん起こってしまうと、非可逆性と考えられていたが、血糖を正常化することにより、腎症の進展が抑制されるのみならず、すでに形成された腎病変が軽快することが明らかとなった。この研究がよりどころとなり、近年、remissionをめざした積極的な治療が試みられている。

そこで今回の特集では、「糖尿病性腎症のRemissionをめざして」と題して、糖尿病性腎症におけるトピックスとremissionをめざした治療の実際を紹介していただいた。先生方の日常臨床の参考となれば幸いである。

なお，筆者らは厚生労働省科学研究費補助金により、Diabetic Nephropathy Remission and Regression Team Trial - Japan（DNETT-Japan）を開始した。本研究は顕性期糖尿病性腎症の患者を対象に、医師と糖尿病療養指導士などのコメディカルスタッフが参加するチーム医療によって、より強力な治療介入を行うことにより、腎症の寛解が可能か否かを検証することを目的とした大規模臨床研究であり、多くの施設からの参加をお願いしたい。

目次

糖尿病性腎症の遺伝因子



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長期間血糖コントロール不良にもかかわらず腎症の進展がほとんどみられない、逆に比較的コントロール良好あるいは罹病期間が短いにもかかわらず腎症が進行している、こういった症例を経験することは決してまれではない。このような違いは、特定の遺伝子内での個人間の微妙な違いに基づくとされており、どの遺伝子のどのような違いが腎症の発症進展に関わるかが明らかになれば、新たな予防法・治療法の開発につながるとともに、個人の病態にあわせた治療（オーダーメイド医療）が可能になると期待されている。ヒトゲノム研究のめざましい進歩を受けて、現在大規模なゲノムワイドアプローチにより糖尿病性腎症の発症進展に関連する遺伝因子同定が試みられている。

糖尿病性腎症の発症進展に関わる遺伝因子探索の現状

1型糖尿病患者における疫学研究では、網膜症の累積頻度は糖尿病罹病期間とともに直線的に増加するのに対し、腎症の累積頻度は罹病期間約25年で頭打ちとなり、以後の発症はほとんど認められていない。結果として、腎症が進行するのは全糖尿病患者の30～40%程度にとどまるとされている。さらに、糖尿病性腎症の家族内集積も知られており、糖尿病性腎症の発症進展にはなんらかの遺伝因子の関与が強く示唆されている。病因に基づく候補遺伝子解析によりアンジオテンシン変換酵素遺伝子多型、アルドース還元酵素遺伝子多型、PKCβ遺伝子多型、アポE遺伝子多型などと腎症との関連が報告されているが、今のところ一定の見解は得られていない。これは、糖尿病性腎症の発症進展には複数の遺伝子が関与しており、個々の遺伝子単独の作用は比較的弱いために、その解明が難しくなっているためと考えられている。

ゲノムワイドアプローチによる試み

ヒトゲノムプロジェクトの完了により30億塩基対に及ぶヒトゲノムDNA配列のほぼすべてが明らかとなった。ヒトゲノム配列の99.9%はすべての人で同一であるが、0.1%の

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ORIGINAL ARTICLE

Combinational effect of genes for the renin–angiotensin system in conferring susceptibility to diabetic nephropathy

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Abstract To elucidate the role of the renin–angiotensin system (RAS) in diabetic nephropathy, we examined the association between diabetic nephropathy in a large cohort of Japanese type 2 diabetic patients and polymorphisms within the genes that encode angiotensin-converting enzyme (*ACE*), angiotensinogen (*AGT*) and angiotensin II receptor type 1 (*AGTR1*). Single nucleotide polymorphisms (SNPs) within these genes were genotyped using invader assay in 747 nephropathy cases and 557 control subjects. Eight SNPs within the *ACE* gene were significantly associated with diabetic nephropathy ($P<0.05$), including five SNPs in almost complete linkage disequilibrium to the insertion/deletion polymorphism in the 16th intron ($P=0.01$, odds ratio =1.34, 95% CI 1.07–1.69). Three SNPs within the *AGT*, including M235T and one SNP in the *AGTR1*, were also significantly associated with nephropathy (M235T $P=0.01$, odds ratio =0.74, 95% CI 0.59–0.94). In addition, we found that the allelic mRNA expression corresponding to the 235M allele was significantly higher than that for the 235T allele in normal kidney tissues. Furthermore, we found a significant additional effect of these three genes by a step-wise logistic regression analysis (final empirical P value =0.00005). We concluded that RAS gene polymorphisms may contribute to the susceptibility to diabetic nephropathy in type 2 diabetes.

Keywords Diabetic nephropathy · Polymorphism · *ACE* · *AGT* · *AGTR1* · Type 2 diabetes

Electronic supplementary material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s10038-006-0090-5 and is accessible for authorized users.

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Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease in Western countries and Japan [excerpts from the United States Renal Data System (USRDS) 2001; Patient Registration Committee 2002]. Several genetic and environmental factors are likely to contribute to its development and progression, although the precise mechanisms for these contributions are unknown. Some studies have indicated that the systemic and/or intrarenal renin–angiotensin system plays a pivotal role in the pathogenesis of diabetic nephropathy (Cooper et al. 2001; Leehey et al. 2000). Local production of angiotensin II is considered to mediate the increase of intraglomerular pressure via

constriction of efferent arterioles and thus contributes to the development and progression of glomerulosclerosis seen in various renal diseases, including diabetic nephropathy. It has also been shown that angiotensin II is able to stimulate the production of several cytokines, such as TGF-β (Kagami et al. 1994) or reactive oxygen species (Jaimes et al. 1998) and to mediate the over-accumulation of extracellular matrix proteins or cause various cellular dysfunctions under diabetic conditions. In addition, some clinical trials have demonstrated that blockage of angiotensin II with either ACE inhibitor or an angiotensin type-I receptor antagonist was able to prevent or delay the progression of renal injury associated with diabetes (Lewis et al. 1993; Brenner et al. 2001), and now these drugs have been recommended as first-choice drugs for the treatment of diabetic subjects with hypertension (American Diabetes Association 2004). Therefore, the importance of the renin–angiotensin system with respect to the pathogenesis of diabetic nephropathy is well established. However, the precise mechanism for the regulation of the renin–angiotensin system in subjects with diabetes mellitus remains unknown.

In contrast, accumulating evidence suggests that genetic susceptibility plays an important role in the development and progression of diabetic nephropathy. The genes encoding some components of the renin–angiotensin system (RAS), such as angiotensin-converting enzyme [*ACE* (MIM 106180)], angiotensinogen [*AGT* (MIM 106150)] and angiotensin II receptor type 1 [*AGTR1* (MIM 106165)], have been reported to be the most probable candidate genes for diabetic nephropathy. An insertion/deletion (I/D) polymorphism in the 16th intron of the *ACE* gene was reported to be associated with blood pressure and plasma levels of ACE (Rigat et al. 1990). The I/D polymorphism has also been reported to be associated with renal expression of ACE (Mizuiri et al. 2001) as well as susceptibility to diabetic nephropathy. The association of CSNP with substitution of methionine at codon 235 to threonine (M235T) in *AGT* and a SNP in the 3′UTR of *AGTR1* (A1166C) with diabetic nephropathy has also been examined. In addition, several reports have demonstrated an interaction among these RAS gene polymorphisms. One study indicated that the *ACE* I/D and *AGT* M235T polymorphisms interacted significantly in subjects with type 1 diabetes (Marre et al. 1997). It was also reported that the number of D (*ACE* I/D)/M (*AGT* M235T)/A (*AGTR1* A1166C) alleles influenced the rate of the deterioration of renal function (Jacobsen et al. 2003). However, to date, the results for the association of these RAS genes with diabetic nephropathy have been inconclusive.

In the present study, we investigated the association between these RAS gene polymorphisms and the development and progression of diabetic nephropathy in type 2 diabetes and provide evidence that shows that the *ACE*, *AGT* and *AGTR1* genes may be independently involved in the susceptibility to diabetic nephropathy.

Materials and methods

DNA preparation and genotyping

DNA samples were obtained from the peripheral blood of patients with type 2 diabetes who regularly attend outpatient clinics at Shiga University of Medical Science, Tokyo Women's Medical University, Juntendo University, Kawasaki Medical School, Iwate Medical University, Toride Kyodo Hospital, Kawai Clinic, Osaka City General Hospital, or Chiba Tokusyukai Hospital. All subjects provided informed consent and were enrolled in this study between 1996 and 2003. DNA extraction was performed according to a standard phenol–chloroform protocol. Diabetic patients were divided into two groups according to the following diagnostic criteria: (1) cases of diabetic nephropathy, i.e., patients with diabetic retinopathy as well as overt nephropathy, indicated by urinary albumin excretion rates (AERs) ≥200 μg/min or urinary albumin-to-creatinine ratios (Alb/Cr) ≥300 mg/g Cr or patients under chronic renal-replacement therapy; (2) control patients with diabetic retinopathy, but showing normal urinary albumin excretion, i.e., AER <20 μg/min or Alb/Cr <30 mg/g Cr. Clinical characteristics of the subjects are shown in Table 1. There are significant differences in gender distribution, duration of diabetes and blood pressures between the case and control groups. SNPs within the *ACE*, *AGT* and *AGTR1* genes were selected from the IMS-JST SNP database

Table 1 Clinical characteristics of the patients

	Case	Control	*P*
n	747	557	
Sex (M:F)	504:243	270:287	<0.0001[a]
Age	60.1±11.7	62.4±10.4	0.0004
Duration (year)	19.3±9.7	15.3±8.9	<0.0001
HbA1c (%)	7.6±3.6	7.6±1.2	0.98
BMI (kg/m^2)	23.8±3.7	23.6±3.3	0.41
SBP (mmHg)	148±20	136±17.0	<0.0001
DBP (mmHg)	80±11	77±10	<0.0001

Data are shown as means ± SD; statistical significance between the two groups was analyzed using the unpaired Student's *t* test

[a] Chi square test

(available at http://www.snp.ims.u-tokyo.ac.jp) and analyzed in 747 cases and 557 controls by Invader assay, as previously described (Ohnishi et al. 2001). We also determined the genotype of I/D polymorphisms within the *ACE* gene in 94 control subjects to calculate the linkage disequilibrium coefficient (Δ) between the *ACE* SNPs and the I/D polymorphisms using a PCR method as described previously (Cambien et al. 1992). The protocol was approved by the Ethics Committee of the Institute of Physical and Chemical Research.

