

Genetic Variations in the Gene Encoding ELMO1 Are Associated With Susceptibility to Diabetic Nephropathy

Atsuyuki Shimazaki,¹ Yoshihiro Kawamura,¹ Akio Kanazawa,¹ Akihiro Sekine,¹ Susumu Saito,¹ Tatsuhiko Tsunoda,¹ Daisuke Koya,² Tetsuya Babazono,³ Yasushi Tanaka,⁴ Masafumi Matsuda,⁵ Koichi Kawai,⁶ Tomohiro Iizumi,⁷ Masahito Imanishi,⁸ Toshihiro Shinosaki,⁹ Toru Yanagimoto,⁹ Minoru Ikeda,⁹ Shigeki Omachi,⁹ Atsunori Kashiwagi,² Kohei Kaku,⁵ Yasuhiko Iwamoto,³ Ryuzou Kawamori,⁴ Ryuichi Kikkawa,² Masatoshi Nakajima,⁹ Yusuke Nakamura,^{1,10} and Shiro Maeda¹

To search for a gene(s) conferring susceptibility to diabetic nephropathy (DN), we genotyped over 80,000 gene-based single nucleotide polymorphisms (SNPs) in Japanese patients and identified that the engulfment and cell motility 1 gene (*ELMO1*) was a likely candidate for conferring susceptibility to DN, in view of the significant association of an SNP in this gene with the disease (intron 18+9170, GG vs. GA+AA, $\chi^2 = 19.9$, $P = 0.000008$; odds ratio 2.67, 95% CI 1.71–4.16). In situ hybridization (ISH) using the kidney of normal and diabetic mice revealed that *ELMO1* expression was weakly detectable mainly in tubular and glomerular epithelial cells in normal mouse kidney and was clearly elevated in the kidney of diabetic mice. Subsequent in vitro analysis revealed that *ELMO1* expression was elevated in cells cultured under high glucose conditions (25 mmol/l) compared with cells cultured under normal glucose conditions (5.5 mmol/l). Furthermore, we identified that the expression of extracellular matrix protein genes, such as type 1 collagen and fibronectin, were increased in cells that overexpress *ELMO1*, whereas the expression of matrix metalloproteinases was decreased. These results indicate that *ELMO1* is a novel candidate gene that both confers susceptibility to DN and plays an important role in the development and progression of this disease. *Diabetes* 54:1171–1178, 2005

From the ¹SNP Research Center, The Institute of Physical and Chemical Research, Yokohama, Kanagawa, Japan; the ²Department of Medicine, Shiga University of Medical Science, Otsu, Shiga, Japan; ³Diabetes Center, Tokyo Women's Medical University, Tokyo, Japan; the ⁴Department of Medicine, Metabolism & Endocrinology, School of Medicine, Juntendo University, Tokyo, Japan; the ⁵Division of Endocrinology and Metabolism, Department of Internal Medicine, Kawasaki Medical School, Kurashiki, Okayama, Japan; the ⁶Kawai Clinic, Tsukuba, Ibaragi, Japan; the ⁷Department of Internal Medicine, Toride Kyodo Hospital, Toride, Ibaragi, Japan; the ⁸Department of Internal Medicine, Osaka City General Hospital, Osaka, Japan; ⁹Discovery Research Laboratories, Shionogi Co. Ltd., Toyonaka, Osaka, Japan; and the ¹⁰Laboratory for Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Tokyo, Japan.

Address correspondence and reprint requests to Shiro Maeda, Laboratory for Diabetic Nephropathy, SNP Research Center, The Institute of Physical and Chemical Research, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan. E-mail: smaeda@src.riken.jp

Received for publication 11 November 2004 and accepted in revised form 20 December 2004.

Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

AER, albumin excretion rate; Alb/Cr, albumin-to-creatinine ratio; DIG, digoxigenine; DN, diabetic nephropathy; ISH, in situ hybridization; LD, linkage disequilibrium; MMP, matrix metalloproteinase; SNP, single nucleotide polymorphism; SSC, sodium chloride–sodium citrate; TGF, transforming growth factor.

© 2005 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in Western countries and Japan (1,2). The pathogenesis of DN appears to be multifactorial. Several genetic and environmental factors are likely to contribute to its development and progression, although the precise mechanisms for this contribution are unknown. Because cumulative epidemiological findings have provided evidence that genetic susceptibility plays an important role in the pathogenesis of this renal disease (3,4), there have been extensive efforts to identify the gene(s) involved in the development and progression of nephropathy, in both type 1 and type 2 diabetes (5,6), but no definitive results have yet emerged.

Single nucleotide polymorphisms (SNPs) are the most common genetic variation and are considered to be potentially useful as markers to identify genetic variants that may confer susceptibility to common etiologically complex diseases.

After developing a high-throughput system for genotyping SNPs that combines the Invader assay with multiplex PCRs (7), we carried out genome-wide association studies using SNPs to identify loci involved in susceptibility to common diseases.

In the study reported here, we performed a genome-wide SNP genotyping analysis of a large panel of Japanese patients with type 2 diabetes in an effort to identify the gene(s) conferring susceptibility to DN. Our data suggest that the engulfment and cell motility 1 gene (*ELMO1*) is likely to contribute to genetic susceptibility to DN.

RESEARCH DESIGN AND METHODS

DNA preparation and SNP 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 Tokusyuikai Hospital. All subjects provided informed consent before enrolling in this study, and DNA extraction was performed according to standard phenol-chloroform procedures. Diabetic patients were divided into two groups according to the following diagnostic criteria: 1) cases of DN, i.e., patients with diabetic retinopathy as well as overt nephropathy, indicated by urinary albumin excretion rates (AERs) ≥ 200 $\mu\text{g}/\text{min}$ or urinary albumin-to-creatinine ratios (Alb/Cr) ≥ 300 mg/gCr , or patients under chronic renal-replacement therapy; and 2) control patients, or patients with diabetic retinopathy but showing no evidence of renal dysfunction, i.e., AER < 20 $\mu\text{g}/\text{min}$ or Alb/Cr < 30 mg/gCr . The SNPs for genotyping were randomly

TABLE 1
Association of landmark SNP in *ELMO1* (intron 18+9170 A/G) during genome-wide screening

Patient groups	<i>n</i>	AA	AG	GG	A	G
Case 1	87	31 (35.6)	40 (46.0)	16 (18.4)	0.59	0.41
Control 1	92	44 (47.8)	45 (48.9)	3 (3.3)	0.72	0.28
Case 2	459	170 (37.0)	216 (47.0)	73 (15.9)	0.61	0.39
Control 2	242	109 (45.0)	120 (49.6)	13 (5.4)	0.70	0.30

		χ^2	<i>P</i>	OR	95% CI
Case 1 vs. control 1	GG vs. AG + AA	10.8	0.001	6.69	1.87–23.85
Case 2 vs. control 2	GG vs. AG + AA	16.3	0.00005	3.33	1.81–6.15

Genotype data are number of subjects (%).

selected from our gene-based Japanese SNP database (available at <http://snp.ims.u-tokyo.ac.jp>) (8,9). The genotype of each SNP locus was analyzed with Invader assays, as previously described (7).

