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SHORT COMMUNICATION

Resequencing of the *CCL5* and *CCR5* genes and investigation of variants for association with diabetic nephropathy

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Chemokine (C–C motif) ligand 5 (*CCL5*) and chemokine (C–C motif) receptor 5 are implicated in the pathogenesis of diabetic nephropathy (DN). We hypothesize that variants in these genes may be associated with DN. The *CCL5* and chemokine receptor type 5 (*CCR5*) genes were resequenced, variants identified (n=58), allele frequencies determined in 46 individuals (92 chromosomes) and efficient haplotype tag single-nucleotide polymorphisms (htSNPs) selected to effectively evaluate the common variation in these genes. One reportedly functional gene variant and eight htSNPs were genotyped in a case–control association study involving Caucasian individuals with type 1 diabetes (267 cases with DN and 442 non-nephropathic diabetic controls). Genotyping was performed using MassARRAY iPLEX, TaqMan, gel electrophoresis and direct capillary sequencing. After correction for multiple testing, there were no statistically significant associations between variants in the *CCL5* and *CCR5* genes and DN.
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Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in the Western world[1] with significant evidence that a subset of type 1 diabetic patients are genetically predisposed to developing DN.[2] Chemokine (C–C motif) ligand 5 (alternatively known as regulated upon activation, normal T cell expressed and secreted; *CCL5*) and its cognate receptor, chemokine receptor type 5 (*CCR5*), are biological candidate genes for DN.

CCL5 is a potent chemoattractant produced by renal mesangial cells, which is active in the recruitment of monocytes and macrophages into the glomeruli and interstitium. CCL5 is nuclear factor -κB dependent and is upregulated by proteinuria[3] and other factors associated with the diabetic milieu.[4–6] Blockade of CCR5 has been shown to substantially reduce monocyte infiltration in experimental glomerulonephritis.[7] *CCL5* and *CCR5* have received considerable attention as candidate genes for DN, although these studies have focused on selected variants with inconsistent findings.[8–15]

The *CCL5* gene locus has been assigned to 17q11.2-q12,[16] and the *CCR5* gene is located on chromosome 3p21.[17] Coding regions, intron–exon boundaries, untranslated and flanking regions of the *CCL5* and *CCR5* genes have been comprehensively screened to identify polymorphisms, and a robust case–control study was performed to assess the association between common haplotype tagging or functional variants and DN in a Caucasian population with type 1 diabetes.

The Irish case– control collection investigated in this paper has been described previously.[18] Briefly, cases have type 1 diabetes with DN, while controls are type 1 diabetic patients without nephropathy. The average age at recruitment of cases and controls was 48.7 (s.d. 9.6) years and 42.2 (s.d. 11.7) years, respectively, with an average disease duration of 32.0 (s.d. 9.6) years for cases and 27.2 (s.d. 9.3) years for controls.

The *CCL5* and *CCR5* reference sequences (accession number chromosome:NCBI35:17:31217613:31236490:1 and chromosome: NCBI35:3:46381637:46396695:1, respectively) were obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Reference mRNA (CCL5, NM_002985, GI:22538813; CCR5, NM_000579, GI:4502638) sequences were used to determine exon–intron boundaries. Overlapping fragments (average length=674 bp) were PCR amplified using genomic DNA from 23 case and 23 control samples. Primers and PCR conditions are available from the authors. Bidirectional sequencing, screening for variants, haplotype tag single-nucleotide polymorphism (htSNP) selection and genotyping were performed as previously described.[18]

The resequenced data have been submitted to GenBank as (a) GQ504011 C–C chemokine ligand 5 (*CCL5*) gene, promoter region

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and complete cds, and as (b) GQ917109 C–C chemokine receptor type 5 (*CCR5*) gene and complete cds. Novel SNPs have been submitted to dbSNP (Table 1).

We screened almost 6 kb of the *CCL5* gene, including all exons, exon–intron boundaries and untranslated regions. Our resequenced data established four differences compared with the GenBank reference sequence for *CCL5* (c.−881G>A, c.−641A>G, c.−534delC and c.−518delA). In all 13 novel SNPs were identified, of which 11 had a minor allele frequency <5%. A total of 27 variants were identified in the screened regions of *CCL5* (Table 1). Genotypes for 10 SNPs with an MAF >5% in 23 cases and 23 controls were used to estimate the haplotype frequencies (Supplementary Figure 1). Eight haplotypes were identified and three htSNPs genotyped using Sequenom MassARRAY iPLEX (Sequenom, San Diego, CA, USA) and direct capillary sequencing in our case–control collection.