Statistical analysis

We assessed the difference in the genotype and/or allele frequencies between case and control groups by chi-square test. To analyze clinical data, the statistical significance between any two groups was analyzed by one-way analysis of variance followed by Sheffe's test. We calculated odds ratios and 95% confidence intervals (CI) with respect to the minor allele compared with the major allele. We calculated LD coefficients (Δ) as described previously (Yamada et al. 2001). We also analyzed the interaction among the SNPs within the RAS genes by step-wise logistic regression analysis according to a previously described method (Cordell and Clayton 2002; Kanazawa et al. 2004). Briefly, the probability, P, of an individual being a case rather than a control is assumed to be affected by a set of SNPs according to the logistic model: for example, $\text{logit}(P) = a_0 + a_1x_1 + a_2x_2$ for single SNP. Here, we use a coding scheme $x_1 = -1, 0, 1$ and $x_2 = -0.5, 0.5, -0.5$ for genotypes 1/1, 1/2 and 2/2, respectively, to represent an additive effect by x_1 and a dominance/recessive effect by x_2 (Cordell and Clayton 2002). The weights are estimated by the maximum-likelihood method and tested by comparison with the null-hypothesis $\text{logit}(P) = a_0$ (constant). For multiple SNPs, interaction effects are added further in addition to the main effects of additional SNPs and tested stepwise whether their effects were significant or not. The tests were performed using *R*.

Measurement of M235T allelic mRNA expression for the *AGT*

One microgram of total RNA prepared from the kidney tissues with informed consent was reverse-transcribed using the SuperScript first-strand synthesis system (Invitrogen), and the cDNA was subjected to PCR reaction with the following primers to amplify target fragments: forward: 5′-GAGAAGATTGACA GGTTCATGCAG-3′; reverse: 5′-GTCACCGAGAA GTTGTCCTGG-3′. For the determination of allelic mRNA expression, the invader assay was performed, and a real-time intensity of fluorescence (FAM for 235M, and Yellow for 235T) was measured using the M×3000P Multiplex Quantitative PCR system (STRATAGENE, La Jolla, CA). Sequential dilution of an amplified product from genomic DNA of the subjects with heterozygousity for the *AGT* gene M235T polymorphism was used for making standard curbs for each allele. The allelic mRNA expression for each allele was compared individually for five subjects, and the statistical difference was analyzed by paired *t*-test. In addition, the ratio of mRNA corresponding to 235M to 235T was compared to that of internal controls; those were amplified products from genomic DNAs for four subjects with heterozygousity of the M235T polymorphism, and the difference was evaluated with an unpaired *t*-test.

Results

Association of SNPs in the RAS genes with diabetic nephropathy

Ten SNPs within the *ACE* gene were successfully genotyped (Fig. 1; Supplementary Table 1; nine intronic SNPs and one synonymous SNP in the 16th exon). As shown in Fig. 1, eight SNPs were significantly associated with diabetic nephropathy ($P<0.05$), and among them, five SNPs were almost in complete linkage disequilibrium with the I/D polymorphism within the 16th intron of the gene (Δ =1, Fig. 1). We found a significant association between several SNPs within the *ACE* gene and diabetic nephropathy, and the allele corresponding to the D allele for the I/D polymorphism was identified as a risk factor for diabetic nephropathy ($P=0.01$, odds ratio =1.34, 95% CI 1.07–1.69, Fig. 1; Table 2, Supplementary Table 1). Interestingly, this association was mainly observed in the female group (Table 2).

Regarding the *AGT* gene, six SNPs including the M235T polymorphism were identified (Fig. 2; Supplementary Table 2), and M235T and two other SNPs were significantly associated with diabetic nephropathy (M235T: $P=0.01$, odds ratio =0.74, 95% CI 0.59–0.94, Fig. 2; Table 2, Supplementary Table 2). We also found a gender-specific association of the M235T polymorphism with diabetic nephropathy in the female group (Table 2).

In the *AGTR1* gene, 26 SNPs were genotyped (Fig. 3; Supplementary Table 3), and 23 SNPs were located in introns. Three SNPs including the A1166C polymorphism were located in exons. None of the mutations were predicted to cause amino acid

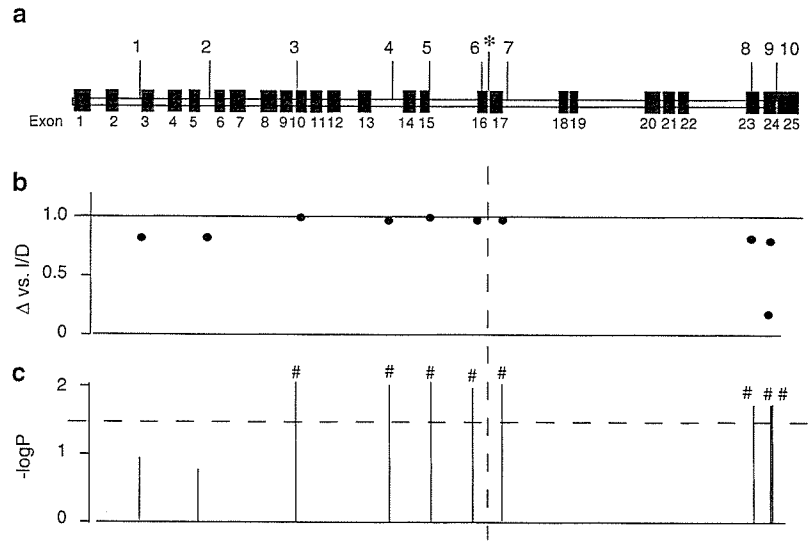

Fig. 1 Analysis of polymorphisms within the *ACE* gene. **a** The structure of the *ACE* gene and localization of 16th intron insertion/deletion polymorphism (*asterisk*) and ten SNPs selected from the IMS-JST SNP database. **b** Linkage disequilibrium coefficient (Δ) between each SNP and the I/D polymorphism. **c** Result of chi-square test. *Bars* over the horizontal breaking line (#) were considered to be significant ($P<0.05$)

Table 2 Association of the RAS polymorphism with diabetic nephropathy

	Genotype	Allele	Dominant	Recessive
[rs4344: *ACE* Δ = 1 vs. I/D intron 17+231 (G > A)]				
Total				
P	0.03	0.049	0.011	0.81
OR (95%CI)		1.18 (1.00–1.40)	1.34 (1.07–1.69)	1.04 (0.74–1.46)
Male				
P	0.29	0.44	0.19	0.69
OR (95%CI)		1.09 (0.87–1.37)	1.22 (0.90–1.66)	0.91 (0.59–1.43)
Female				
P	0.01	0.01	0.004	0.44
OR (95%CI)		1.38 (1.06–1.79)	1.69 (1.18–2.44)	1.24 (0.72–2.12)
[rs699 (M235T), *AGT* exon2 + 806 (T > C)]				
Total				
P	0.029	0.039	0.012	0.77
OR (95%CI)		0.81 (0.66–0.99)	0.74 (0.59–0.94)	1.10 (0.57–2.15)
Male				
P	0.59	0.34	0.83	0.31
OR (95%CI)		0.88 (0.67–1.15)	0.85 (0.62–1.16)	0.92 (0.41–2.03)
Female				
P	0.03	0.03	0.012	0.77
OR (95%CI)		0.71 (0.52–0.98)	0.63 (0.43–0.90)	1.20 (0.34–4.20)
[rs388915: *AGTR1* intron2 + 21,942 (T > C)]				
Total				
P	0.029	0.59	0.19	0.06
OR (95%CI)		0.94 (0.76–1.17)	0.85 (0.67–1.08)	1.93 (0.95–3.92)
Male				
P	0.22	0.92	0.53	0.17
OR (95%CI)		0.99 (0.74–1.31)	0.90 (0.65–1.25)	1.99 (0.73–5.42)
Female				
P	0.06	0.42	0.15	0.17
OR (95%CI)		0.87 (0.62–1.22)	0.75 (0.51–1.11)	2.02 (0.72–5.64)

substitutions. One SNP within the second intron was significantly associated with diabetic nephropathy (P=0.029, Fig. 3; Table 2). In our study, however, the A1166C polymorphism was not associated with diabetic nephropathy (P=0.17, Fig. 3).

