

Risk of Diabetic Nephropathy in Type 1 Diabetes Is Associated With Functional Polymorphisms in RANTES Receptor Gene (*CCR5*)

A Sex-Specific Effect

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Chemokines and their receptors have been implicated in the development of diabetic nephropathy. To determine whether the risk of diabetic nephropathy is influenced by two functional polymorphisms in the regulated upon activation normal T-cell expressed and secreted (RANTES) receptor gene (*CCR5*), we recruited patients with type 1 diabetes, including 496 case subjects with overt proteinuria or end-stage renal disease and 298 control subjects with normoalbuminuria. Male carriers of the 59029G allele, which is associated with diminished expression of *CCR5* on the surface of immunocompetent cells, had significantly higher risk of developing diabetic nephropathy than noncarriers (OR [95% CI] 1.9 [1.2–3.0]). Similarly, male carriers of the 32-bp deletion, which causes truncation of the protein, had significantly higher risk of diabetic nephropathy than noncarriers (2.3 [1.3–4.2]). Combining both polymorphisms, three haplotypes were distinguished: one nonrisk haplotype carrying the 59029A allele and the 32-bp insertion and two risk haplotypes carrying the 59029A allele with the 32-bp deletion and carrying the 59029G allele with the 32-bp insertion. The distribution of these haplotypes differed significantly ($P < 0.00001$) in men with and without diabetic nephropathy but was not associated with diabetic nephropathy in women. In conclusion, two functional polymorphisms in *CCR5* that decrease expression of the RANTES receptor on immunocompetent cells are associated with increased risk of diabetic nephropathy in type 1 diabetes, but only in men. *Diabetes* 54:3331–3335, 2005

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ACR, albumin-to-creatinine ratio; ESRD, end-stage renal disease; RANTES, regulated upon activation normal T-cell expressed and secreted.

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In diabetic nephropathy, the infiltration of the glomeruli and interstitium by immunocompetent cells is a common finding both in rodent models of diabetes and in biopsy specimens collected from diabetic patients (1,2). Moreover, the increased synthesis of chemokines and other inflammatory mediators by glomerular and tubular cells has been reported in hyperglycemic conditions (2,3). This includes such chemokines as regulated upon activation normal T-cell expressed and secreted (RANTES) and macrophage inflammatory protein-1 α and macrophage inflammatory protein-1 β (4–5). These molecules are able to recruit to kidney monocytes by binding to chemokine receptors such as CCR1, CCR3, and CCR5 (6).

Recent studies have reported that polymorphisms in the RANTES receptor gene (*CCR5*) were associated with the increased risk of nephropathy in type 2 diabetes in a Japanese population (7) and with a higher risk of kidney rejection among nondiabetic individuals with kidney transplants (8). The present study aims to examine the association of two functional polymorphisms in *CCR5* with the risk of diabetic nephropathy in Caucasians with type 1 diabetes.

CCR5 has been mapped to the short arm of chromosome 3 within the chemokine receptor gene cluster (9). Recent studies established that this gene comprises three exons spanning a region of about 6 kb. The clinical and functional relevance of many polymorphisms and haplotypes in *CCR5* has been reported (10–13). The most consistent data have been published for the insertion/deletion of 32 bp in exon 3 (11). The deletion changes the open reading frame of *CCR5* and results in a nonfunctional truncated protein. Deletion homozygotes are protected against HIV-1 infection (13). Carriers are relatively common, ~10–15% of healthy individuals in Caucasian populations (14,15). In East Asian populations, however, it is very rare (15). The heterozygous carriers of the deletion have decreased *CCR5* expression on the surface of immunocompetent cells.

Another sequence difference in intron 1, A59029G, also affects expression of the receptor (16). Carriers of the 59029G allele have decreased *CCR5* expression on the surface of immunocompetent cells and, if infected by HIV, develop clinical symptoms more slowly than noncarriers

TABLE 1
Selected clinical characteristics of individuals with type 1 diabetes according to study group and sex