In the approximately 9 kb of *CCR5* screened, six novel SNPs were identified, five of which had an MAF <5%. Thirty-one variants were identified in total (Table 1), 22 of which were used to estimate the haplotype frequencies (Supplementary Figure 1). Haplotype frequencies were obtained from *snphap* and input into Stata, in which the *htsearch* command was used to identify htSNPs. Twelve haplotypes were observed and five htSNPs selected, including *CCR5*_rs333, a 32-bp deletion reported to confer resistance to HIV.[19] One additional SNP (*CCR5*_rs1799987) was selected for genotyping on the basis of previous association with DN,[8–13] as well as putative functionality.[19] The selected SNPs were genotyped in an Irish case–control collection of sufficient size to provide approximately 80% power to detect an odds ratio (OR) of 1.6, with an MAF of 10%. No SNP exhibited deviation from Hardy–Weinberg equilibrium ($P>0.001$). Our case–control collection was selected using strict phenotypic criteria and has been previously used to investigate association with DN.[18,20,21]

Greater than 95% completion was achieved for all genotyped SNPs. Contingency table χ^2 tests were used to compare genotype and allele frequencies between cases and controls.

No *CCL5* variants were found to be associated with DN (Table 2). The *CCR5*_rs7637813 AA genotype was more common in controls than in cases (50.5 vs 42.1%; $P=0.04$, Table 2). Haplotype analysis using Haploview[18] revealed that the *CCR5* haplotypes rs7637813A, rs10577983Ins, rs2227010A, rs333Ins and rs17765882T were also more common in the control group than in the case group (8.9 vs 5.8%; $P=0.03$, Supplementary Table 1). Adjustment for multiple comparisons using Bonferroni correction rendered these *P*-values non-significant.

Table 1 Minor allele frequencies for *CCL5* (*n*=27) and *CCR5* (*n*=31) variants derived from 92 chromosomes

SNP	*rs/ss number*	*Base change*	*MAF* (n=*92*)	*SNP*	*rs/ss number*	*Base change*	*MAF* (n=*92*)
1	*CCL5*_rs4239253[a]	A>G	<0.05	1	*CCR5_ss161639230*	*ATC Ins/Del*	*0.08*
2	**CCL5_rs2107538**[a]	**G>A**	**0.17**	2	*CCR5*_rs2040388	A>G	0.43
3	*CCL5*_rs1800825[a]	T>C	<0.05	3	*CCR5_ss161639233*	*G>T*	*<0.05*
4	*CCL5*_rs2280788[a]	C>G	<0.05	4	*CCR5*_rs3136535	G>A	0.14
5	*CCL5*_rs3817655[a]	T>A	0.18	**5**	**CCR5_rs7637813**	**A>G**	**0.29**
6	*CCL5_ss161639192*	*C>T*	*<0.05*	6	*CCR5*_rs41490645	A>C	0.15
7	*CCL5*_rs3817656	T>C	0.12	7	*CCR5*_rs2856757	A>C	0.40
8	*CCL5*_rs1065341[a]	A>G	<0.05	8	*CCR5*_rs41412948	C>T	NA[b]
9	*CCL5*_rs28914816	A>C	<0.05	**9**	**CCR5_rs10577983**	**CTAT Ins/Del**	**0.41**
10	*CCL5*_rs9909416[a]	G>A	0.13	10	*CCR5_ss161639236*	*T>A*	*<0.05*
11	*CCL5_ss161639197*	*T>C*	*<0.05*	11	*CCR5*_rs2734225	G>T	0.38
12	*CCL5*_rs9908907	G>T	0.13	**12**	**CCR5_rs2227010**	**A>G**	**0.46**
13	**CCL5_ss161639200**	**T>A**	**0.12**	13	*CCR5*_rs2856758	A>G	0.15
14	*CCL5*_rs4795095[a]	T>C	0.12	14	*CCR5*_rs2734648	G>T	0.39
15	*CCL5_ss161639203*	*G>A*	*<0.05*	15	*CCR5*_rs1799987	A>G	0.48
16	*CCL5_ss161639206*	*T>C*	*<0.05*	16	*CCR5*_rs1799988[a]	C>T	0.48
17	*CCL5_ss161639209*	*G>A*	*<0.05*	17	*CCR5*_rs1800023	A>G	0.39
18	*CCL5*_rs16971600[a]	A>G	<0.05	18	*CCR5*_rs1800024	C>T	0.07
19	*CCL5_ss161639212*	*G>C*	*<0.05*	19	*CCR5*_rs1799863	T>A	<0.05
20	*CCL5_ss161639215*	*A>G*	*<0.05*	**20**	**CCR5_rs333**	**32 bp Ins/Del**	**0.10**
21	*CCL5_ss161639218*	*T>G*	*0.12*	**21**	**CCR5_rs17765882**	**C>T**	**0.09**
22	*CCL5*_rs9898152[a]	C>T	0.11	22	*CCR5*_rs1800874	G>T	0.39
23	*CCL5_ss161639221*	*C>T*	*<0.05*	23	*CCR5*_rs41526948	A>G	<0.05
24	*CCL5_ss179321721*	*TAAA Ins/Del*	*<0.05*	24	*CCR5_ss161639239*	*C>T*	*<0.05*
25	**CCL5_rs9898132**[a]	**C>T**	**0.13**	25	*CCR5*_rs41442546	C>A	<0.05
26	*CCL5_ss161639224*	*A>G*	*<0.05*	26	*CCR5*_rs746492	G>T	0.47
27	*CCL5_ss161639227*	*C>T*	*<0.05*	27	*CCR5*_rs3087251	G>A	0.47
				28	*CCR5*_rs3087252	C>T	0.39
				29	*CCR5*_ss161639242	G>T	<0.05
				30	*CCR5*_ss161639245	C>T	<0.05
				31	*CCR5*_rs3087253[a]	T>C	0.47