Our first screening involved genotyping 94 DN patients (63 men and 31 women, age 57.9 ± 12.5 years, duration of diabetes 18.6 ± 9.7 years, HbA_{1c} $7.7 \pm 1.3\%$, mean \pm SD) and 94 control patients (37 men and 57 women, age 62.7 ± 9.9 years, duration of diabetes 16.2 ± 8.4 years, HbA_{1c} $7.4 \pm 1.1\%$) for 81,315 SNP loci. By evaluating the statistical data using 2×3 or 2×2 contingency tables, we selected SNPs that showed significant differences in genotypic or allelic frequencies between the DN and control groups. Then we analyzed the SNPs in a larger number of subjects—466 DN and 266 control patients (DN patients: 305 men and 161 women, age 59.6 ± 13.5 years, duration of diabetes 17.3 ± 10.4 years, HbA_{1c} $7.8 \pm 1.1\%$; control patients: 125 men and 141 women, age 62.9 ± 12.0 years, duration of diabetes 14.6 ± 9.3 years, HbA_{1c} $7.1 \pm 1.2\%$) (10). The ethics committees of the Institute of Physical and

Chemical Research and each participating institution approved the study protocol.

Identification of polymorphisms in *ELMO1* and genotyping. PCR primers were designed using GenBank *ELMO1* sequence data (accession nos: AC078843, AC083861, AC007444, AC009196, AC078844, and AC007349) to amplify specific regions of *ELMO1*. Repetitive elements were excluded from the search by invoking the Repeat Masker computer program (available at <http://www.repeatmasker.org>) in the manner described by Bedell et al. (11). PCR experiments and DNA sequencing were carried out as previously described (12). Genotyping of each SNP in the critical region was performed with the Invader assay or, in some cases, by the TaqMan assay using maximum number of the subjects.

ISH.

Tissue preparation. Under pentobarbital anesthesia, the 30-week-old mice were flushed with PBS through the abdominal aorta followed by perfusion

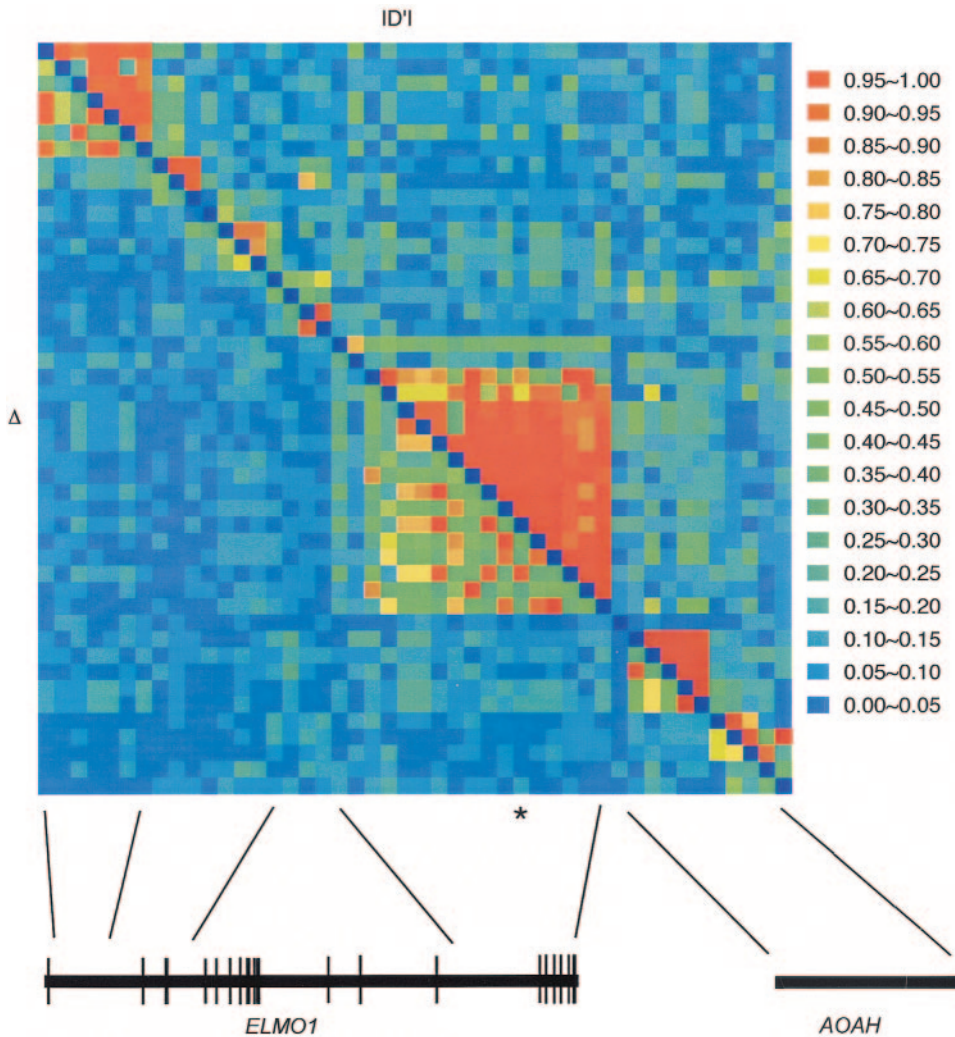


FIG. 1. LD mapping around *ELMO1*. LD coefficients (D' and Δ) between every two SNPs were calculated. SNPs with minor allele frequencies <0.15 were not included in the calculations. An asterisk denotes the landmark SNP at intron 18+9170 (A/G).