Clinical characteristics at time of examination	Control subjects		Case subjects	
	Men	Women	Men	Women
<i>n</i>	136	162	275	221
Age at diabetes diagnosis (years)	12.1 ± 7.4	11.8 ± 6.8	12.2 ± 7.7	11.7 ± 6.9
Duration of diabetes (years)	22.6 ± 8.7	23.0 ± 7.8	26.5 ± 8.3*	26.9 ± 7.9†
HbA _{1c} (%)	7.9 ± 1.1	8.0 ± 1.4	8.8 ± 1.9*	8.7 ± 1.6*
Systolic blood pressure (mmHg)	120 ± 13	118 ± 15	135 ± 12†	139 ± 20†
Diastolic blood pressure (mmHg)	74 ± 7	73 ± 2	79 ± 10*	79 ± 15†
Treated for hypertension (%)	10.2	9.7	73.2†	69.4†
ACE inhibitor (%)	6.8	7.0	48.9†‡	49.1†‡
Proliferative retinopathy (%)	21	24	78†	77†

Data are means ± SD or %. * $P < 0.05$ for comparison with control subjects; † $P < 0.02$ for comparison with control subjects. ‡A majority of the cases were enrolled in the study between 1991 and 1998. During that time, an ACE inhibitor was not prescribed to all patients with diabetic nephropathy.

(16). Whether this polymorphism influences gene transcription directly or is a marker of another sequence difference that affects the mRNA level of *CCR5* is unclear (13,17). Interestingly, some data indicate that expression of *CCR5* on the surface of immunocompetent cells is influenced by sex hormone levels and that the recruitment of these cells to damaged kidneys may differ between men and women (18,19). Our examination of a sex-specific effect of RANTES polymorphisms was also prompted by the observation that the risk of end-stage renal disease (ESRD) in the U.S. population of patients with type 1 diabetes is twice as high in men as in women (20).

To examine the association between functional polymorphisms in *CCR5* and the risk of diabetic nephropathy in type 1 diabetes, we recruited case subjects and control subjects from among type 1 diabetic patients attending the Joslin Diabetes Clinic. We examined 496 case subjects with advanced diabetic nephropathy (251 patients with ESRD and 245 patients with persistent proteinuria) and 298 control subjects with normoalbuminuria and a minimum of 15 years duration of diabetes. Selected clinical characteristics of the patients are presented in Table 1 according to study group and sex. Age at diabetes onset was similar in all study groups, but duration of diabetes was slightly longer for case subjects than control subjects. Treatment with antihypertensive medication (including ACE inhibitors) was more frequent, and HbA_{1c} and blood pressure were higher in the group of case subjects than in the control subject group.

DNA from case subjects and control subjects was genotyped for the two functional polymorphisms of *CCR5*: A59029G in intron 1 and the 32-bp insertion/deletion in exon 3. Genotype distributions for both polymorphisms are presented in Table 2 according to study group and sex. None deviated significantly from Hardy-Weinberg equilibrium. Among men, the genotype distributions for both the A59029G polymorphism and the 32-bp insertion/deletion differed significantly between control subjects and case subjects ($P = 0.005$ and 0.009 , respectively). The risk of diabetic nephropathy for male carriers of the 59029G allele was nearly twofold that for noncarriers (odds ratio [OR] [95% CI] 1.9 [1.2–3.0]), whereas the risk for male carriers of the 32-bp deletion was more than twofold that for noncarriers (2.3 [1.3–4.2]). Among women, the risk of diabetic nephropathy was unrelated to the genotype for either *CCR5* polymorphism.

To test whether the observed associations were independent or resulted from linkage disequilibrium between

the two functional polymorphisms, we performed a haplotype analysis. Using Haplo.Stat (21) software, we identified three haplotypes that accounted for 99% of the genetic variation at these two loci. The distribution of these haplotypes is shown in Table 3 according to study group and sex. Among women, haplotype frequencies were almost identical in case subjects and control subjects (global statistics 5.24, $P = 0.15$). Among men, however, the distribution of these haplotypes was strikingly different between case subjects and control subjects (global statistics 22.94, $P = 0.00004$). The haplotype containing the 59029A allele in intron 1 and the 32-bp insertion in exon 3 was protective and occurred with a frequency of 54.2% in control subjects and 37.6% in case subjects (empirical $P < 0.00001$). The haplotype containing the 59028A allele in intron 1 and the 32-bp deletion in exon 3 was a risk haplotype and occurred with a frequency of 7.3% in control subjects and 12.2% in case subjects (empirical $P = 0.03$). The haplotype containing the 59028G allele in intron 1 and the 32-bp insertion in exon 3 was a risk haplotype and occurred with a frequency of 38.6% in control subjects and 49.5% in case subjects (empirical $P = 0.0008$). When this analysis was stratified according to duration of diabetes, the results were similar, although the difference between case subjects and control subjects was more pronounced

TABLE 2
A52029G and 32 bp insertion/deletion *CCR5* genotype distributions among case subjects and control subjects according to sex

