Brief Genetics Report

Polymorphism Screening of Four Genes Encoding Advanced Glycation End-Product Putative Receptors

Association Study With Nephropathy in Type 1 Diabetic Patients

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Advanced glycation end-products (AGEs) may play an important role in the pathogenesis and progression of cardiovascular and renal complications of diabetes. Four putative AGE receptors (RAGEs), AGE-R1, AGE-R2, and AGE-R3 have been described. In this study, we scanned the sequence of the genes encoding these AGE receptors in 48 patients with type 1 diabetes and investigated the identified polymorphisms (n = 19) in 199 type 1 diabetic patients with nephropathy and 193 type 1 diabetic patients without nephropathy. Overall, none of the polymorphisms was strongly associated with nephropathy. The minor allele of a polymorphism located in the promoter region of the RAGE gene (C-1152A) conferred a weak protective effect ($P < 0.05$) and was associated with a longer duration of nephropathy-free diabetes ($P = 0.08$). *Diabetes* 50:1214–1218, 2001

Microvascular lesions and accelerated atherosclerosis are the major causes of morbidity and early mortality in diabetes (1). There are data showing that genetic factors may influence the risk of developing both micro- and macrovascular complications in diabetic patients (2–5). The mechanisms by which chronic hyperglycemia exerts its adverse effects are not completely understood, but the role of advanced glycation end-products (AGEs), the reactive derivatives of nonenzymatic glucose-protein, and glucose-lipid condensation reaction are now well established (6). In vivo and in vitro studies indicate that AGEs have a role in the pathogenesis of diabetic nephropathy and the progression of renal failure (7). AGEs have a number of deleterious effects, including cross-linking of proteins, modification of matrix components, platelet aggregation, defective vascular relaxation, abnormal lipoprotein metabolism, and induction of cytotoxic pathways that may affect the pathophysiology of the vascular wall (7). The inhibition of AGE formation by aminoguanidine treatment improves the microvascular lesions found in diabetic animals within both the glomerulus and the retina (6) and prevents diabetes-induced arterial wall protein cross-linking (8).

AGE-specific cellular receptor (AGE-R) complexes have been identified on different cell types, including endothelial cells, smooth muscle cells, lymphocytes, and monocytes, where they mediate AGE removal as well as multiple biological responses, including the triggering of inflammatory genes. They include (see Table 1 for nomenclature) the AGE receptor (RAGE) (9), the Dolichyl-diphosphooligosaccharide-protein glycosyltransferase (AGE-R1) (10), the Protein kinase C substrate, 80KH phosphoprotein (AGE-R2) (11), and Galectin-3 (AGE-R3) (12). Recent studies in the NOD mouse further demonstrated that increased levels of AGE in kidneys were associated with reduced AGE-R sites on mesangial cells; this suggests that impaired kidney AGE-R function may contribute to renal disease in this type of mouse (13).

Because polymorphisms in genes encoding RAGEs might affect the genetic susceptibility to vascular complications of diabetes, we screened the four AGE-R genes for sequence variation and investigated the identified variants in type 1 diabetic patients with and without nephropathy.

To identify polymorphisms, DNA from 48 type 1 diabetic patients with or without nephropathy was amplified by polymerase chain reaction (PCR). A total of 62 pairs of primers were designed to generate overlapping fragments of <300 bp, spanning each exon of the four genes as well as their promoter and intronic sequences flanking exons (Table 1). Mutation detection was performed using single-strand conformational polymorphism (SSCP) analysis and direct sequencing of the allelic PCR fragments. A few polymorphisms may not have been detected by the SSCP method we used. SSCP variants were detected in the four genes, five in RAGE, five in AGE-R1, four in AGE-R2, and five in AGE-R3. Sequencing of the PCR fragments revealed

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AGE, advanced glycation end-product; AGE-R, AGE-specific cellular receptor; apo, apolipoprotein; ASO, allele-specific oligonucleotides; PCR, polymerase chain reaction; RAGE, AGE receptor; SSCP, single-strand conformational polymorphism.