TABLE 2
Genotype distribution of SNPs in the *ELMO1*

Position (major/minor)	Major/ major	Major/ minor	Minor/ minor	Total
Intron 16+101856 (A/G)				
Case group	326 (51.9)	252 (40.1)	50 (8.0)	628
Control group	242 (57.6)	165 (39.3)	13 (3.1)	420
Intron 16+104353 (A/G)				
Case group	296 (46.7)	269 (42.4)	69 (10.9)	634
Control group	211 (49.6)	195 (45.9)	19 (4.5)	425
Intron 16+105608 (C/T)				
Case group	355 (56.2)	237 (37.5)	40 (6.3)	632
Control group	263 (62.8)	148 (35.3)	8 (1.9)	419
Intron 16+114454 (C/T)				
Case group	229 (41.0)	262 (47.0)	67 (12.0)	558
Control group	161 (44.0)	184 (50.3)	21 (5.7)	366
Intron 17+5495 (A/G)				
Case group	286 (45.1)	278 (43.9)	69 (10.9)	633
Control group	212 (49.9)	197 (46.3)	16 (3.8)	425
Intron 18+9170 (A/G)*				
Case group	244 (37.7)	304 (47.0)	99 (15.3)	647
Control group	178 (41.8)	221 (51.9)	27 (6.3)	426
Intron 19+2430 (G/A)				
Case group	239 (37.6)	306 (48.1)	91 (14.3)	636
Control group	182 (42.9)	215 (50.7)	27 (6.4)	424
Intron 20+1239 (A/G)				
Case group	311 (48.7)	265 (41.5)	63 (9.9)	639
Control group	214 (51.0)	189 (45.0)	17 (4.0)	420
3' flanking + 735 (A/T)				
Case group	316 (50.1)	257 (40.7)	58 (9.2)	631
Control group	221 (52.5)	183 (43.5)	17 (4.0)	421
Intron 1+5853 (T/G)				
Case group	310 (48.7)	278 (43.7)	48 (7.5)	636
Control group	195 (46.2)	174 (41.2)	53 (12.6)	422

Genotype data are presented as number of subjects (%). *Landmark SNP used for the genome-wide screening.

with 4% paraformaldehyde buffered with 0.1 mol/l PBS (pH 7.4). The kidneys were swiftly removed and cut into small pieces. The renal cortex tissue was immediately dissected and immersed into a fresh portion of the same fixative at 4°C overnight. All steps were carried out with care to avoid contamination with RNase. Diethylpyrocarbonate-treated water was used at 0.1% to prepare each buffer. Fixed samples were thoroughly rinsed with 0.1 mol/l PBS (pH 7.4), subsequently dehydrated by passage through an alcohol series, and cleared in xylene. ISH was performed on paraffin-embedded sections.

ISH study. Antisense and sense single-strand cRNAs were synthesized from cDNA fragments encoding *ELMO1* or encoding *ELMO2* using RT-PCR. The *ELMO1* cDNA fragment consisted of 525 bp (mouse sequence nucleotides 51–575, GenBank accession no. AY406934). The *ELMO2* cDNA fragment consisted of 501 bp (mouse sequence nucleotides 51–551, GenBank accession no. AF398884). Each fragment was subcloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA). The template of *ELMO1* subunit was linearized with the restriction enzyme *HindIII*, and the labeled RNA probe was synthesized with T7 RNA polymerase in the presence of digoxigenin (DIG)-labeled UTP (DIG Labeling Kit; Roche Diagnostics, Basel, Switzerland) for antisense-probe, or it was linearized with the restriction enzyme *EcoRV*, and the labeled RNA probe was synthesized with SP6 RNA polymerase for sense-probe. The template of *ELMO2* subunit was linearized with the restriction enzyme *EcoRV*, and the labeled RNA probe was synthesized with SP6 RNA polymerase for antisense-probe, or the template of *ELMO2* subunit was linearized with the restriction enzyme *HindIII*, and labeled RNA probe was synthesized with T7 RNA polymerase for sense-probe.

The probes were precipitated and DIG incorporation was assessed by dot blotting. After dewaxing, the tissue sections were dipped into 0.01 mol/l citrate buffer (pH 6.0) at 95°C for 30 min, rinsed with 0.1 mol/l PBS (pH 7.4), and permeabilized with 2.5 µg/ml proteinase K (Roche Diagnostics) at 37°C for 10 min. Next, they were briefly washed with 0.1 mol/l PBS, rinsed in 0.1 mol/l triethanolamine (pH 8.0), and then acetylated with 0.1 mol/l triethanolamine containing 0.25% acetic anhydride for 10 min. Hybridization was conducted by the method described previously (13). Briefly, sections were prehybridized

and then hybridized with 500 ng/ml DIG-labeled antisense or sense cRNA probe for 17 h at 50°C in a hybridization buffer containing 50% deionized formamide, 1 × Denhardt's solution, 10% dextran sulfate, 600 mmol/l NaCl, 0.025% SDS, 5 mmol/l EDTA (pH 8.0), 0.25 mg/ml yeast tRNA, and 10 mmol/l Tris-HCl (pH 7.6). During hybridization, the slides were covered with parafilm and kept in a closed, moist chamber. After hybridization, the samples were rinsed three times with 5 × sodium chloride–sodium citrate (SSC) for 10 min at 50°C. The samples were stringently washed with 2 × SSC containing 50% formamide for 20 min at 50°C and thoroughly washed twice with 0.2 × SSC for 20 min at 50°C. Then the samples were 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, preincubated in normal rabbit serum at a dilution of 1:500 in DIG buffer 1 for 30 min, and subsequently incubated in anti-DIG sheep polyclonal antibodies at a dilution of 1:1,000 in DIG buffer 1 for 30 min at room temperature and rinsed with DIG buffer 1. These samples were then incubated in biotinylated anti-sheep rabbit polyclonal antibodies at a dilution of 1:1,000 in DIG buffer 1 for 30 min at room temperature and rinsed again with DIG buffer 1. The samples were next treated with avidin-biotinylated horseradish peroxidase complex solution (Vector Laboratories, Burlingame, CA) at room temperature for 60 min. After immunological incubation, the samples were extensively washed with DIG buffer 1, then processed using 0.1% 3,3'-diaminobenzidine hydrochloride substrate dissolved in 50 mmol/l Tris-HCl (pH 7.4) containing 0.05% H₂O₂ for 5 min at room temperature in the dark and examined for expression of the specific gene of interest. After a brownish color developed, 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).