Genotypes	Men		Women	
	Control subjects	Case subjects	Control subjects	Case subjects
A59029G				
AA	44 (32)	55 (20)	46 (28)	63 (28)
AG	77 (57)	164 (60)	75 (46)	110 (50)
GG	15 (11)	56 (20)	41 (26)	48 (22)
χ^2	10.55		0.77	
P	0.005		NS	
32 bp insertion/ deletion				
Ins/Ins	120 (88)	210 (76)	130 (81)	190 (86)
Ins/Del	13 (10)	60 (22)	28 (17)	28 (13)
Del/Del	3 (2)	5 (2)	4 (2)	3 (1)
χ^2	8.70		2.36	
P	0.009		NS	

Data are *n* (%) unless otherwise indicated. P value calculated for 2 degrees of freedom. NS, not significant.

TABLE 3
Estimated frequencies of *CCR5* haplotype distribution among studied groups stratified by sex

	Haplotype*		Frequency		Score	<i>P</i>
	A59029G	32 bp (Ins/Del)	Control subjects	Case subjects		
Men	A	Ins	0.54	0.38	-4.7	<10 ⁻⁵
	A	Del	0.07	0.12	2.2	0.03
	G	Ins	0.39	0.50	3.3	0.0008
			Global statistic		22.9	<10 ⁻⁵
Women	A	Ins	0.45	0.46	1.1	NS
	A	Del	0.09	0.08	-1.0	NS
	G	Ins	0.46	0.46	-0.2	NS
			Global statistic		5.2	NS

*Haplotypes with frequency <0.01 were not considered. NS, not significant.

among men with diabetes duration <24 years than among men with diabetes duration ≥24 years (data not shown). Among women, the distribution of haplotypes was similar in case subjects and control subjects, regardless of duration of diabetes.

Complex genotypes were assigned to case subjects and control subjects based on these haplotype frequencies so that the risk of diabetic nephropathy could be examined according to the number of risk haplotypes (Fig. 1). Individuals who carried neither risk haplotype were considered the reference group. Among men, the OR for carriers of one risk haplotype was 2.3 (95% CI 1.3–4.1) and increased to 4.8 (2.5–9.3) for carriers of two risk haplotypes. When case subjects with proteinuria and case subjects with ESRD were analyzed separately, the pattern of ORs was very similar for each stage of diabetic nephropathy (data not shown). Among women, the ORs were not significantly different from 1.0, regardless of the number of risk haplotypes.

The results shown in Fig. 1 were examined with logistic models to test the significance of the difference between men and women in the effect of genotype on the OR for

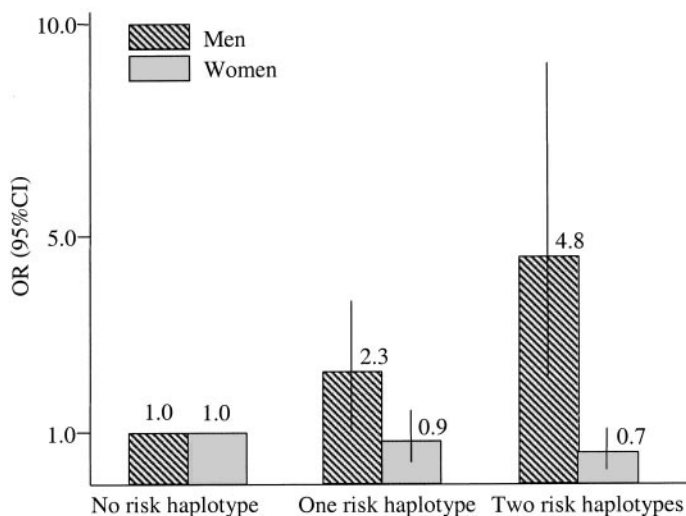


FIG. 1. The risk of developing advanced diabetic nephropathy according to the number of *CCR5* risk haplotypes and sex. The difference between the sexes in the effect of the number of risk haplotypes was statistically significant ($P < 0.0001$).

nephropathy in type 1 diabetes. The effect of the number of risk haplotypes was multiplicative so it could be fitted by a linear term, with a relative odds of 2.2 per risk haplotype in men and 0.8 per risk haplotype in women. The difference between slopes was highly significant statistically ($P < 0.0001$).

In conclusion, the two functional polymorphisms in *CCR5* that decrease expression of the receptor on immunocompetent cells are associated with an increased risk of diabetic nephropathy in type 1 diabetes; however, this association is present only in men.