TABLE 1
Description of the explored genes

Gene	OMIM name	OMIM accession number	Chromosome	Number of exons	Length of explored sequences (bp)						Nb fragments for SSCP analysis
					5′	5′ nt	Exons	Flanking intronic region	3′ nt	3′	
RAGE	AGER	600214	6p21.3	11	1084	—	1212	1531	176	60	18
AGE-R1	OST48	602202	1p36.1	11	114	—	1371	1157	—	150	11
AGE-R2	PRKCSH	177060	19	18	1200	137	1736	2202	388	63	23
AGE-R3	LGALS3	153619	14q21-q22	6	382	52	753	1296	—	101	10

AGER, Advanced glycosylation end-product–specific receptor; LGALS3, galectin 3; nt, nontranslated; OMIM, Online Mendelian Inheritance in Man (site available at: http://www.ncbi.nlm.nih.gov/Omim/); OST48, oligosaccharyltransferase; PRKCSH, protein kinase C substrate, 80 kDa protein, heavy chain.

the presence of missense mutations in exon 3 of RAGE (G82S), exon 15 of AGE-R2 (I452V), and exon 3 of AGE-R3 (P64H and T98P). Silent mutations were identified in exon 1 of RAGE (A2A), exon 3 of AGE-R1 (G120G), and exon 15 of AGE-R2 (L445 l). Five polymorphisms were found in the 5′ untranslated regions of RAGE and AGE-R3 genes, and the remaining ones were found in introns or 3′ untranslated regions (Table 2). All polymorphisms were further genotyped using allele-specific amplification in two groups of Caucasian type 1 diabetic patients from Denmark, matched for age, sex, and duration of diabetes. One group consisted of subjects with nephropathy (n = 199), and the other group consisted of subjects with longstanding diabetes without nephropathy (n = 193). Genotype and minor allele frequencies for the two groups of patients are reported in Tables 3 and 4 (when two polymorphisms were in complete concordance, only one of them was entered in the table). In patients with or without nephropathy, there was no significant deviation from Hardy-Weinberg equilibrium for any genotype. A weak association was observed for the C-1152A polymorphism located in the promoter region of the RAGE gene; the minor allele at this locus is less frequent in patients with than without nephropathy (odds ratio of heterozygotes 0.49 [0.25–0.94]; P = 0.031). In addition, a larger number of the glutamic acid repeats in the AGE-R2 gene was associated with the presence of nephropathy; long alleles corresponding to 10, 11, and 12 repeats were more frequent in the presence of nephropathy than were the short 8 and 9 repeat alleles (P = 0.041). A possible protective effect of the RAGE-1152A allele was consistent with a trend for longer duration of diabetes before onset of nephropathy in C-1152A heterozygotes

TABLE 2
Description of the polymorphisms

Gene	Polymorphism	Position	Type of variation	Minor allele frequency
RAGE				
	C-1152A	5′	C/a	0.06
	T-388A	5′	T/a	0.32
	A2A	Exon 1	A/t	0.01
	G82S	Exon 3	G/a	0.04
	G+196A (196th nucleotide after codon Stop)	3′	G/a	0.06
AGE-R1				
	G120G	Exon 3	T/c	0.39
	C+17/in4T (17th nucleotide of intron 4)	Intron 4	C/t	0.20
	T-16/in6C (16th nucleotide before exon 7)	Intron 6	T/c	0.41
	+32/in8/ins/del (32nd nucleotide of intron 8)	Intron 8	ins/del	0.13
	G+40A	3′	G/a	0.12
AGE-R2				
	C+9/in5T (9th nucleotide of intron 5)	Intron 5	C/t	0.01
	AA312rpt	Exon 11	GAG repeats (8–12)	0.013/0.528/0.379/0.079/0.001
	L445L	Exon 15	T/c	0.01
	I452V	Exon 15	A/g	0.01
AGE-R3				
	G-716A	5′	G/a	0.429
	–698ins/del	5′	±12 bp	0.408
	–496rpt	5′	TA repeats (7, 8, or 9)	0.674/0.217/0.109
	P64H	Exon 3	C/a	0.408
	T98P	Exon 3	A/c	0.425