Photomicrographic evaluation. The slides were examined under bright-field microscope Optiphot-equipped Coolpix digital camera system (Nikon), with particular attention paid to evaluation of the glomerular cross views.

Real-time quantitative RT-PCR. Total RNA was prepared from COS cells cultured under normal (5.5 mmol/l) or high (25 mmol/l) glucose conditions. Quantitative real-time PCR was performed using the primers described below. For *ELMO1*: sense 5'-CCG GAT TGT GCT TGA GAA CA-3', antisense 5'-CTC ACT AGG CAA CTC GCC CA-3'; fibronectin: sense 5'-GCT CAG AAT CCA AGC GGA GA-3', antisense 5'-CTT TCC CAA GCA ATT TTG ATG G-3'; for collagen I (α1): sense 5'-CAC CAA CCT CGC TAC AGA-3', antisense 5'-TCA CAG ATC ACG TCA TCG CAC-3'; for transforming growth factor (TGF)-β1: sense 5'-AGG TCA CCC GCG TGC TAA T-3', antisense 5'-GGT TCA GGT ACC GCT TC TCG -3'; for matrix metalloproteinase (MMP)-2: sense 5'-GAT GCC GCC TTT AAC TGG AG-3', antisense 5'-CAT CTG CGA TGA GCT TGG G-3'; for MMP3: sense 5'-TTT CTC GTT GCT GCT CAT GAA-3', antisense 5'-GAG ACA GGC GGA ACC GAG T-3'; and for GAPDH: sense 5'-GCT CAG AAT CCA AGC GGA GA-3', antisense 5'-CTT TCC CAA GCA ATT TTG ATG G-3'. The amplifications were carried out in a 25-µl reaction volume containing 1 × EX Taq buffer, 200 mmol/l dNTPs, 1/20,000 SYBR Green, 800 nmol/l of each primer, 0.05 units EX TaqDNA polymerase, 2.75 ng TaqStart antibody (Clontech), and 5 ng of template. The thermal profile used was 50°C for 2 min and 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 amplification and quantification was performed in an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems) and normalized to GAPDH.

Statistical analysis. Statistical analyses for the association study, haplotype frequencies, and calculation of linkage disequilibrium (LD) coefficients (D' or Δ) were described previously (14). Analysis of haplotype structure was carried out by estimating haplotype phasing using an expectation maximization algorithm (15) and by constructing haplotype blocks as previously described (10,16). For quantitative RT-PCR experiments, comparisons among three or more groups were analyzed by one-way ANOVA, followed by Scheffe's tests to evaluate statistical differences between two groups.

RESULTS

Genome-wide association study. Among the 81,315 SNP loci we tested in our original screening, 1,615 SNP loci had significant *P* values <0.01 between DN and control patients (see supplementary Table 1 in the online appendix available at <http://diabetes.diabetesjournals.org>) and were analyzed further in another larger group of patients (10). Of these SNPs, one SNP locus in the 18th intron of *ELMO1* on chromosome 7p14 was strongly associated with DN (GG vs. GA+AA, $\chi^2 = 16.3$, *P* = 0.00005; odds ratio [OR] 3.33, 95% CI 1.81~6.15) (Table 1). Correction of multiple

TABLE 3
Association of SNPs in the *ELMO1* with DN

Position	Genotype (2×3)	Allele	Major/major vs. others	Minor/minor vs. others
Intron 16+101856 (A/G)				
χ^2	11.5	7.3	3.3	10.5
<i>P</i>	0.003	0.007	0.07	0.001
Intron 16+104353 (A/G)				
χ^2	13.7	5.3	0.9	13.7
<i>P</i>	0.001	0.02	0.3	0.0002
Intron 16+105608 (C/T)				
χ^2	13.0	8.7	4.5	11.3
<i>P</i>	0.002	0.003	0.03	0.0008
Intron 16+114454 (C/T)				
χ^2	10.1	4.2	0.8	10.1
<i>P</i>	0.006	0.04	0.4	0.002
Intron 17+5495 (A/G)				
χ^2	17.7	8.4	2.3	17.5
<i>P</i>	0.0001	0.004	0.13	0.00002
Intron 18+9170 (A/G)*				
χ^2	19.9	9.4	1.8	19.9
<i>P</i>	0.00005	0.002	0.2	0.000008
Intron 19+2430 (G/A)				
χ^2	16.6	9.8	3.0	16.2
<i>P</i>	0.0003	0.002	0.08	0.00006
Intron 20+1239 (A/G)				
χ^2	12.3	4.0	0.5	12.3
<i>P</i>	0.002	0.04	0.5	0.0005
3' flanking + 735 (A/T)				
χ^2	10.1	3.6	0.6	10.1
<i>P</i>	0.006	0.06	0.4	0.001
Intron 1+5853 (T/G)				
χ^2	7.4	3.4	0.7	7.4
<i>P</i>	0.02	0.07	0.4	0.007

	OR (95% CI)		
	Allele	Major/major vs.	Minor/minor vs.
Intron 16+101856 (A/G)	1.32 (1.08–1.62)	1.26 (0.98–1.62)	2.71 (1.45–5.05)
Intron 16+104353 (A/G)	1.25 (1.03–1.51)	1.13 (0.88–1.44)	2.61 (1.55–4.41)
Intron 16+105608 (C/T)	1.38 (1.11–1.70)	1.32 (1.02–1.69)	3.47 (1.61–7.49)
Intron 16+114454 (C/T)	1.23 (1.01–1.50)	1.13 (0.86–1.47)	2.24 (1.35–3.73)
Intron 17+5495 (A/G)	1.33 (1.10–1.61)	1.21 (0.94–1.54)	3.13 (1.79–5.47)
Intron 18+9170 (A/G)*	1.32 (1.11–1.60)	1.19 (0.92–1.52)	2.67 (1.71–4.16)
Intron 19+2430 (G/A)	1.34 (1.12–1.61)	1.25 (0.97–1.60)	2.46 (1.57–3.84)
Intron 20+1239 (A/G)	1.22 (1.00–1.48)	1.10 (0.80–1.41)	2.59 (1.50–4.50)
3' flanking + 735 (A/T)	1.21 (0.99–1.47)	1.10 (0.86–1.41)	2.41 (1.38–4.19)
Intron 1+5853 (T/G)	0.83 (0.70–1.01)	0.90 (0.71–1.16)	0.57 (0.38–0.86)

*Landmark SNP used for the genome-wide screening.

testing error was made using the following calculation: Overall *P* values ($P_{1st} \times P_{2nd}$) \times number of tests ($1st + 2nd$) = $0.001 \times 0.00005 \times (81,315 \times 4 + 1,615 \times 4) = 0.017$. After performing this correction, we concluded that the association of this landmark SNP with DN was statistically significant.