We further evaluated interactions among the SNPs within the RAS genes by step-wise logistic regression analysis. The analysis was based on a full genotype model that includes all effects of additive, dominance/recessive and interaction between SNPs (see Materials

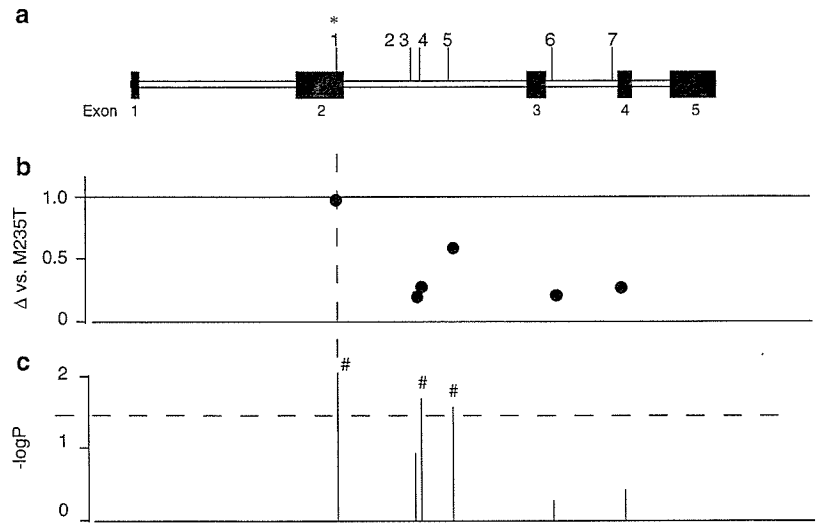

Fig. 2 Analysis of SNPs within the *AGT* gene. **a** The structure of the *AGT* gene and localization of each SNP. An *asterisk* indicates the M235T polymorphism. **b** Linkage disequilibrium coefficient (Δ) between each SNP and the M235T polymorphism. **c** Result of chi-square test for the association study

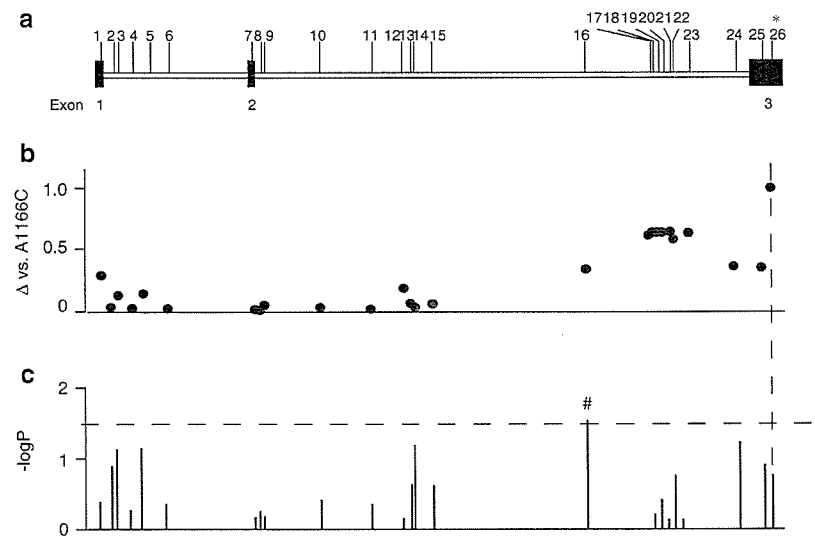

Fig. 3 Analysis of SNPs within the *AGTR1* gene. **a** The structure of the *AGTR1* gene and localization of each SNP. An *asterisk* indicates the A1166C polymorphism. **b** Linkage disequilibrium coefficient (Δ) between each SNP and A1166C polymorphism. **c** Result of chi-square test for the association study

and methods). The result indicated that there was no significant synergistic interaction among any of the SNPs examined. However, the significant additional effect of several SNPs (SNP at intron 17 + 231 and intron 24 + 97 of the *ACE* gene, M235T SNP of the *AGT* gene and SNP at intron 2 + 21,942 of the *AGTR1* gene) was detected in this population (final empirical *P* value =0.00005, Table 3).

The allelic mRNA expression of the *AGT* corresponding to each allele for the M235T polymorphism

We measured the allelic mRNA expression for each allele using invader assay (see Materials and methods). As a result, we could find that the mRNA expression corresponding to the 235M allele was higher than the mRNA corresponding to the 235T allele in normal kidney tissues from all five individuals (Fig. 4). Also, the expression ratio for 235M to 235T in the kidney was shown to be significantly increased compared to that for the genomic DNA from heterozygous subjects, which was considered to be an internal control (3.20±1.41, 0.98±0.11, kidney mRNA, and genomic DNA, respectively, P=0.017).

Discussion

In this study we found a significant association of the *ACE* gene with diabetic nephropathy in a large cohort

Table 3 Results of step-wise logistic regression analysis

Combination of SNPs	Significance of addition	Combined empirical *P* value
rs388915	–	–
rs388915 + rs2242638	0.01	0.00075
rs388915 + rs2242638 + rs699	0.02	0.00017
rs388915 + rs2242638 + rs699 + rs4344	0.03	0.00005

Number of risk genotype[a]					
	0	1	2	3	4
Case (*n*=704)	5 (0.7%)	103 (14.6%)	319 (45.3%)	264 (37.5%)	13 (1.8%)
Control (*n*=508)	13 (2.6%)	114 (22.4%)	217 (42.7%)	162 (31.3%)	2 (0.4%)
OR	0.27	0.59	1.11	1.28	4.76
95% CI	0.1–0.77	0.44–0.80	0.88–1.40	1.01–1.63	1.07–21.2

[a] Risk genotype: CC for rs388915 (AGTR1); TT, TC for rs2242638 (ACE), TT for rs699 (AGT); GA, AA for rs4344 (ACE)

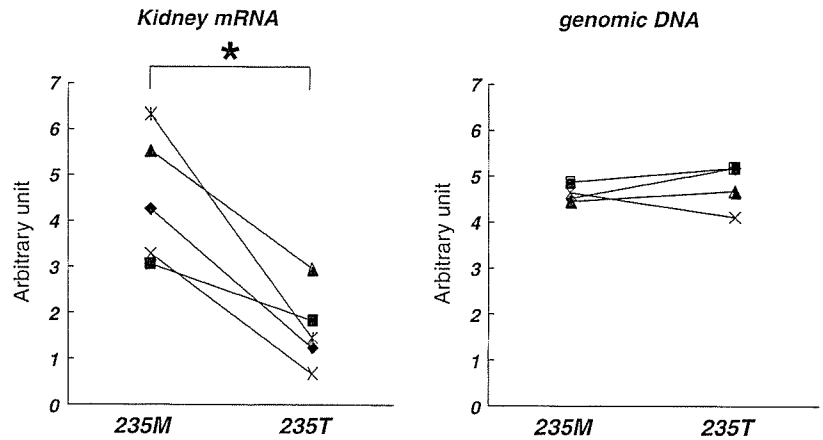

Fig. 4 Allelic expression of the AGT mRNA corresponding to M235T polymorphism in the kidney. The allelic mRNA expression for each allele in the kidney tissue (*left*). Quantification of the control products amplified from genomic DNAs of heterozygous subjects (*right*). *$P<0.05$

of Japanese subjects with type 2 diabetes. Our present results also suggest that the *AGT* gene and the *AGTR1* gene also contribute to susceptibility to diabetic nephropathy, and these three genes may have a significant additional effect in conferring susceptibility to the disease.

The existence of genetic factors for the development and progression of diabetic nephropathy is well accepted, and the gene-encoding components of RAS are considered to be one of the most probable candidates. Furthermore, the association of RAS genes with diabetic nephropathy has been extensively examined, especially with respect to an insertion deletion polymorphism within the 16th intron of the *ACE* gene. Although many reports have provided evidence that the D allele of the *ACE* I/D polymorphism is a risk allele for the development and progression of diabetic nephropathy (Ng et al. 2005; Tomino et al. 1999), several conflicting results have also been reported (Grzeszczak et al. 1998; Kimura et al. 1998). In the present study, we identified several SNPs that were almost in complete linkage disequilibrium with the I/D polymorphism, and these SNPs were significantly associated with diabetic nephropathy. The allele corresponding to the D allele for I/D polymorphism was identified as a risk factor for diabetic nephropathy in our study population (Fig. 1; Table 2). Therefore, the results presented here are consistent with most previous reports.

The association of the *AGT* and *AGTR1* genes with diabetic nephropathy has also been extensively examined, but, to date, the results have not been conclusive. The M235T polymorphism of the *AGT* gene was originally reported to be associated with hypertension (Jeunemaitre et al. 1992), and the TT genotype of this polymorphism was also shown to confer a risk for the progression of diabetic nephropathy (Freire et al. 1998). However, another study failed to identify the distinct role of the *AGT* gene in conferring susceptibility to the disease (Zychma et al. 2000). From the results presented here, the carrier of the M allele was thought to be at risk for nephropathy (Table 2).

The functional implications of this substitution are not fully understood. In this study, we examined the

allelic *AGT* gene expression corresponding to each allele for the M235T polymorphism in the kidney and found that the expression of the allelic mRNA corresponding to the 235M allele was consistently higher than mRNA corresponding to the 235T allele in the kidney tissues (Fig. 4). Since we examined the difference in the allelic mRNA expression in the individual kidney samples from heterozygous subjects, we could exclude the effects of environmental and/or other non-genetic factors, and then the present measurement of allelic mRNA expression was considered to directly reflect the genetic contribution itself. Therefore, the carrier of the M allele, rather than the T allele, may be more susceptible to diabetic nephropathy. In addition, Jacobsen et al. (2003) identified a combinational effect of the D allele of the *ACE* gene and the M allele on the progression of diabetic nephropathy, further supporting our present finding. Regarding the *AGTR1* gene, the A1166C polymorphism in the 3′UTR of the gene was reported to be associated with the progression of diabetic nephropathy in the Japanese population (Tomino et al. 1999), although most case–control studies did not identify a significant association of this polymorphism with diabetic nephropathy. The A1166C polymorphism was also not associated with the disease in the present population. Instead, we found a significant association between the SNP in the second intron of the *AGTR1* gene and nephropathy. However, the association of this SNP with nephropathy was modest (Table 2), and further study will be required to evaluate a distinct effect of this polymorphism.

Because there were several differences in the clinical parameters between the case and control subjects (Table 1), we also analyzed the association of the RAS gene polymorphisms using control subjects with longer diabetes duration (≥10 years), those whose diabetes duration was not different from the case group (case 19.3±9.7, control 19.0±7.5; P=0.58), and identified the same association as the original finding (Supplementary Table 4). We also evaluated the effects of the RAS gene polymorphisms in male and in female groups separately, since gender distribution was significantly different. Interestingly, some of the RAS gene polymorphisms contributed to the susceptibility to the disease in a gender-specific manner (Table 2). The gender-specific effects of the RAS genes have also been shown in previous reports (Tomino et al. 1999; Friere et al. 1998; Fradin et al. 2002), although the results were not always consistent, probably because of the differences in the ethnicity or in the number of the subjects evaluated.