Positions in the 5′ untranslated regions are given from the ATG for the RAGE gene and from the +1 transcription initiation site for the AGE-R3 gene. All polymorphisms in the AGE-R1 gene were in complete or nearly complete linkage disequilibrium. The C-1152A and G+196A polymorphisms in the RAGE gene were completely associated. All polymorphisms of AGE-R2 except the repeat polymorphism were completely associated. All biallelic polymorphisms of the AGE-R3 gene were in nearly complete concordance (overall <7% recombinants).

TABLE 3
Genotypes and minor allele frequencies in diallelic polymorphisms, according to the absence or presence of nephropathy

Gene	Polymorphism	Absence of nephropathy (n = 193) Genotype frequency* 11	12	22	Rare allele frequency	Presence of nephropathy (n = 199) Genotype frequency* 11	12	22	Rare allele frequency	P†
RAGE										
	C-1152A	84.7	15.3	0.0	0.076	91.5	8.5	0.0	0.043	0.043
	T-388A	48.7	42.2	9.1	0.302	43.6	44.6	11.8	0.341	0.52
	G82S	90.6	9.4	0.0	0.047	93.6	5.9	0.5	0.035	0.27
AGE-R1										
	G120G	35.1	49.2	15.7	0.403	38.1	49.2	12.7	0.373	0.65
	C+17/in4T	66.3	30.0	3.7	0.187	62.7	33.2	4.1	0.207	0.77
	T-16/in6C	33.0	53.2	13.8	0.404	35.1	47.9	17.0	0.410	0.53
	+32/in8/ins/del	78.7	20.3	1.0	0.112	72.9	23.5	3.6	0.153	0.17
	G+40A	79.2	18.2	2.6	0.117	78.0	20.5	1.5	0.118	0.67
AGE-R3										
	T98P	31.9	53.4	14.7	0.414	30.3	52.3	17.4	0.436	0.75

Odds ratio (95% CI) for nephropathy associated with RAGE/A-1152 carrying 0.49 (0.25–0.94); P = 0.031. RAGE/A2A: rare allele carriers, 5 subjects without and 6 subjects with nephropathy. *The major allele is coded 1, and the minor allele is coded 2; †difference in genotype frequency between absence versus presence of nephropathy (2 df).

(21.4 ± 9.6 years) as compared with C-1152C homozygotes (17.8 ± 7.3 years) (P = 0.08). In patients with nephropathy, RAGE-1152A heterozygotes had a higher mean level of plasma HDL cholesterol (n = 16; mean 1.75 ± 0.12) than did RAGE-1152C homozygotes (n = 172; 1.44 ± 0.04) (P = 0.02); a similar result was observed for plasma apolipoprotein (apo)-A1 (2.00 ± 0.09 and 1.72 ± 0.03, respectively; P = 0.005). No such association was observed in patients without nephropathy. Plasma levels of triglycerides, total cholesterol, apoB, LDL cholesterol, and lipoprotein-a did not differ according to RAGE-1152 genotypes (data not shown).