Limited literature exists regarding the role of these polymorphisms in diseases other than AIDS. A recent study of renal allograft recipients found that acute and chronic rejections were more frequent in carriers of the 59028G allele than in noncarriers (22). Moreover, carriers of the 32-bp deletion in exon 3 of *CCR5* are at higher risk of developing biliary lesions after liver transplant (23). None of the above studies examined the effects according to sex. The sex effect and the RANTES gene polymorphism was previously described among male patients with uveitis (24). The sex differences in the expression of the chemokine receptors, including *CCR5*, have been observed in some studies. These differences are related to the level of sex steroid hormones that were shown in a very elegant study on transsexuals treated with estrogens and androgens (18). Therefore, our results suggest that sex hormones, together with genetic variability in the RANTES receptor, may affect the natural history of diabetic nephropathy.

Our study did not confirm the results of the Japanese study, which revealed an association between the 59029A allele in *CCR5* and diabetic nephropathy in individuals with type 2 diabetes (7). There can be several reasons for this discrepancy. For example, our study group members had type 1 diabetes rather than type 2 diabetes and was much larger: 298 control subjects and 496 case subjects compared with 269 control subjects but only 132 case subjects in the Japanese study. Moreover, the majority of case subjects in the latter study had microalbuminuria rather than advanced nephropathy. Finally, the Japanese data were not analyzed according to sex, and the 32-bp deletion does not occur in the Japanese population (15).

The molecular mechanisms underlying the findings of our study are only speculative at this time. Recent literature has suggested that chemokines, cytokines, and their receptors are involved in the pathogenesis of diabetic nephropathy (3,5,6). It is postulated that the increased synthesis of certain chemokines and cytokines in diabetic kidneys indicates damage to glomeruli and tubular cells. In turn, these chemokines and cytokines mediate the recruitment of immunocompetent cells to the specific areas of kidney damage (4). Thus, a genetically determined impairment in the function of the chemokine receptor *CCR5* may change the profile of recruited cells, and this may promote, for example, renal fibrosis instead of normal tissue repair (2). This effect may be more visible in men since it is known that testosterone may promote apoptotic damage of renal cells and also may diminish the expression of chemokine receptors on immunocompetent cells (18,25). Alternatively, the increased risk of diabetic nephropathy associated with functional polymorphisms in *CCR5* may be secondary to increased blood pressure in carriers of the risk alleles (26,27). Several authors have shown that carriers of the 32-bp deletion have higher blood pressure than with noncarriers (26,28). In the present study, neither

HbA_{1c} nor blood pressure differed between carriers and noncarriers of the risk haplotypes (online appendix [available at <http://diabetes.diabetesjournals.org>]). However, our study was cross-sectional, not longitudinal, so we do not have the ability to examine directly the effect of risk haplotypes on the risk of diabetic nephropathy after controlling for the effects of these covariates.

The significant interaction between sex and the effect of functional polymorphisms in *CCR5* on the risk of diabetic nephropathy deserves special consideration. This finding cannot plausibly be due to survival bias. First, the study population was relatively young, and the effect of mortality on the findings would be most evident in the case subjects with ESRD, the group most affected by high mortality. However, the association of these polymorphisms with the risk of ESRD was similar to their association with that for the risk of proteinuria, suggesting the absence of an effect of mortality on the genotype distribution. Furthermore, the effect of these polymorphisms in men with long diabetes duration (when the effects of mortality would be greatest) was slightly less than in men with short duration. Finally, women with advanced nephropathy are also affected by mortality, but the functional polymorphisms in *CCR5* were not associated in women with the risk of diabetic nephropathy regardless of diabetes duration or stage of nephropathy (proteinuria or ESRD).

How much the sex-specific effect of functional polymorphisms in *CCR5* contributes to the observation that men have twice the risk of ESRD compared with women in the U.S. population with type 1 diabetes is unknown (20). Further studies are needed to confirm our findings regarding a sex-specific effect of functional *CCR5* polymorphisms and to assess the contribution of these polymorphisms to the different levels of risk of ESRD in men and women with type 1 diabetes.

In summary, *CCR5* may be the first immune-related gene that affects the natural history of diabetic nephropathy in a sex-related manner.