Subsequent mapping of LD around the landmark SNP in *ELMO1* revealed that LD in this region extended ~100 kb upstream and 100 kb downstream of the landmark site (Fig. 1). Therefore, the critical region for susceptibility to DN seemed likely to lie within this 200-kb high-LD block. Because *ELMO1* is the only gene within this LD block, it was concluded that *ELMO1* was the most likely candidate for conferring susceptibility to DN.

We next screened for additional polymorphisms in *ELMO1* and identified another 516 polymorphisms (448

SNPs, 49 insertion/deletion polymorphisms, 18 tandem repeat polymorphisms, and 1 other polymorphism) (see supplementary Fig. 1 in the online appendix). Genotyping of these variations using the maximum number of DN ($n = 640$) and control ($n = 426$) subjects showed that several of them were associated with DN (Tables 2 and 3; information on those SNPs can be found in supplementary Table 2 in the online appendix); in particular, SNP in intron 18 (+9170 A/G) had the strongest association with DN (GG vs. AG+AA, $\chi^2 = 19.9$, $P = 0.000008$; OR 2.67, 95% CI 1.71–4.16). We also analyzed haplotype structure using the expectation maximization algorithm and found that nine SNPs in *ELMO1*, with an allelic frequency of >0.15 , constituted one haplotype block, and that the five common haplotypes accounted for $>90\%$ of the population (Fig. 2). Subsequent association studies with DN for each haplo-

	1 2 3 4 5 6 7 8 9	case	control	χ^2	<i>P</i>	odds ratio (95% CI)
Haplotype 1	T C C T A G A T G	0.297	0.322	1.4	0.24	
Haplotype 2	C C C C G A G T A	0.296	0.256	3.8	0.05	1.22 (1.00~1.49)
Haplotype 3	C T A C A G A C A	0.217	0.240	1.4	0.23	
Haplotype 4	C C C C G A A T A	0.060	0.051	0.7	0.40	
Haplotype 5	C C C C A G A T A	0.056	0.072	2.2	0.14	
Haplotype 6	C C C C G A A C A	0.019	0.006	5.9	0.015	3.14 (1.19~8.29)
Haplotype 7	C C A C A G A C A	0.011	0.009	0.2	0.63	
Haplotype 8	C T A C A G A C G	0.005	0.013	2.46	0.12	

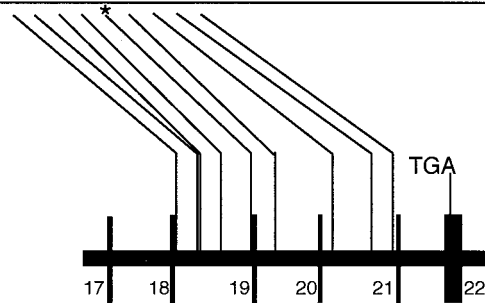


FIG. 2. Analysis of haplotype structure and estimated haplotype frequencies within *ELMO1*. Nine variations including landmark SNP constituted one haplotype block. An asterisk highlights the landmark SNP at intron 18+9170 (A/G). 1: intron18+324 (C/T); 2: intron18+2583 (C/T); 3: intron 18+2634 (C/A); 4: intron 18+5417 (C/T); 5: intron 18+9170 (A/G); 6: intron 19+2430 (G/A); 7: intron 20+1239 (A/G); 8: intron 20+5475 (T/C); 9: intron 20+8419 (A/G). *P* values for comparing haplotype frequencies between case and control groups were generated by χ^2 test using a 2×2 contingency table composed of the number of one haplotype and the sum of other haplotypes (e.g., haplotype 2/other haplotypes).

type identified a significant association of haplotypes 2 and 6 with DN. However, the association of these haplotypes was not stronger than that found at the +9,170 A/G locus. **ISH.** To identify a possible role of *ELMO1* in the pathogenesis of DN, we examined the expression pattern of this gene in normal (*db/+m*) and diabetic (*db/db*) mouse kidney by ISH and identified that the expression of *ELMO1* could be observed mainly in glomerular epithelial cells and tubular epithelial cells (Fig. 3). The signal of *ELMO1* mRNA was remarkably increased in the kidney of diabetic mice (*db/db* 30 weeks old) (Fig. 3E and F) compared with that of controls (*db/+m* 30 weeks, Fig. 3B and C), whereas the expression of *ELMO2* was not different between these mice (Fig. 3H, I, K, and L). We also examined *ELMO1* expression by RT-PCR in various kinds of cultured cells, including glomerular mesangial cells, proximal tubular epithelial cells, COS cells, and retinal pigment epithelial cells, and could only detect definite expression in COS cells.

Real-time quantitative RT-PCR for *ELMO1*. We next examined the effects of glucose on the expression of *ELMO1* in COS cells cultured under either normal (5.5 mmol/l) or high (25 mmol/l) concentrations of glucose. As shown in Fig. 4, the expressions of *ELMO1* were significantly increased in the cells cultured under high glucose conditions compared with those in the cells under normal conditions or conditions in which osmolarity had been adjusted with mannitol (+19.5 mmol/l). These results suggested that the increase in *ELMO1* expression under high glucose conditions might contribute to the development and progression of DN.

Expression of extracellular matrix genes in *ELMO1*-overexpressing cells. From the results described above, it was suggested that the excess expression of *ELMO1* under high glucose conditions might contribute to the development and progression of DN. Although the precise mechanism of the development and progression of DN is still unknown, it has been suggested that TGF- β 1-induced overaccumulation of extracellular matrix proteins is, at least in part, involved in the pathogenesis of DN (17).