Polymorphisms in the RAGE gene have recently been identified and shown to not be associated with type 2 diabetes and macrovascular complications (14) or with diabetic microangiopathy (15). However, in these studies, only the coding regions of the RAGE gene were screened for mutations. Because RAGE expression and induction of cellular oxidant stress are closely linked in diabetic tissue, where AGEs accumulate, polymorphisms in the regulatory regions are therefore candidates for microvascular disease. We identified a C-1152A polymorphism in the promoter region of the RAGE gene. This region overlaps with the 3′ untranslated region of the PBX2 gene on chromosome 6 (16). A PBX2 pseudogene (φPBX2), which has a highly conserved sequence, is present on chromosome 3 (17); this could result in the erroneous assignment of the C-1152A polymorphism to the RAGE gene promoter. However, two arguments rule out this possibility: *1*) the 3′ primer (ataggatcgggggctctgag) used to amplify the promoter region of the RAGE gene for genotyping the C-1152A and T-388A polymorphisms was chosen outside the region homologous to φPBX2; and *2*) complete concordance, based on the observation of 346 common homozygotes and 46 heterozygotes, was observed between the C-1152A polymorphism and the RAGE G+196A polymorphism located downstream of the RAGE gene in a region not homologous to φPBX2. This implies that the C-1152A polymorphism is specific for the RAGE gene. Our results show that the C-1152A polymorphism is weakly associated with the presence of nephropathy in type 1 diabetes. This association might be spurious because of the number of statistical tests performed and should be confirmed in another study. If the association is real, the mechanism by which this C-1152A allele could exert its protective role is not clear. The region –1543/–587 has been identified as important in mediating promoter responsiveness to lipopolysaccharide stimulus, probably because of the presence of two necrosis factor-κB–like binding sites (18). However, the role of negative regulatory elements within this promoter, which explains variable basal expression and inducibility in diverse cell types, is not elucidated. The C-1152A polymorphism creates a potential Islet-1 transcription factor–like binding site that could modify RAGE expression. Interestingly, the apoA1 and HDL cholesterol levels are higher in the C-1152A

TABLE 4
Allele frequencies in repeat polymorphisms according to the absence or presence of nephropathy

Gene	Polymorphism (rpt)	Absence of nephropathy (n = 193) Allele frequency (rpt) 7	8	9	10	11	12	Presence of nephropathy (n = 199) Allele frequency (rpt) 7	8	9	10	11	12	P*
AGE-R2	AA312	—	0.021	0.563	0.345	0.068	0.003	—	0.005	0.492	0.413	0.090	0.000	0.041
AGE-R3	–496	0.709	0.202	0.089	—	—	—	0.640	0.232	0.128	—	—	—	0.09

AGE-R2/C+9/in5T, L445L, 1452V: rare allele carriers, 4 subjects without and 4 subjects with nephropathy. *Difference in allele frequency between absence versus presence of nephropathy (2 df).

carriers among diabetic patients with nephropathy, thus supporting a protective role of this polymorphism against deleterious effects of RAGE-AGEs interaction on lipid profile. Indeed, it has been shown that glycation of the protein component of HDL induces a greater transfer of cholesteryl ester from HDL to lighter-density lipoproteins (19). Further studies are necessary to understand better the function of the RAGE promoter. In addition, the association observed with the C-1152A polymorphism could be caused either by its complete concordance with the G+196A polymorphism located in the 3′ untranslated region of the gene (whose function is unknown) or by another polymorphism not yet identified in the HLA region on chromosome 6. Although no association or linkage with diabetic complications per se has been identified with markers located in the HLA region, putative loci for metabolic factors potentially affecting the occurrence of vascular complications, such as triglyceride levels or fasting glucose, have been described in Finnish families (20) and in Pima Indians (21).

The role of the glutamic acid repeat in exon 11 of the AGE-R2 gene in the pathogenesis of nephropathy is unknown. Little is known about the structure/function relationship of that acidic protein. However, similar polymorphic glutamic acid repeat elements have recently been described in the α2B-adrenergic receptor gene and have been shown to be associated with reduced basal metabolic rate in obese subjects (22). Because these repeat elements were shown to be important for agonist-dependent receptor desensitization, allelic proteins might have different properties. Further studies are necessary to understand fully the role of AGE-R2.

We identified several polymorphisms in the genes encoding putative AGE receptors, and none of these polymorphisms was strongly associated with diabetic nephropathy. However, the C-1152 polymorphisms located in the promoter of the RAGE gene should be investigated further in relation to this pathology.

RESEARCH DESIGN AND METHODS

The patients participating in this study have been previously described in detail (23). All albuminuric type 1 diabetes patients (n = 242) attending the outpatient clinic at the Steno Diabetes Center, Gentofte, Denmark, in 1993 who were >18 years of age and had their glomerular filtration rate measured during the same year were invited to participate in the study; 199 patients were included. A total of 193 type 1 diabetic patients with persisting normoalbuminuria recruited from our outpatient clinic and matched for sex, age, and duration of diabetes served as control subjects.