Susceptibility to common diseases such as diabetic nephropathy is thought to be determined by several genetic factors. Therefore, the combinational effect of individual genes should be more emphasized. Regarding the RAS genes, several reports on type 1 diabetes suggested that there were some interactive effects among RAS gene polymorphisms in the development and progression of diabetic nephropathy (Marre et al. 1997; Jacobsen et al. 2003), although the results are not conclusive. To evaluate the interaction of the three genes examined in the present study more accurately, we applied a step-wise logistic regression analysis as previously reported (Cordell and Clayton 2002). Using this analysis, we were able to determine the significance of interactive and/or additional effects of all combinations, and therefore, we were able to evaluate the interactions of each gene more precisely. As a result, we found significant additional effects among SNPs at intron 17 + 231 (Δ =1 vs. I/D), intron 24 + 97 of the *ACE* gene, M235T SNP of the *AGT* gene and SNP at intron 2 + 21,942 of the *AGTR1* gene. Since we were unable to identify a synergistic effect of the genes, the effect of each of the three genes is likely to be independent, and simple additional effects could be observed. The results of logistic regression analysis also indicated that the increased number of risk alleles (alleles corresponding to the D allele, C allele for rs2242638 in the *ACE*, M allele for the *AGT* and C allele of rs388915 for the *AGTR1*) was an independent

Table 4 Result of logistic regression analysis

	β	SE (b)	OR	95% CI	P
Sex (male 1; female 2)	0.821	0.177	2.27	1.61–3.22	<0.0001
Age	–0.040	0.008	0.96	0.95–0.98	<0.0001
BMI	0.016	0.024	1.02	0.97–1.07	0.51
Duration of diabetes	0.060	0.010	1.06	1.04–1.08	<0.0001
HbA1c	0.011	0.031	1.01	0.95–1.07	0.73
Hypertension	2.169	0.215	8.75	5.74–13.34	<0.0001
Number of risk genotype	0.348	0.111	1.42	1.14–1.76	0.0017

β Regression coefficient, *SE* standard error, *OR* odds ratio, *CI* confidence interval

risk for the development and progression of diabetic nephropathy (P=0.0017, OR =1.42, 95% CI: 1.14–1.76; Table 4).

The mechanism by which the RAS gene polymorphisms contributes to the susceptibility to diabetic nephropathy should be elucidated. Although all SNPs within the RAS genes examined did not have any significant association with blood pressure or prevalence of hypertension in the present cross-sectional analysis (data not shown), further studies, such as a longitudinal prospective study or in vitro functional analysis, are necessary to elucidate the precise mechanism of the effect of the RAS gene in conferring susceptibility to diabetic nephropathy.

In conclusion, we identified significant associations between RAS gene polymorphisms and diabetic nephropathy in type 2 diabetes. Our present result from a step-wise logistic regression analysis also indicated that the *ACE*, *AGT* and *AGTR1* genes may be independently involved in the susceptibility to diabetic nephropathy.

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ELMO1 increases expression of extracellular matrix proteins and inhibits cell adhesion to ECMs

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We have previously identified the engulfment and cell motility 1 (*ELMO1*) as a susceptibility gene for diabetic nephropathy. To elucidate the role of *ELMO1* in the pathogenesis of chronic renal injury, we examined the expression of *Elmo1* in the kidney of a rat model for chronic glomerulonephritis (uninephrectomy plus anti-Thy1.1 antibody [E30] injection). We found that the expression of the *Elmo1* was significantly increased in the renal cortex and glomeruli of uninephrectomized rats injected with E30 compared to controls. By *in situ* hybridization, the expression of *Elmo1* was shown to be elevated in the diseased kidney, especially in glomerular epithelial cells. In COS cells, the overexpression of *ELMO1* resulted in a substantial increase in fibronectin expression, whereas the depletion of the *ELMO1* by small interfering RNA (siRNA) targeting *ELMO1* significantly suppressed the fibronectin expression in *ELMO1* overexpressing and control cells. We also found that the expression of integrin-linked kinase (ILK) was significantly increased in *ELMO1* overexpressing cells, and the *ELMO1*-induced increase in fibronectin was partially, but significantly, inhibited by siRNA targeting ILK. Furthermore, we identified that the cell adhesion to ECMs was considerably inhibited in cells overexpressing *ELMO1*. These results suggest that the *ELMO1* contributes to the development and progression of chronic glomerular injury through the dysregulation of ECM metabolism and the reduction in cell adhesive properties to ECMs.

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KEYWORDS: chronic glomerulonephritis; extracellular matrix; cell adhesion; fibronectin; gene expression

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In progressive renal diseases such as chronic glomerulonephritis or diabetic nephropathy, its pathological lesion is characterized by the accumulation of extracellular matrix (ECM) proteins in the glomeruli.[1–4] Recent studies have indicated that the abnormal functions of glomerular cells, such as glomerular epithelial cells (GEC) and glomerular mesangial cells, may play a key role in the development and progression of chronic glomerular injury.[5–7] The detachment of GEC from the glomerular basement membranes (GBMs),[8,9] and/or excess of the production of ECM proteins in glomerular cells,[10,11] is thought to mediate the establishment of the chronic glomerular lesions. However, the precise mechanisms that cause these abnormalities have not yet been elucidated.

We previously identified the engulfment and cell motility 1 gene (*ELMO1*) as a novel candidate for diabetic nephropathy.[12] We also demonstrated that the expression of *ELMO1* could be observed mainly in the GEC, and was elevated in cells cultured in high glucose conditions as well as in the glomeruli of diabetic db/db mice. These results suggest that *ELMO1* may play a role in the development and progression of chronic glomerular injury, such as diabetic glomerulosclerosis. However, the contribution of the *ELMO1* gene to the pathogenesis of these glomerular diseases still remains uncertain.

In the present study, we examined the role of the *ELMO1* gene in the pathogenesis of chronic glomerular injury, and provide evidence that the expression of the rat *Elmo1* gene was increased in the kidney of an animal model for chronic glomerulonephritis. Furthermore, we suggest that *ELMO1* may contribute to the development of the disease by increasing the production of ECM proteins or by the reduction in cell adhesive properties to the ECM.

RESULTS

Expression of the rat *Elmo1* gene is increased in the kidney of unilaterally nephrectomized rats injected with anti-Thy1.1 antibody [E30]

We first examined the expression of the *Elmo1* gene in the kidney of a rat model for chronic glomerulonephritis by real-time quantitative polymerase chain reaction (PCR). As shown in Figure 1a, the expression of the *Elmo1* gene in the renal cortex was significantly higher in uninephrectomized

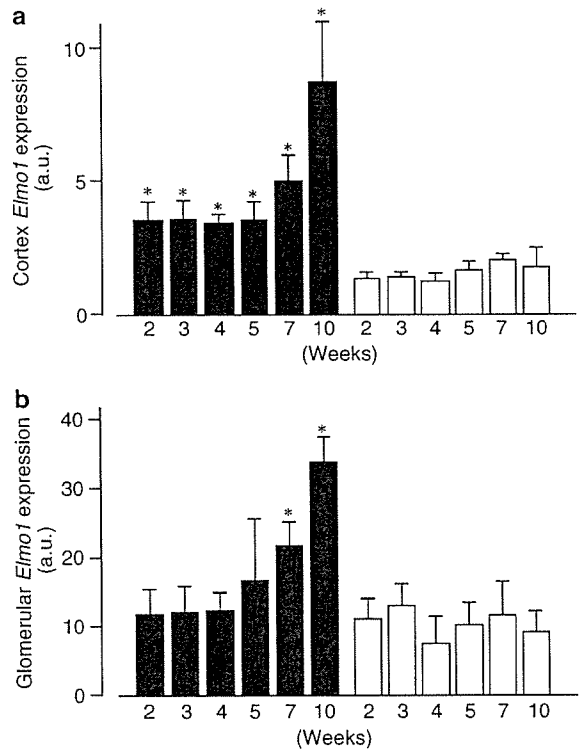

Figure 1 | The expression of *Elmo1* in the kidney of a rat model for chronic glomerulonephritis analyzed by real-time quantitative RT-PCR. Total RNAs were prepared from (**a**) renal cortex and (**b**) glomeruli 2, 3, 4, 5, 7, and 10 weeks after E30 or vehicle injection. ■, Uninephrectomy + E30 injection; □, uninephrectomy and vehicle injection (control). Each bar indicates mean ± s.d., *$P<0.01$ vs control.

rats injected with E30 (monoclonal antibody for Thy-1.1) than in control rats during the observation period. In isolated glomeruli, *Elmo1* expression was significantly increased at seven and 10 weeks compared to control rats (Figure 1b). In uninephrectomized rats injected with E30, the expression of *Elmo1* was gradually increased according to the progression of the disease, and significantly increased at 10 weeks compared to those at 2 weeks after injection with E30 (cortex: 8.7 ± 2.1 and 3.5 ± 0.8; glomeruli: 33.9 ± 3.8 and 11.9 ± 3.8, mean ± s.d., 10 and 2 weeks, respectively, $P<0.0001$), whereas those of control rats remained unchanged during the observation period.

Results of *in situ* hybridization

By *in situ* hybridization, we also found that the expression of *Elmo1* was clearly elevated in the kidney of uninephrectomized rats injected with anti-Thy-1.1 antibody [E30] (Figure 2e–g) compared to that of control rats (Figure 2a–c). The elevation of *Elmo1* expression was observed mainly in GEC and in interstitial infiltrating cells (Figure 2j), especially in the damaged glomeruli (Figure 2j and l) compared to relatively unaffected glomeruli (Figure 2n). The expression of the *elmo1* could be detected also in the peritubular capillaries (Figure 2j).