Therefore, we investigated the effects of excess expression of *ELMO1* on the expression of extracellular matrix protein genes, as well as on that of the TGF- β 1 gene, using cells that overexpressed *ELMO1*. As shown in Fig. 5, the expression of these genes in cells stably transfected with pcDNA3.1-*ELMO1* were remarkably increased compared with those in the cells transfected with pcDNA3.1-LacZ (TGF- β 1 1.36-fold vs. 1.5-fold; collagen type 1 [α 1] 5.5-fold vs. 5.8-fold; fibronectin 11.1-fold vs. 12.1-fold; *ELMO1*-line 1 and *ELMO1*-line 2 vs. LacZ, respectively) (Fig. 5B–D), whereas the expression of MMPs were reduced (MMP2 0.66 vs. 0.27 and MMP3 0.54 vs. 0.57; *ELMO1*-line 1 and *ELMO1*-line 2 vs. LacZ, respectively) (Fig. 5E and F).

DISCUSSION

In a genome-wide case-control association study using SNPs as genetic markers, we identified *ELMO1* as a candidate gene conferring susceptibility to DN. Our data also suggest that one intronic SNP (intron 18+9170) might affect susceptibility to DN.

The results presented here, as well as in recent publications (10,18,19), provide evidence that a genome-wide case-control association study using gene-based SNPs as genetic markers is a powerful strategy for identifying genes associated with susceptibility to common diseases. Epidemiological findings had strongly suggested a contribution of genetic factors to the development and progression of DN (3,4), but worldwide efforts have so far failed to identify any solid evidence to indicate a genetic susceptibility to the disease. In many cases, the results were conflicting, probably because sample sizes were often inappropriate. However, the major cause of failures to obtain solid conclusions is possibly that multiple genetic factors are involved in DN in a complex manner and the influence of each individual factor is too weak to be identified. Therefore, approaches other than standard candidate-gene analysis or family-based linkage analysis are required to identify genes that are involved in susceptibility to common diseases such as DN. As we began this

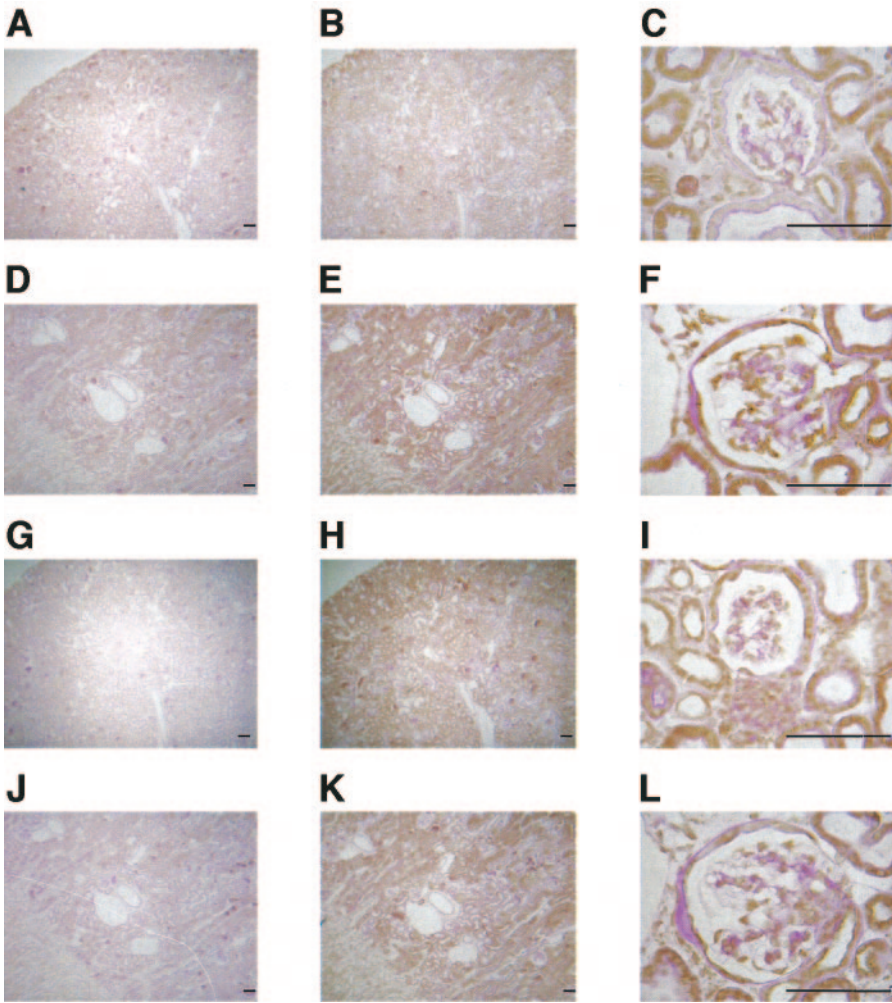


FIG. 3. Results of ISH using the kidney of 30-week-old nondiabetic (*db/+m*) and diabetic (*db/db*) mice. **A:** Sense probe for *ELMO1* (*db/+m*). **B:** Antisense probe for *ELMO1* (*db/+m*). **C:** Antisense probe for *ELMO1* (*db/+m*). **D:** Sense probe for *ELMO1* (*db/db*). **E:** Antisense probe for *ELMO1* (*db/db*). **F:** Antisense probe for *ELMO1* (*db/db*). **G:** Sense probe for *ELMO2* (*db/+m*). **H:** Antisense probe for *ELMO2* (*db/+m*). **I:** Antisense probe for *ELMO2* (*db/+m*). **J:** Sense probe for *ELMO2* (*db/db*). **K:** Antisense probe for *ELMO2* (*db/db*). **L:** Antisense probe for *ELMO2* (*db/db*). Scale bar = 100 μ m.

study by analyzing a huge number of loci (81,315 SNPs) on a genome-wide basis, using a high-throughput genotyping system developed in our institute, and because all of the SNPs contributing to this report are the gene-based SNPs from the Japanese population (8,9), we were able to screen for candidate genes for disease susceptibility more efficiently than would be possible using other SNP databases.

The *ELMO1* gene, on chromosome 7p14, is a known mammalian homologue of the *C. elegans* gene, *ced-12*, which is required for engulfment of dying cells and for cell migration (20). *ELMO1* has also been reported to cooperate with CrkII and Dock180, which are homologues of *C. elegans* *ced-2* and *ced-5*, respectively, to promote phagocytosis and cell shape changes (20,21). However, until now no evidence has been reported to suggest a role for this gene in the pathogenesis of DN.