Genomic DNA was prepared from leukocytes. Primers were designed to span all exon and exon-intron boundary sequences as well as promoter regions from RAGE and AGE-R3. Mutation detection was performed using radioactive SSCP analysis with electrophoresis of samples on 6% acrylamide/7.5% glycerol nondenaturing gels at 1,200 V for 5 h at room temperature. Only this assay condition for SSCP analysis was used because we previously observed (24) that using three other conditions did not improve the sensitivity of the technique, which we estimate may be as high as 90% (25). DNA from patients presenting different SSCPs was reamplified by PCR, and subsequent sequencing in both directions using the big-dye sequencing kit (Pharmacia) was performed. Sample electrophoresis was performed on an automated DNA sequencer (ABI). Genotyping of relevant polymorphisms in 392 subjects was performed using allele-specific oligonucleotides (ASO). Primer sequences, ASOs, and assay conditions are available on our web site at http://genecanvas.idf.inserm.fr. For biallelic polymorphisms, comparison of genotype frequencies between patients with or without nephropathy was performed using a χ^2 test with 2DF, except when one genotype was absent (1 df). For the two repeat polymorphisms, the differences in allele frequencies were considered. For RAGE/C-1152A where a difference was significant at $P < 0.05$, a logistic regression (SAS Proc Logist) adjusted for duration of diabetes was performed to calculate the odds ratio and 95% CI for the complication. Calculations were performed with SAS (Cary, NC).

REFERENCES

1. Andersen AR, Christiansen JS, Andersen JK, Kreiner S, Deckert T: Diabetic nephropathy in type 1 (insulin-dependent) diabetes: an epidemiological study. *Diabetologia* 25:496–501, 1993
2. Seaquist ER, Goetz FC, Rich S, Barbosa J: Familial clustering of diabetic kidney disease: evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 320:1161–1165, 1989
3. 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
4. Earle K, Walker J, Hill C, Viberti GC: Familial clustering of cardiovascular disease in patients with insulin-dependent diabetes and nephropathy. *N Engl J Med* 326:673–677, 1992
5. Fioretto P, Steffes MW, Barbosa J, Rich SS, Miller ME, Mauer M: Is diabetic nephropathy inherited? Studies of glomerular structure in type 1 diabetic sibling pairs. *Diabetes* 48:865–869, 1999
6. Brownlee M: Lilly lecture 1993: Glycation and diabetic complications. *Diabetes* 43:836–841, 1994
7. Raj DS, Choudhury D, Welbourne TC, Levi M: Advanced glycation end-products: a nephrologist's perspective. *Am J Kidney Dis* 35:365–380, 2000
8. Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A: Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232: 1629–32, 1986
9. Schmidt AM, Hasu M, Popov D, Zhang JH, Chen J, Yan SD, Brett J, Cao R, Kuwabara K, Costache G, Simionescu N, Stern D: Receptor for advanced glycation end-products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. *Proc Natl Acad Sci U S A* 91:8807–8811, 1994
10. Li YM, Mitsuhashi T, Wojciechowicz D, Shimizu N, Li J, Stitt A, He C, Banerjee D, Vlassara H: Molecular identity and cellular distribution of advanced glycation endproduct receptors: relationship of p60 to OST-48 and p90 to 80K-H membrane proteins. *Proc Natl Acad Sci U S A* 93:11047–11052, 1996
11. Goh KC, Lim YP, Ong SH, Siak CB, Cao X, Tan YH, Guy GR: Identification of p90, a prominent tyrosine-phosphorylated protein in fibroblast growth factor-stimulated cells, as 80 K-H. *J Biol Chem* 271:5832–5838, 1996
12. Vlassara H, Li YM, Imani F, Wojciechowicz D, Yang Z, Liu FT, Cerami A: Identification of galectin-3 as a high-affinity binding protein for advanced glycation end-products (AGE): a new member of the AGE-receptor complex. *Mol Med* 1:634–646, 1995
13. He CJ, Zheng F, Stitt A, Striker L, Hattori M, Vlassara H: Differential expression of renal AGE-Receptor genes in NOD mice: possible role in NOD diabetic renal disease. *Kidney Int* 58:1931–1940, 2000
14. Hudson BI, Stickland MH, Grant PJ: Identification of polymorphisms in the receptor for advanced glycation end-products (RAGE) gene: prevalence in type 2 diabetes and ethnic groups. *Diabetes* 47:1155–1157, 1998
15. Liu L, Xiang K: RAGE Gly82Ser polymorphism in diabetic microangiopathy (Letter). *Diabetes Care* 22:646, 1999
16. Sugaya K, Fukagawa T, Matsumoto K, Mita K, Takahashi E, Ando A, Inoko H, Ikemura T: Three genes in the human MHC class III region near the junction with the class II: gene for receptor of advanced glycosylation end-products, PBX2 homeobox gene and a notch homolog, human counterpart of mouse mammary tumor gene int-3. *Genomics* 15:408–419, 1994
17. Aguado B, Campbell RD: The novel gene G17, located in the human major histocompatibility complex, encodes PBX2, a homeodomain-containing protein. *Genomics* 25:650–659, 1995
18. Li J, Schmidt AM: Characterization and functional analysis of the promoter of AGER, the receptor for advanced glycation end-products. *J Biol Chem* 272:16498–16506, 1997
19. Passarelli M, Catanozi S, Nakandakare ER, Rocha JC, Morton RE, Shimabukuro AF, Quintao EC: Plasma lipoproteins from patients with poorly controlled diabetes mellitus and "in vitro" glycation of lipoproteins enhance the transfer rate of cholesteryl ester from HDL to apo-B-containing lipoproteins. *Diabetologia* 40:1085–1093, 1997
20. Pajukanta P, Nuotio I, Terwilliger JD, Porkka KV, Ylitalo K, Pihlajamaki J, Suomalainen AJ, Syvanen AC, Lehtimaki T, Viikari JS, Laakso M, Taskinen MR, Ehnholm C, Peltonen L: Linkage of familial combined hyperlipidaemia to chromosome 1q21-q23. *Nat Genet* 18:369–373, 1998
21. Pratley RE, Thompson DB, Prochazka M, Baier L, Mott D, Ravussin E, Sakul H, Ehm MG, Burns DK, Foroud T, Garvey WT, Hanson RL, Knowler