RESEARCH DESIGN AND METHODS

Patients for this study were selected from the Joslin Study on the Genetics of Diabetic Nephropathy in Type 1 Diabetes. The Joslin Study was conducted at the Joslin Clinic between 1991 and 2003. During that period, patients with type 1 diabetes attending the clinic were screened regularly for urinary albumin excretion. Patients with ESRD and persistent proteinuria were recruited as case subjects; patients with persistent normoalbuminuria and at least 15 years of diabetes were recruited as control subjects into this study. Overall, 694 case subjects (including 372 with persistent proteinuria and 315 with ESRD) and 730 control subjects were recruited and examined in the Joslin Study. For the present study, we selected only patients for whom DNA had already been extracted. This included 320 control subjects and 520 case subjects.

After consenting to participate in the study, each subject had a standardized physical examination and provided a diabetes history (diagnosis, treatment, and complications). Each individual provided a blood sample for biochemical measurements and DNA extraction. Systolic and diastolic blood pressure (fifth Korotkoff sound) were measured twice with a standard sphygmomanometer while the individual was seated after a 5-min rest. The Committee on Human Subjects of the Joslin Diabetes Center approved the protocol and informed consent procedures.

Diagnosis of diabetic nephropathy. The diabetic nephropathy status of each patient was determined on the basis of questionnaires, medical records of the Joslin Clinic, and measurements of the urinary albumin-to-creatinine ratio (ACR). Methods for measuring the ACR have been described previously (29). Patients were classified as control subjects if they had a diabetes duration >15 years and an ACR <17 mg/g (men) or <25 mg/g (women) in at least two of three consecutive urine specimens taken at least 2 months apart. Patients with microalbuminuria or intermittent proteinuria were excluded from the study. Patients were considered case subjects if they had persistent proteinuria or if they had ESRD due to diabetic nephropathy. Persistent proteinuria was defined as two of three successive urinalyses positive by

reagent strip (>2+ on Multistix; Bayer, Elkhart, IN) or an ACR >250 mg/g (men) or >355 mg/g (women). The individuals who had begun dialysis or received a renal transplant were considered case subjects with ESRD.

Genotyping protocol. The functional DNA polymorphisms in *CCR5*, the A59029G in intron 1, and the 32-bp insertion/deletion in exon 3 have been previously described (1,2). The A59029G polymorphism was determined by PCR followed by digestion with restriction endonuclease *Bsp1286I*. PCR (250- μ l reaction volume) was performed on 20 ng of genomic DNA using 0.25 units of *Taq* polymerase (PGC Scientific) with the following primers: 59029CCR5F: CCCGTG AGC CCA TAG TTA AAA CTC and 59029CCR5R: GAT TTC AGC TAA GAC TCA TCT CTC TGC.

Cycling parameters were denaturation at 95°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 40 s, with final extension at 72°C for 10 min. PCR products (976 bp) were digested with *Bsp1286I* (New England Biolabs, Ipswich, MA) at 37°C for a minimum of 3 h and then fractionated on a 2% agarose gel. An additional restriction site appears when the G allele is present and three fragments are seen in heterozygotes (976 bp and 844 bp + 132 bp).

The 32-bp insertion/deletion *CCR5* polymorphism was determined by PCR followed by fractionation of amplification product at 2.5% fine-resolution agarose (MetaPhor; Cambrex Bio Science, Rockland, ME). PCR (25- μ l reaction volume) was performed on 20 ng of genomic DNA using 0.25 units of *Taq* polymerase (PGC Scientific) with the following primers: CCR5F: GATAGG TACTGGCTGTGCTCCAT and CCR5R: ACCAGCCCCAAGATGACTATCT.

Cycling parameters were denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and an extension at 72°C for 30 s, with a final extension at 72°C for 10 min. Two fragments were seen on the gel: 239 bp corresponding to insertion and 207 bp corresponding to deletion.

Altogether, 93% of control subjects and 95% of case subjects were successfully genotyped for the above described polymorphisms; the remainder could not be determined due to technical difficulties with PCR amplification.

Analytic methods. The data from the study were analyzed using SAS (version 8.02 for Windows; SAS Institute, Cary, NC). Haplotype frequencies were estimated using the Estimated Haplotypes program (30). Differences in haplotype distribution between the studied groups were evaluated by scoring the haplotypes using generalized linear models. Empirical *P* values for this approach were computed using the Haplo.Stat program (21). Haplotypes with an estimated frequency <0.01 were not considered. Logistic models were used first to test the effect of the number of risk haplotypes on the risk of diabetic nephropathy separately according to sex. Then the sexes were combined in a single model with interaction terms for sex to test the significance of the difference in haplotype effect according to sex.

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