ELMO1 increases the expression of fibronectin

In order to ascertain the possible involvement of *ELMO1* in the development of chronic glomerular injury, we investigated the effects of *ELMO1* on the expression of fibronectin by real-time quantitative reverse transcriptase (RT)-PCR and enzyme-linked immunosorbent assay. As shown in Figure 3a and b, the expression of fibronectin was markedly increased in cells overexpressing *ELMO1* compared to control cells. The small interfering RNA (siRNA) treatment targeting *ELMO1* resulted in significant silencing of *ELMO1* expression in *LacZ*-(control) and *ELMO1*-overexpressing cells (15 ± 5.4 and 35 ± 7.7% of those in cells treated with control siRNA for LacZ- and *ELMO1*-overexpressing cells, respectively). The treatment of siRNA for *ELMO1* resulted in a significant reduction of the expression of fibronectin in *LacZ*- and *ELMO1*-overexpressing cells (fibronectin mRNA, Figure 3a: 60 ± 5.0% and 64 ± 12%; fibronectin protein excreted into culture media, Figure 3b: 32 ± 3.6 and 61 ± 7.7% of those in cells treated with control siRNA for LacZ- and *ELMO1*-overexpressing cells, respectively).

ELMO1 overexpression inhibits cell adhesion to ECMs

To further understand the involvement of *ELMO1* in the development of chronic glomerular-injury, we examined the effect of *ELMO1* overexpression on the cell adhesion to the extracellular matrices using COS cells stably overexpressing *ELMO1*. As shown in Figure 4a–f, cell adhesion to extracellular matrices was considerably inhibited in cells overexpressing *ELMO1* compared to control cells (collagen type I: 0.26 ± 0.048 and 0.20 ± 0.045; collagen type IV: 0.47 ± 0.062 and 0.58 ± 0.041; fibronectin: 0.71 ± 0.011 and 0.35 ± 0.087; vitronectin: 0.19 ± 0.075 and 0.094 ± 0.014; laminin: 0.73 ± 0.032 and 0.67 ± 0.033, of control cells for ELMO1-line1 and ELMO1-line2, respectively).

The expression of ILK was increased in the *ELMO1* overexpressing cells

We next examined the expression of integrin-linked kinase (ILK), focal adhesion kinase (FAK) and Src, which were considered to be involved in the regulation of cell adhesion or ECM metabolism, in cells overexpressing *ELMO1*. As shown in Figure 5a, the expression of ILK was significantly increased in *ELMO1* overexpressing cells compared with control cells (1.75 ± 0.11- and 1.82 ± 0.077-fold increase compared to control cells for *ELMO1*-line1 and *ELMO1*-line2, respectively), whereas expression of FAK and Src did not differ significantly between the *ELMO1*-overexpressing and control cells.

The effects of ILK knockdown on the expression of ECM genes

As shown in Figure 6, the treatment with ILK siRNA could partially, but significantly, inhibit the increase in the expression of collagen type I (α1) and fibronectin in cells stably overexpressing *ELMO1*. The effects of *ELMO1* overexpression or knockdown of the ILK on the expression of ILK

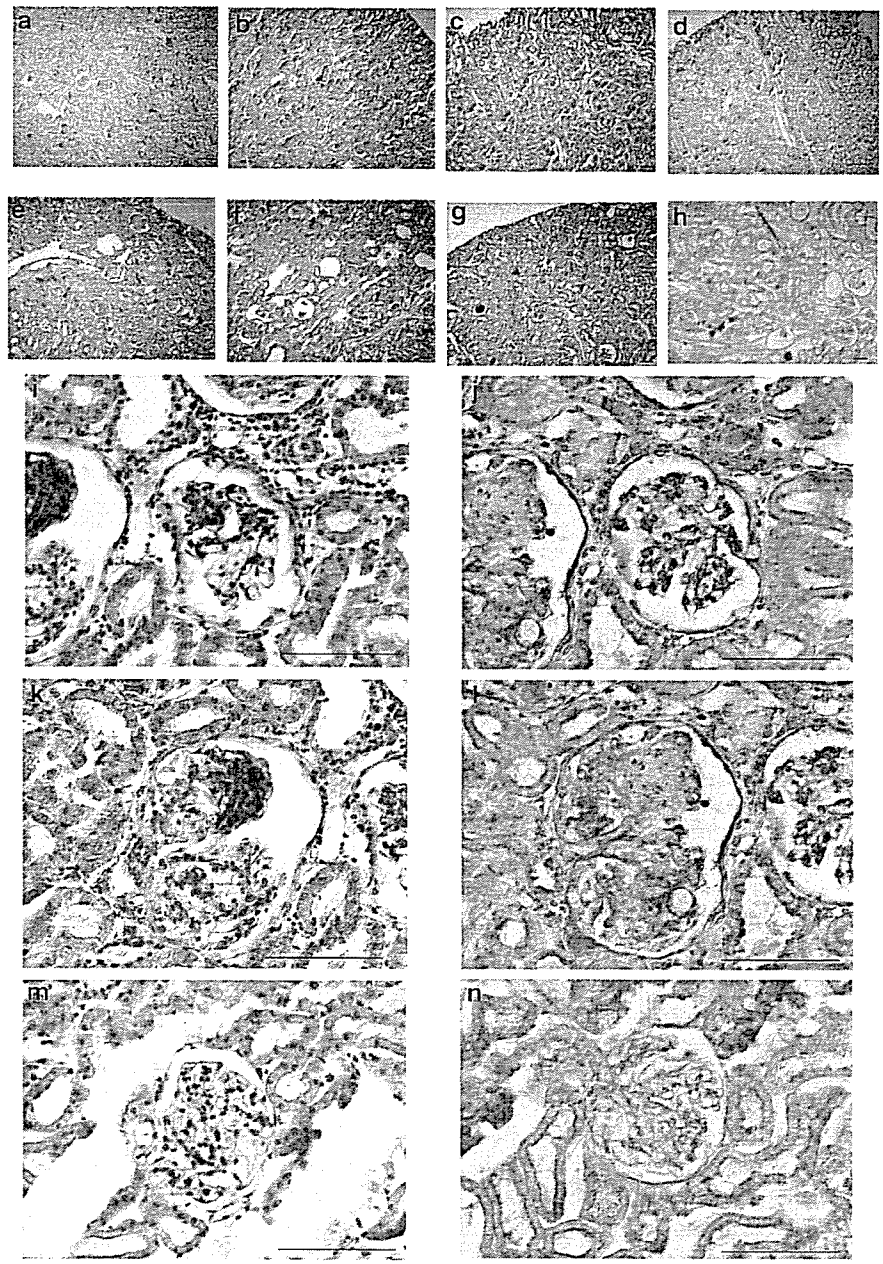

Figure 2 | Results of *in situ* hybridization using the kidney of an uninephrectomized rat injected with E30. (**a–h**) *In situ* hybridization for *Elmo1*, (**a–d**) uninephrectomy with vehicle (control), (**e–h**) uninephrectomy + E30 injection, (**a, e**) 3 weeks, (**b, f**) 5 weeks, (**c, d, g, h**) 7 weeks after vehicle or E30 injection. (**a–c, e–g**) Anti-sense, (**d, h**) sense probe. (**i, k, m**) Periodic acid Schiff stain and hematoxylin staining, (**j, l, n**) *in situ* hybridization for *Elmo1*, glomerulus of nephrectomized rat 7 weeks after E30 injection. Bar = 100 μm.

could be confirmed by Western blot analysis (Figure 6c) or by ILK kinase assay (Figure 6d).

DISCUSSION

In this study, we demonstrated that the expression of rat *Elmo1* was increased in the kidney of an animal model for chronic glomerular injury. Our data also suggest that the overexpression of *ELMO1* resulted in the accumulation of ECM proteins and in the reduction in cell adhesion properties to the ECM.

Although the precise mechanism of the development and progression of chronic glomerulosclerosis remains uncertain, cumulative evidence suggests that an abnormality of the function of glomerular cells, such as GECs or mesangial cells, plays a pivotal role in the pathogenesis of chronic glomerular injury.

We have recently identified the *ELMO1* gene as a novel candidate for conferring susceptibility to diabetic nephropathy, and we also demonstrated that the expression of *Elmo1* was mainly observed in the GEC in the normal mouse kidney.[12] We show here, for the first time, that the expression

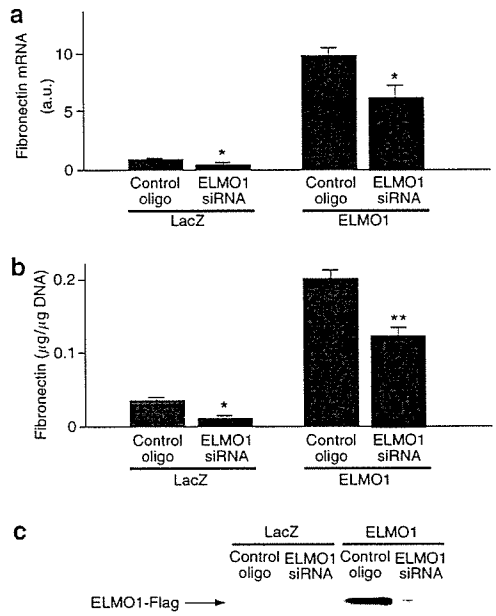

Figure 3 | The expression of fibronectin in the cells stably transfected with *LacZ* or *ELMO1*. (**a**) Fibronectin mRNA determined with real-time quantitative RT-PCR (**b**) excretion of fibronectin protein into culture media measured by enzyme-linked immunosorbent assay. (**c**) Western blot analysis using anti-FLAG monoclonal antibody. LacZ: LacZ overexpressing cells, ELMO1: *ELMO1* overexpressing cells, Control: cells treated with control oligonucleotide, ELMO1 siRNA: cells treated with siRNA targeting *ELMO1*. Each bar indicates mean ± s.d. of three independent experiments. $*P<0.05$, $**P<0.01$.vs cells treated with control siRNA.