WC, Bennett PH, Bogardus C: An autosomal genomic scan for loci linked to prediabetic phenotypes in Pima Indians. *J Clin Invest* 15 101:1757–1764, 1998
22. Heinonen P, Koulu M, Pesonene U, Karvonene MK, Rissanene A, Laakso M, Valve R, Uusitupa M, Scheinin M: Identification of a three-amino acid deletion in the α2B-adrenergic receptor that is associated with reduced basal metabolic rate in obese subjects. *J Clin Endocrinol Metab* 84:2429–2433, 1999
23. Tarnow L, Cambien F, Rossing P, Nielsen FS, Hansen BV, Lecerf L, Poirier O, Danilov S, Parving HH: Lack of relationship between an insertion/deletion polymorphism in the angiotensin I–converting enzyme gene and diabetic nephropathy and proliferative retinopathy in IDDM patients. *Diabetes* 44:489–494, 1995
24. Poirier O, Ricard S, Behague I, Souriau C, Evans AE, Arveiler D, Marques-Vidal P, Luc G, Roizes G, Cambien F: Detection of new variants in the Apolipoprotein B gene by PCR-SSCP. *Human Mutation* 8:282–285, 1996
25. Cambien F, Poirier O, Nicaud V, Herrmann SM, Mallet C, Ricard S, Behague I, Hallet V, Blanc H, Loukaci V, Thillet J, Evans A, Ruidavets JB, Arveiler D, Luc G, Tiret L: Sequence diversity in 36 candidate genes for cardiovascular disorders. *Am J Hum Genet* 65:183–191, 1999