In this study, we showed that the expression of *ELMO1* was increased in the kidney of diabetic mice compared with that of control mice, whereas the expression of *ELMO2* was not different between diabetic and nondiabetic mice. We also identified that the increased expression of *ELMO1* could be observed in the glomeruli isolated from diabetic mice by real-time quantitative PCR (data not shown). These results suggested some role of *ELMO1* in the pathogenesis of DN.

We next examined the effects of glucose concentrations on the expression of this gene in COS cells, and we found

that the expression of *ELMO1* was significantly elevated in cells under high glucose conditions compared with cells under normal glucose conditions. The effect of glucose on the expression of *ELMO1* was not due to increased osmolality, because the expression was not elevated in cells under normal glucose conditions with 19.5 mmol/l mannitol. From these observations, we speculated that increases in the expression of *ELMO1* under high glucose

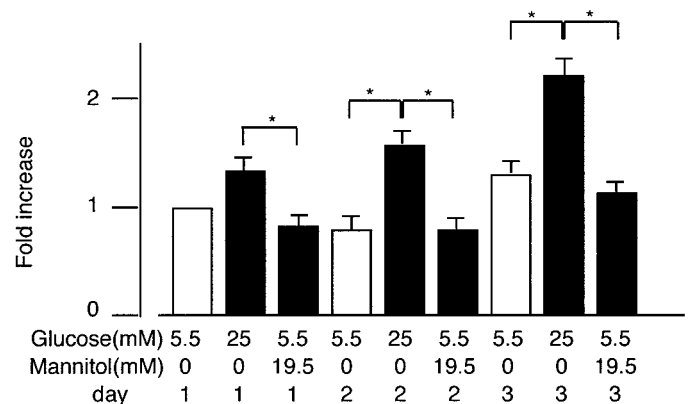


FIG. 4. Effects of glucose on the expression of *ELMO1* in COS cells. Data are presented as fold increase compared with the cells cultured under normal glucose conditions for 1 day. Results were obtained from three independent experiments. Data are means \pm SD. **P* < 0.0001.

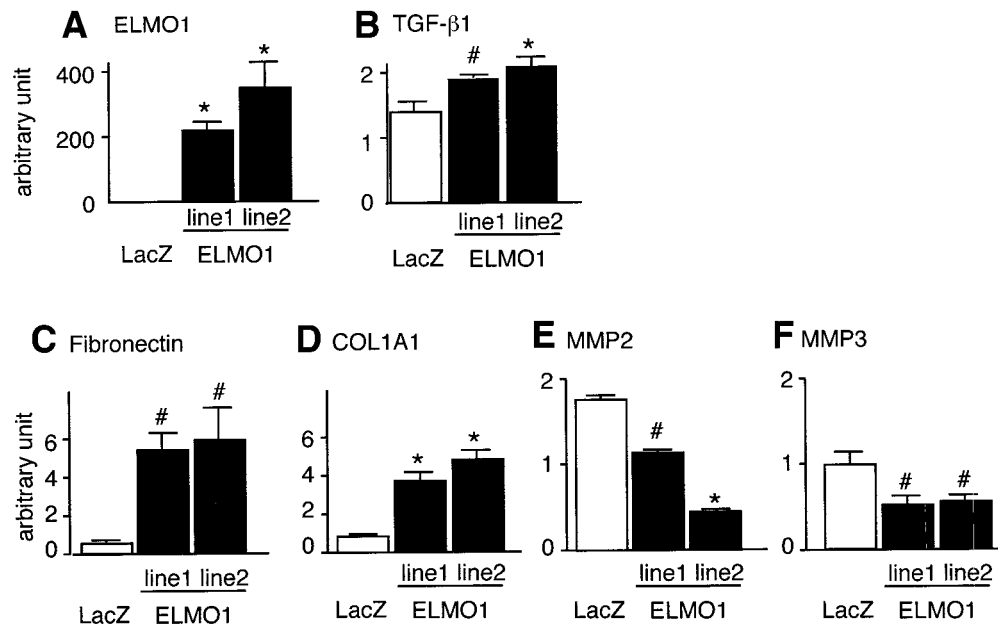


FIG. 5. The effects of *ELMO1* overexpression in COS cells. The expression of each gene was analyzed by real-time quantitative RT-PCR. A: *ELMO1*. B: TGF- β 1. C: Fibronectin. D: Collagen type 1 (α 1). E: MMP-2. F: MMP-3. \square , cells transfected with pcDNA-LacZ; \blacksquare , cells transfected with *ELMO1*. * $P < 0.0001$ vs. LacZ; # $P < 0.005$ vs. LacZ.

conditions might contribute to the development and progression of DN. Interestingly, the excess expression of *ELMO1* resulted in increased expression of extracellular matrix genes and in the decreased expression of MMP genes. Therefore, it is suggested that persistent excess of *ELMO1* leads to the overaccumulation of extracellular matrix proteins and to the development and progression of diabetic glomerulosclerosis. Because the increase in the expression of extracellular matrix genes is much greater than that of TGF- β 1, a TGF- β 1-independent mechanism was thought to exist.

The precise mechanism of *ELMO1* influence on the expression of these genes is still unknown. Previous reports suggested that *ELMO1* cooperates with Dock180 to function as an activator for Rac-1 (21). Accordingly, we next examined the effects of Rac-1 and coexpression of Dock180 with *ELMO1* on the expression of both extracellular matrix genes and MMPs. However, no significant additional effect of these factors on the expression of these genes was detected (data not shown), suggesting that the effects of *ELMO1* on the regulation of expression of these genes were independent of Rac-1.

The mechanism whereby *ELMO1* polymorphisms affect the susceptibility to DN also requires further examination. *ELMO1* is >500 kb, and the polymorphisms associated with DN are located far from the transcription start site. Because we could not identify any variations within the coding region or splicing area, we thought that the identified variations might in fact affect *ELMO1* gene expression under a high glucose milieu. Indeed, several modulators for transcriptional activity were reported to be located as far as hundreds of kilobasepairs from the transcription initiation site. We then searched for specific protein binding to the DNA corresponding to several candidate regions within *ELMO1* and identified high glucose-induced specific protein binding at the landmark SNP site (data not shown). Because the sequence including this landmark

SNP site (intron 18+1970) was compatible with the binding site for GCR-1 (see supplementary Table 2 in the online appendix), which could control the transcriptional activity of glycolytic enzyme genes in response to the alteration of extracellular concentrations of glucose in *Saccharomyces cerevisiae* (22), this region might be directly involved in conferring susceptibility to DN. However, the elucidation of the precise mechanism requires further study.