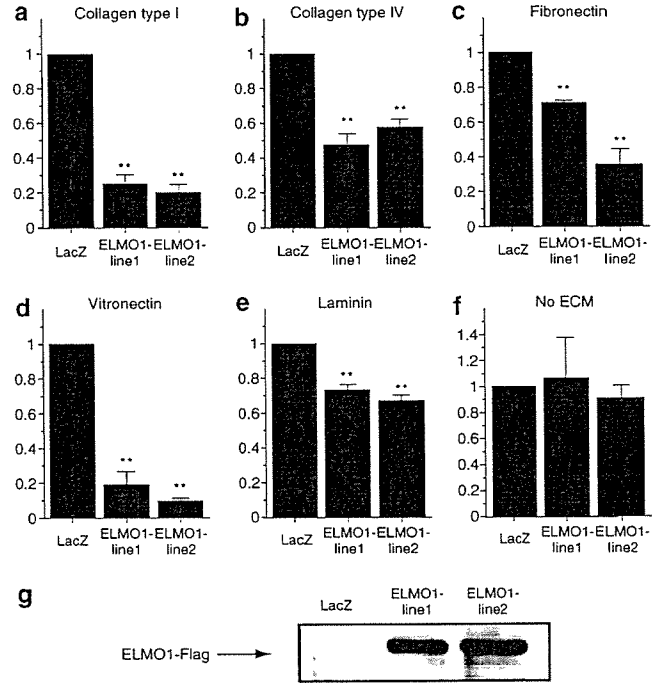

Figure 4 | *ELMO1* overexpression inhibits cell-adhesion to ECMs. The ability of *LacZ* and *ELMO1* overexpressing cells to adhere to ECMs was examined as described in the Materials and Methods section. (**a**) Collagen type I, (**b**) collagen type IV, (**c**) fibronectin, (**d**) vitronectin, (**e**) laminin, (**f**) no ECM. Results are presented as mean ± s.d. from four or five independent experiments. $**P<0.01$ vs *LacZ*. (**g**) Western blot analysis using anti-FLAG monoclonal antibody.

of the *Elmo1* gene is significantly increased in the glomeruli and cortex of uninephrectomized rats injected with E30 (an animal model for chronic glomerular injury). The results of *in situ* hybridization revealed that the elevation of *Elmo1* gene expression was mainly observed in the GEC, suggesting that the increase in *ELMO1* levels may contribute to the development and progression of chronic glomerular injury.

To test this hypothesis, we examined the effects of siRNA targeting *ELMO1* on cells stably overexpressing *ELMO1* and *LacZ* (control). As we reported previously, the expression of fibronectin was considerably increased in the cells overexpressing *ELMO1*. In addition, the suppression of *ELMO1* expression resulted in the significant reduction in the expression of ECM gene, both in the cells overexpressing *ELMO1* and in control cells. From these observations, the *ELMO1* gene can be considered to be a potent regulator for ECM gene expression, and the increase in the expression of *ELMO1* gene may contribute to the development and progression of glomerulosclerosis via the excess accumulation of ECM in the renal glomeruli.

Interestingly, we also identified that cells overexpressing *ELMO1* showed lower adhesive properties to the ECM proteins compared to control cells. The GBM is considered to function as a filtration barrier to maintain the homeostasis of blood components, such as albumin. Since the GEC were known to play a central role in maintaining the barrier function of the GBM by tight adhesion to the GBM, the detachment of the cells from the GBM may cause the loss of the barrier function of the GBM, and consequently result in the leakage of albumin into the urine.[13–16] Therefore, it can be speculated that the decrease in adhesive properties to the ECM proteins by *ELMO1* also contributes to the development of proteinuria during chronic glomerular injury.

The precise mechanism for the *ELMO1*-induced reduction in cell adhesive properties to the ECMs should be investigated. It is well known that transmembrane receptors, integrins, play a central role in the interaction between cells and ECM proteins.[17,18] Consequently, we examined the expression of members of the integrin family in cells overexpressing *ELMO1*, and identified a significant reduction in $\alpha 2$ and $\beta 3$ integrin mRNA in *ELMO1* overexpressing cells (data not shown). Therefore, we suggest that *ELMO1* decreases the cell adhesion properties to the ECMs through, at least in part, the reduction in the expression of these integrins.

In order to elucidate the mechanism of the *ELMO1*-induced increase in ECM expression and reduction in cell adhesive properties further, we investigated the effects of *ELMO1* on the expression of several protein kinases, such as FAK, ILK, and Src, which have all been reported to contribute to the regulation of ECM protein metabolism and/or cell adhesion properties.[19–23] We found that the expression of ILK

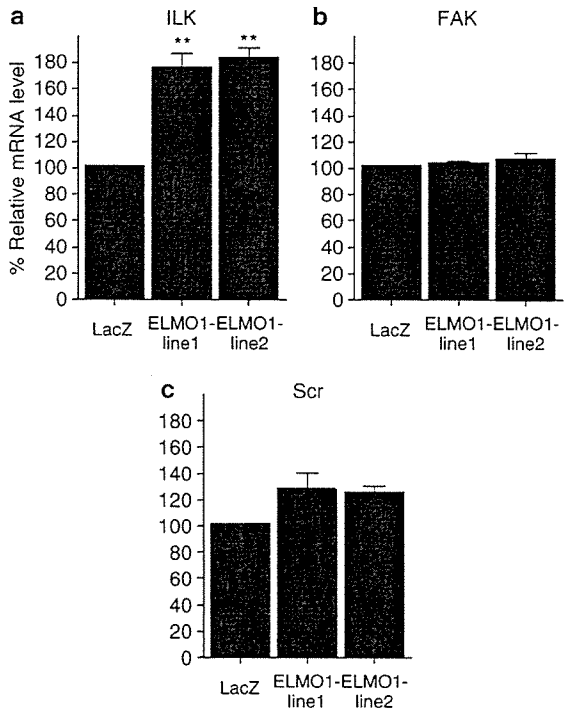

Figure 5 | The expression of several protein kinases in *ELMO1* and *LacZ* overexpressing cells. (**a**) ILK, (**b**) FAK, (**c**) Src. Data are presented as means ± s.d. of three independent experiments. $^{**}P < 0.01$ vs *LacZ*.

increased significantly in *ELMO1* overexpressing cells, whereas the expression of FAK and Src did not differ between *ELMO1* overexpressing and control cells. Since ILK is known to be responsible for the regulation of cell adhesion, and for the accumulation of ECM proteins by several types of cytokines, such as TGF-β, the increase in ILK was considered to mediate the effects of *ELMO1* in the expression of ECMs and the changes in the cell adhesion properties. Moreover, in further support of this hypothesis, the results of siRNA experiments targeting ILK indicated that the suppression of ILK mRNA partially, but significantly, abolished the *ELMO1*-induced increase in the expression of type 1 collagen (α1) and fibronectin (Figure 6).

The molecular mechanism of the influence of *ELMO1* on the expression of the genes reported here and/or cell adhesion properties is still unknown. Previous reports suggested that *ELMO1* cooperates with Dock180 to function as an activator for Rac-1.[24,25] Accordingly, we next examined the effects of Rac-1 and coexpression of Dock180 with *ELMO1* on the expression of ECM genes. However, no significant additional effects of these factors on the expression of these genes were detected (data not shown), suggesting the effects of *ELMO1* on the regulation of expression of these genes and/or cell adhesion properties were independent of Rac-1. The elucidation of the precise mechanisms requires further study.

In conclusion, we have shown that the expression of *Elmo1* was increased in the kidney of an animal model for

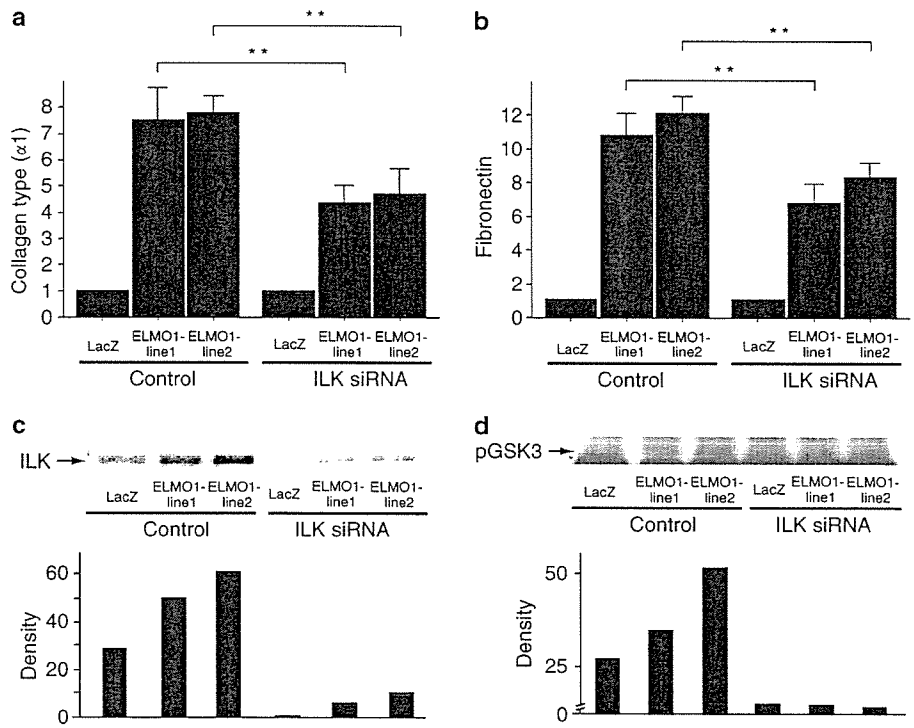

Figure 6 | The effects of ILK knockdown on the *ELMO1*-induced increase in ECM gene expression. (**a**) Collagen type 1 (α1), (**b**) fibronectin. Control: cells treated with control siRNA. ILK siRNA: cells treated with siRNA targeting ILK. Results were expressed as means ± s.d. of three independent experiments. $^{**}P < 0.01$ vs cells treated with control siRNA. (**c**) Western blot analysis for ILK. (**d**) Result of ILK activity assay. Representative results of three independent experiments were presented.

chronic glomerular injury. The examination of cells overexpressing *ELMO1* also indicated that the increase in *ELMO1* expression causes the increase in expression of ECMs and the reduction in cell adhesive properties. These results suggest that *ELMO1* plays a pivotal role in the development and progression of chronic glomerular injury, and may be a new target for the treatment of chronic glomerular disease.

MATERIALS AND METHODS

Materials

COS-7 cells were obtained from the Japan Health Sciences Foundation (Osaka, Japan), Dulbecco's modified Eagle medium, phosphate-buffered saline (PBS), penicillin G, streptomycin, and Trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, CA, USA). SYBR Green I and Crystal violet were obtained from CAMBREX Bio Science (Rockland, ME, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Ex Taq Buffer, dNTPs, and Ex Taq DNA polymerase were purchased from TaKaRa Bio (Otsu, Shiga, Japan).

Induction of E30 (monoclonal antibody against Thy-1.1 antigen)-induced chronic nephritis model in unilaterally nephrectomized rats

Seven-week-old male Wistar rats (Japan SLC, Inc., Shizuoka) were subjected to ligation of the left renal artery and renal vein, and then the left kidney was removed under sodium pentobarbital anesthesia. One week after the unilateral nephrectomy, the animals were divided into two groups that received either E30 (70 μg in 400 μl of saline) or saline vehicle via the tail vein.[26]

Isolation of total RNA from renal cortex and glomerulus

On the indicated days after injection of E30 or saline vehicle, animals were anesthetized with sodium pentobarbital, and the kidneys were perfused with PBS through the abdominal aorta. Next, the kidneys were removed, and the renal cortex was extracted and the isolated glomeruli were prepared by standard sieving techniques. Total RNA was extracted from the renal cortex and glomeruli by a standard technique followed by ethanol precipitation. Briefly, a small piece of the renal cortex or isolated glomeruli was homogenized in 500 μl of ISOGEN (Nippon Gene, Tokyo, Japan). After addition of 400 μl chloroform, the mixture was centrifuged for 15 min at 4°C (10 000 r.p.m.). The supernatant was mixed with an equal volume of isopropylalcohol and centrifuged for 10 min at 4°C (10 000 r.p.m.). The supernatant was precipitated with 70% ethanol three times and dried under reduced pressure.

In situ hybridization

For tissue preparation, the kidneys were perfused through the abdominal aorta with 4% paraformaldehyde in PBS under pentobarbital anesthesia. The renal tissue was immediately dissected and immersed in a fresh portion of the same fixative at 4°C overnight. Fixed samples were thoroughly rinsed with PBS, dehydrated by passage through an alcohol series, and cleared in xylene. *In situ* hybridization was performed on paraffin-embedded sections. Probes for *in situ* hybridization studies were prepared from cDNA fragments encoding *Elmo1* as previously described.[12] The tissue sections were permeabilized with proteinase K (Roche Diagnostics, Basel, Switzerland), and acetylated with 0.1 mol/l triethanolamine containing 0.25% acetic anhydride. Next, the sections were hybridized with 500 ng/ml digoxigenin (DIG)-labeled cRNA probe for 17 h at 50°C in a hybridization buffer (50% deionized formamide, 1 × Denhardt's solution, 10% dextran sulfate, 600 mmol/l NaCl, 0.025% sodium dodecyl sulfate, 5 mmol/l EDTA pH 8.0, 0.25 mg/ml yeast tRNA, and 10 mmol/l Tris-HCl pH 7.6). After hybridization, the samples were rinsed with 5 × sodium chloride–sodium citrate, then stringently rinsed with 2 × sodium chloride–sodium citrate containing 50% formamide, and finally rinsed thoroughly with 0.2 × sodium chloride–sodium citrate. The samples were then immersed in 1.5% blocking reagent dissolved in DIG buffer 1 (100 mmol/l Tris-HCl, pH 7.5, containing 150 mmol/l NaCl) for 60 min at room temperature. After preincubation in normal rabbit serum at a dilution of 1:500 in DIG buffer 1 for 30 min, the samples were incubated in anti-DIG sheep polyclonal antibodies in DIG buffer 1 for 30 min at room temperature and then rinsed with DIG buffer 1. These samples were incubated in biotinylated anti-sheep rabbit polyclonal antibodies in DIG buffer 1 for 30 min at room temperature and rinsed again with DIG buffer 1. Next, the samples were treated with avidin-biotinylated horseradish peroxidase complex solution (Vector Laboratories, Burlingame, CA, USA) at room temperature for 60 min, followed by extensive washing with DIG buffer 1. After treatment with 0.1% 3,3′-diaminobenzidine hydrochloride substrate dissolved in 50 mmol/l Tris-HCl (pH 7.4) containing 0.05% H_2O_2 for 5 min at room temperature in the dark, the sections were examined for expression of the specific gene of interest. The reaction was stopped by rinsing in Tris-EDTA buffer (10 mmol/l Tris-HCl, pH 7.6, containing 1 mmol/l EDTA), and the target mRNA signals were visualized. The sections were counterstained with periodic acid-Schiff reagent, dehydrated, and finally mounted in Entellan New (Merck, Whitehouse Station, NJ, USA).

Preparation of cells stably overexpressing *ELMO1*

A full-length cDNA for human *ELMO1* was amplified from a human spleen cDNA library using appropriate sense (5′-ACGCTGTAG GATCCTCATTC-3′) and anti-sense (5′-TTTCAGTTACAGTCATA GACGAAGT-3′) primers. The amplified product was purified and subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) at its multiple cloning site. The expression vectors for *hELMO1* and *LacZ* (control) were introduced into COS-7 cells using the liposome transfection procedure (FuGene 6, Roche Diagnostics, Basel, Switzerland), and the cells were selected in the media containing neomycin analog, G418 (Invitrogen, Carlsbad, CA, USA). The cloned cell lines were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA), penicillin G (50 U/ml), and streptomycin (50 μg/ml).

siRNA experiments

A 21-nucleotide synthetic siRNA duplex was prepared by FASMAC (Atsugi, Kanagawa, Japan). The siRNA sequences used for targeting *ELMO1* or *ILK* were as follows. For *ELMO1*: 5′-GTG CTC ACC TTT AAC CTC CTT-3′, which corresponds to nucleotide positions 1147–1165 in *ELMO1* cDNA (Genbank Accession NM_014800). For *ILK*: 5′-CCC GGC TCA GGA TTT TCT CTT-3′, which corresponds to nucleotide positions 875–893 in *ILK* cDNA (Genbank Accession NM_004517). As a control, an siRNA with a non-silencing oligonucleotide sequence (QIAGEN, Hilden, Germany) was used. COS-7 cells were transfected with the siRNA using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after siRNA transfection, total RNA was extracted for real-time quantitative RT-PCR.

Table 1 | Gene-specific primers for real-time quantitative RT-PCR

Gene	Forward	Reverse
ELMO1	5′-CCGGATTGTGCTTGAGAACA-3′	5′-CTCACTAGGCAACTCGCCCA-3′
COL1A1	5′-CACCAATCACCTGCGTACAGA-3′	5′-TCACAGATCACGTCATCGCAC-3′
FNTN	5′-CCTCAATTGTTGTTCGCTGGA-3′	5′-GACGGAGTTTGCAGTTTCAGG-3′
ILK	5′-CGGCTCAGGATTTTCTCGC-3′	5′-GGTCCACGACGAAATTGGTG-3′
FAK	5′-CACACTTGGAGAGCTGAGGTCA-3′	5′-AACATTCCGAGCAGCAATGTC-3′
Src	5′-AGCTGGTGCAGTTGTATGCTGT-3′	5′-GCGATCTGAGCAGCCATGT-3′
GAPDH	5′-AGGTGAAGGTCGGAGTCAACG-3′	5′-GCTCCTGGAAGATGGTGATGG-3′

Real-time quantitative RT-PCR

Total RNA was extracted from COS-7 cells using Rneasy® Mini columns (QIAGEN, Hilden, Germany), and first-strand cDNA was prepared with the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time quantitative RT-PCR was carried out in an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems Japan, Tokyo, Japan) using SYBR Green I. The amplifications were performed in 25 μl reaction volumes containing Ex Taq Buffer, 200 nM dNTPs, 1/20 000 diluted SYBR Green I, 800 nM of each primer, 0.05 U Ex Taq DNA polymerase, and 2.75 ng TaqStart Antibody (BD Biosciences, San Jose, CA, USA). The thermal profile used was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 30 s. The data were normalized by glyceraldehyde-3-phosphate dehydrogenase to account for differences in reverse transcriptional efficiencies. The gene-specific primers we used in this study are described in Table 1.

Enzyme-linked immunosorbent assay for fibronectin

A QuantiMatrix™ enzyme-linked immunosorbent assay strips (Chemicon, Temecula, CA, USA) was used for the measurement of fibronectin secreted to the conditioned media collected for 24 h. Samples were incubated with rabbit anti-fibronectin antibody for 1 h at room temperature in the strips. After the plate was washed four times with wash buffer, goat anti-rabbit IgG conjugated with horseradish peroxidase was added and incubated for 30 min, and the strips were reacted with TMB/E substrate for 10 min. The concentrations of fibronectin were determined to measure the absorbance at 450 nm. DNA concentrations were determined using CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA).

Western blot analysis

Cells were lysed with cell lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM ethyleneglycol-bis-aminoether-*N*,*N*′-tetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate), and the extracted proteins were analyzed on 12.5% sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred to Immobilion membrane (Millipore, Bedford, MA, USA). The membrane was blocked using 1% skim milk (Becton Dickinson, MD,, USA) in PBS, and reacted with the anti-FLAG monoclonal antibody (Sigma-Aldrich, St Louis, USA) or anti-ILK polyclonal antibody (Upstate, Lake Placid, NY, USA), followed by incubating with horseradish peroxidase conjugated second antibody. Immunoreactive bands were detected in ECL plus Western Blotting Detection System (Amersham Biosciences, UK).