In summary, we identified *ELMO1* as a candidate gene for conferring susceptibility to DN based on results from a genome-wide case-control association study using gene-based SNPs. The evidence presented here suggests that *ELMO1* could be a useful target for new drugs to aid in the prevention and treatment of DN.

ACKNOWLEDGMENTS

This work was supported by a grant from the Japanese Millennium Project.

The authors would like to thank Shuichi Tsukada, Kana Ohashi, and the technical staff at the SNP Research Center and the Institute of Physical and Chemical Research.

REFERENCES

- Excerpts from the United States Renal Data System (USRDS): 2001 Annual Data Report: atlas of end-stage renal disease in the United States. *Am J Kidney Dis* 38 (Suppl. 3):S1-S176, 2001
- Patient Registration Committee, Japanese Society for Dialysis Therapy: The current state of chronic dialysis treatment in Japan. *J Jpn Soc Dial Ther* 35:1155-1184, 2002
- Krolewski AS, Warram JH, Rand LI, Kahn CR: Epidemiologic approach to the etiology of type 1 diabetes mellitus and its complications. *N Engl J Med* 317:1390-1398, 1987
- Quinn M, Angelico MC, Warram JH, Krolewski AS: Familial factors determine the development of diabetic nephropathy in patients with IDDM. *Diabetologia* 39:940-945, 1996
- Moczulski DK, Rogus JJ, Antonellis A, Warram JH, Krolewski AS: Major susceptibility locus for nephropathy in type 1 diabetes on chromosome 3q. *Diabetes* 47:1164-1169, 1998
- Imperatore G, Hanson RL, Pettitt DJ, Kobes S, Bennett PH, Knowler WC,

- the Pima Diabetes Genes Group: Sib-pair linkage analysis for susceptibility genes for microvascular complications among Pima Indians with type 2 diabetes. *Diabetes* 47:821–830, 1998
7. Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y: A high-throughput SNP typing system for genome-wide association studies. *J Hum Genet* 46:471–477, 2001
 8. Hirakawa M, Tanaka T, Hashimoto Y, Kuroda M, Takagi T, Nakamura Y: JSNP: a database of common gene variations in the Japanese population. *Nucleic Acid Res* 30:158–162, 2002
 9. Haga H, Yamada R, Ohnishi Y, Nakamura Y, Tanaka T: Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. *J Hum Genet* 47:605–610, 2002
 10. Tanaka N, Babazono T, Saito S, Sekine A, Tsunoda T, Haneda M, Tanaka Y, Fujioka T, Kaku K, Kawamori R, Kikkawa R, Iwamoto Y, Nakamura Y, Maeda S: Association of solute carrier family 12 (sodium/chloride) member 3 with diabetic nephropathy, identified by genome-wide analyses of single nucleotide polymorphisms. *Diabetes* 52:2848–2853, 2003
 11. Bedell JA, Korf I, Gish W: MaskerAid: a performance enhancement to RepeatMasker. *Bioinformatics* 16:1040–1041, 2000
 12. Saito S, Iida A, Sekine A, Miura Y, Sakamoto T, Ogawa C, Kawauchi S, Higuchi S, Nakamura Y: Identification of 197 genetic variations in six human methyltransferase genes in the Japanese population. *J Hum Genet* 46:529–537, 2001
 13. Lan HY, Mu W, NG YY, Nikolic-Paterson DJ, Atkins RC: A simple, reliable, and sensitive method for nonradioactive in situ hybridization: use of microwave heating to improve hybridization efficiency and preserve tissue morphology. *J Histochem Cytochem* 44:281–287, 1996
 14. Yamada R, Tanaka T, Unoki M, Nagai T, Sawada T, Ohnishi Y, Tsunoda T, Yukioka M, Maeda A, Suzuki K, Tateishi H, Ochi T, Nakamura Y, Yamamoto K: Association between a single-nucleotide polymorphism in the promoter of the human interleukin-3 gene and rheumatoid arthritis in Japanese patients, and maximum-likelihood estimation of combinational effect that two genetic loci have on susceptibility to the disease. *Am J Hum Genet* 68:674–685, 2001
 15. Excoffier L, Slatkin M: Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* 12:921–927, 1995
 16. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D: The structure of haplotype blocks in the human genome. *Science* 296:2225–2229, 2002
 17. Sharma K, Ziyadeh FN: Hyperglycemia and diabetic kidney disease: the case for transforming growth factor-beta as a key mediator. *Diabetes* 44:1139–1146, 1995
 18. Ozaki K, Ohnishi Y, Iida A, Sekine A, Yamada R, Tsunoda T, Sato H, Sato H, Hori M, Nakamura Y, Tanaka T: Functional SNPs in the lymphotoxin- α gene that are associated with susceptibility to myocardial infarction. *Nat Genet* 32:650–654, 2002
 19. Suzuki A, Yamada R, Chang X, Tokuhira S, Sawada T, Suzuki M, Nagasaki M, Nakayama-Hamada M, Kawaida R, Ono M: Ohtsuki M, Furukawa H, Yoshino S, Yukioka M, Tohma S, Matsubara T, Wakitani S, Teshima R, Nishioka Y, Sekine A, Iida A, Takahashi A, Tsunoda T, Nakamura Y, Yamamoto K: Functional haplotypes of *PADI4*, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet* 34:395–402, 2003
 20. Gumienny TL, Brugnera E, Tosello-Tramont AC, Kinchen JM, Haney LB, Nishiwaki K, Walk SF, Nemergut ME, Macara IG, Francis R, Schedl T, Qin Y, Van Aelst L, Hengartner MO, Ravichandran KS: CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. *Cell* 107:27–41, 2001
 21. Brugnera E, Haney L, Grimsley C, Lu M, Walk SF, Tosello-Tramont AC, Macara IG, Madhani H, Fink GR, Ravichandran KS: Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. *Nat Cell Biol* 4:574–582, 2002
 22. Herrero P, Flores L, de la Cera T, Moreno F: Functional characterization of transcriptional regulatory elements in the upstream region of the yeast *GLK1* gene. *Biochem J* 343:319–325, 1999