ILK activity assay

Cells were solubilized with lysis buffer 2 (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml Leupeptin). The cell lysate was centrifuged at 10 000 *g* for 15 min at 4°C. Two hundred and fifty micrograms of extracted protein was immunoprecipitated overnight with 1 μg of rabbit anti-ILK polyclonal antibody (Upstate), and then 30 μl of protein A agarose beads were added followed by 3 h rotation at 4°C. The beads were washed twice with lysis buffer 2 and kinase buffer (25 mM Tris (pH7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$), and were suspended in kinase buffer containing 200 μM ATP and 1 μg of glycogen synthase kinase-3 fusion protein. Phospholylated-glycogen synthase kinase-3 was detected by Western blot analysis using rabbit anti-phospho-glycogen synthase kinase-3α/β ($Ser^{21}/^{9}$) polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA).

Cell-adhesion assays

The cell-adhesion assays were performed in CytoMatrix Cell Adhesion Strips (Chemicon). Cells were detached with Trypsin-EDTA, resuspended in non-enzymatic Dulbecco's modified Eagle medium containing 10% fetal bovine serum and plated on each well (5×10^5 cells per well). After incubation at 37°C for 60 min in a CO_2 incubator, the plates were washed with PBS containing Ca^{2+} and Mg^{2+} to remove the non-adherent cells. The adherent cells were fixed and stained with 0.2% crystal violet in 10% ethanol solution for 5 min. After washing twice with PBS, the cell-bound stain was extracted by incubating the cells in a solubilization buffer (0.05 M NaH_2PO_4, pH 4.5 in 50% ethanol), and the absorbance was measured at 540 nm.

Statistical analysis

For statistical testing, comparisons among three or more groups were analyzed by one-way analysis of variance, followed by Scheffe's tests to evaluate statistical differences between the experimental and control groups. $P<0.05$ was considered to be significant.

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INTERNAL MEDICINE

□ CASE REPORT □

A Case with Membranous Lupus Nephritis Developing after a Twenty-year Remission of Membranoproliferative Glomerulonephritis

Qiong Wu, Yoko Koike, Makoto Nishina, Masao Toyoda,
Daisuke Suzuki and Masayuki Endoh

Abstract

A 30-year-old woman who showed remission of membranoproliferative glomerulonephritis (MPGN) 20 years previously developed membranous lupus nephritis (MLN). She had photosensitivity, facial erythema, proteinuria of 2.59 g/24 hr, anti-nuclear antibody and anti-ds-DNA antibody. To confirm whether a misdiagnosis of MPGN was made 20 years ago, the clinical data at that time were evaluated retrospectively. She had only mild proteinuria and hematuria but no photosensitivity or facial erythema. Anti-nuclear antibody was negative. Renal biopsy showed occasional lobulation and glomerular capillary double contour. The diagnosis of MPGN was definite. This might be a rare case of one person suffering from two types of glomerulonephritis, MPGN and MLN.

Key words: membranoproliferative glomerulonephritis, membranous lupus nephritis, immune complex, antinuclear antibody, hypocomplementemia

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by overproduction of a multitude of different autoantibodies and immune complex formation. The deposition or in situ formation of immune complexes in the kidneys causes histopathological changes in the glomeruli, interstitium and blood vessels, which is termed lupus nephritis and is divided into six classes according to WHO criteria. Recently, some rebiopsy studies have shown that lupus nephritis was found to transform into other histologic patterns (1-4). Some patients with "full-house" nephropathy were found to develop SLE several years later (5, 6). In some SLE patients the histopathologic features of lupus nephritis on renal biopsy were not observed, while accompanying IgA nephropathy was diagnosed (7). It was also reported that a SLE patient with focal proliferative lupus nephritis (WHO III) showed minimal change nephrotic syndrome (MCNS) in the remission period (8). However, there is no report of de novo lupus nephritis developing after remission of primary glomerulonephritis. Here, we report a case of membranous lupus nephritis developing after a 20-year remission of membranoproliferative glomerulonephritis (MPGN).

Case Report

A 30-year-old Japanese woman was referred to the Tokai University Hospital because of proteinuria discovered in a post-partum health check. Her medical history included cured membranoproliferative glomerulonephritis 20 years previously and she recently experienced photosensitivity. Her grandmother had diabetes mellitus and her grandfather had renal disease (no detailed data). On physical examination, facial butterfly erythema and edema in her legs were observed. Her blood pressure was 124/80 mmHg and electrocardiogram, chest X-ray and abdominal ultrasonography were unremarkable. Red blood cell count was 3.7×10^6/μl, hemoglobin 11.5 g/dl, white blood cell 4.4×10^3/μl and platelet 25.2×10^4/μl. Serum total protein (TP) was 6.4 g/dl, albumin (Alb) 2.7 g/dl, blood urea nitrogen (BUN) 9 mg/dl and

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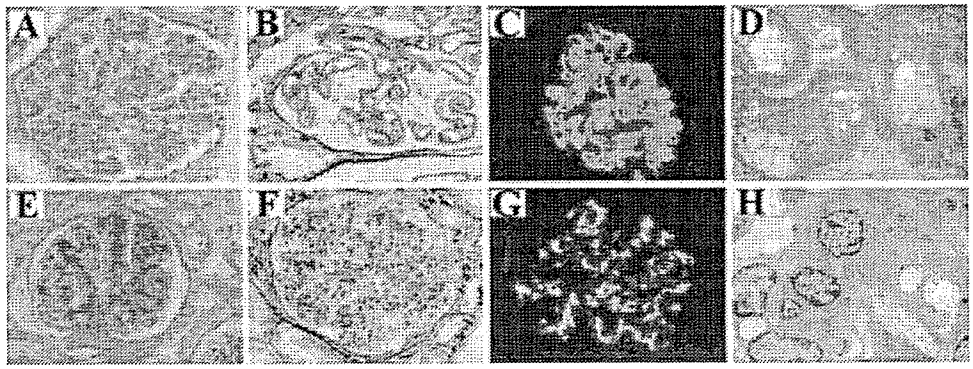

Figure 1. Membranous lupus nephritis at 30 years of age (A-D). Renal biopsy specimens showed diffusely thickened glomerular capillary walls by PAS staining (A. ×200), numerous spike formations by PASM staining (B. ×400), granular deposits of IgG along the capillary walls by immunofluorescence (C. ×200) and numerous mesangial, subepithelial and intramembranous dense deposits by electron microscopy (D. ×2000). Membranoproliferative glomerulonephritis at 10 years of age (E-H). Renal biopsy specimens showed increased mesangial matrix with infiltrating cells by PAS staining (E. ×200), few mesangial interpositions or double contours by PASM staining (F. ×200), mesangial deposits of C3 by immunofluorescence (G. ×200) and fewer mesangial dense deposits by electron microscopy (H. ×3000).

creatinine (Cr) 0.6 mg/dl.

Urinalysis revealed 2.59 g/24 hr of proteinuria, one plus hematuria and various types of casts including red cell casts. Anti-nuclear antibody (ANA) was 40-fold positive, and anti-ss-DNA was 185.0 AU/ml (normal range <25 AU/ml), anti-ds-DNA 40.8 IU/ml (normal range <12 IU/ml), and serum immune complex C1q (C1qIC) 6.4 μg/ml (normal range < 3.0 μg/ml). Anti-Sm antibody, anti-SSA and anti-SSB antibody were negative. Serum immunoglobulin G (IgG) was 1822 mg/dl, IgA 333 mg/dl, IgM 153 mg/dl, serum complement 3 (C3) 43.2 mg/dl (normal range 55-90 mg/dl), C4 2.7 mg/dl (normal range 15-40 mg/dl) and CH50 12.0 U/ml (normal range 30-40 U/ml).

She fulfilled 5 of the 11 criteria of the American Rheumatism Association for SLE: photosensitivity, facial butterfly erythema, renal disorder, antinuclear antibody and anti-ds-DNA antibody.

Renal biopsy showed diffuse thickening of capillary walls in all glomeruli under light microscopy (Fig. 1A). Subepithelial spike formation and partial double contoured basement membrane were observed by PAM staining (Fig. 1B). Immunofluorescence microscopy showed marked granular deposition of IgG along the glomerular capillary wall (Fig. 1C) and similar distributions for C1q and C3 deposits. A trace deposition of IgA and mild deposition of IgM were also observed in glomeruli. IgG subclasses deposition was demonstrated strong intensity along the glomerular capillary (Fig. 2). Electron microscopy showed increased mesangial matrix with electron-dense deposits, epithelial foot process effacement, and thickening of basement membrane with a large number of uniform subepithelial and intramembranous electron-dense deposits (Fig. 1D). These findings led to a diagnosis of membranous lupus nephritis.

She was then given two cycles of methylprednisolone pulse treatment (1 g/day×3 days/cycle) and oral prednisolone which were begun at 30 mg/day and gradually tapered. As a result, proteinuria decreased slowly to less than 1.0 g/day. ANA and anti-ds-DNA titers returned to normal. Serum complements also showed an increasing tendency. However, ten months later, the titers of autoantibodies elevated again and hypocomplementemia became more severe than before although proteinuria remained at a low level. The clinical data of one month and ten months after treatment are shown in Table 1. After 14 months of follow-up, the patient dropped out with about 1 g/day proteinuria, strongly positive ANA and severe hypocomplementemia but negative anti-ds-DNA.

This patient had a past history of membranoproliferative glomerulonephritis when she was ten years old. She was admitted to the department of pediatrics because of proteinuria and hematuria found in a school health check. Her physical examination was unremarkable. Blood pressure was normal. Chest and abdomen x-rays, IVP and abdominal ultrasonography were normal. Laboratory tests showed hemoglobin 12.9 g/dl, white blood cell count 6.5×10^3/μl and platelet count 28.1×10^4/μl. Proteinuria was 1+ and hematuria 2+. Renal function and serum immunoglobulin level were normal. Serum CH50 was in the normal range of 33.8 U/ml (30-40 U/ml). CRP, anti-streptolysin O (ASO) and ANA were negative.

A renal biopsy sample contained 18 glomeruli, which were placed under observation. Most of the glomeruli were markedly swollen with distinct lobulation and numerous infiltrating cells. Mesangial proliferation was marked with occasional mesangial interposition and double track formation of capillary loops (Figs. 1E, 1F). Immunofluorescence