Abbreviations: *CCL5*, chemokine (C–C motif) ligand 5; *CCR5*, chemokine receptor type 5; SNP, single-nucleotide polymorphism.
htSNPs are highlighted in bold font. Novel SNPs are highlighted in italic font and carry an ss identifier.
[a]HapMap genotype data available (release 27, phase II+III, Feb 09).
[b]as *CCR5*_rs41412948 is situated within *CCR5*_rs10577983 deletion, no frequency data are available.

Table 2 Genotype and allele counts for *CCL5* and *CCR5* variants (*n*=9) genotyped in the case (*n*=267) control (*n*=442) collection

		Cases (n=*267*)		*Controls* (n=*442*)			
SNP	*Genotype/allele*	n	*(%)*	n	*(%)*	P	*Genotyping method*
*CCL5*_rs2107538	GG	181	(67.8)	316	(71.8)	0.09	MassARRAY iPLEX
	GA	83	(31.1)	111	(25.2)		
	AA	3	(1.1)	13	(3.0)		
	G	445	(83.3)	743	(84.4)	0.58	
	A	89	(16.7)	137	(15.6)		
*CCL5*_ss161639200	TT	201	(75.6)	346	(78.8)	0.46	Direct capillary sequencing
	TA	62	(23.3)	86	(19.6)		
	AA	3	(1.1)	7	(1.6)		
	T	464	(87.2)	778	(88.6)	0.43	
	A	68	(12.8)	100	(11.4)		
*CCL5*_rs9898132	CC	223	(83.8)	364	(82.4)	0.86	MassARRAY iPLEX
	CT	41	(15.4)	75	(17.0)		
	TT	2	(0.8)	3	(0.7)		
	C	487	(91.5)	803	(90.8)	0.65	
	T	45	(8.5)	81	(9.2)		
*CCR5*_rs7637813	AA	112	(42.1)	222	(50.5)	0.04	TaqMan
	AG	130	(48.9)	172	(39.1)		
	GG	24	(9.0)	46	(10.5)		
	A	354	(66.5)	616	(70.0)	0.17	
	G	178	(33.5)	264	(30.0)		
*CCR5*_rs10577983	Ins/Ins	79	(30.2)	156	(35.5)	0.34	Direct capillary sequencing
	Ins/Del	133	(50.8)	208	(47.4)		
	Del/del	50	(19.1)	75	(17.1)		
	Ins	291	(55.5)	520	(59.2)	0.18	
	Del	233	(44.5)	358	(40.8)		
*CCR5*_rs2227010	AA	66	(26.0)	109	(25.3)	0.95	Direct capillary sequencing
	AG	127	(50.0)	221	(51.3)		
	GG	61	(24.0)	101	(23.4)		
	A	259	(51.0)	439	(50.9)	0.98	
	G	249	(49.0)	423	(49.1)		
*CCR5*_rs1799987[a]	AA	78	(29.7)	127	(29.1)	0.99	TaqMan
	AG	129	(49.0)	216	(49.4)		
	GG	56	(21.3)	94	(21.5)		
	A	285	(54.2)	470	(53.8)	0.88	
	G	241	(45.8)	404	(46.2)		
*CCR5*_rs333	Ins/Ins	220	(83.0)	349	(80.0)	0.47	Agarose gel electrophoresis
	Ins/Del	43	(16.2)	80	(18.3)		
	Del/Del	2	(0.8)	7	(1.6)		
	Ins	483	(91.1)	778	(89.2)	0.25	
	Del	47	(8.9)	94	(10.8)		
*CCR5*_rs17765882	CC	224	(88.2)	357	(82.8)	0.17	Direct capillary sequencing
	CT	28	(11.0)	69	(16.0)		
	TT	2	(0.8)	5	(1.2)		
	C	476	(93.7)	783	(90.8)	0.06	
	T	32	(6.3)	79	(9.2)		

Abbreviations: *CCL5*, chemokine (C–C motif) ligand 5; *CCR5*, chemokine receptor type 5; SNP, single-nucleotide polymorphism.
[a]*CCR5*_rs1799987 is a putatively functional SNP.

The rationale behind this investigation is supported by both *in vitro* and *in vivo* studies, together with previous reports of association for DN. Investigations in Japanese and Asian Indian populations have suggested an association between *CCR5*_rs1799987 (alias 59029G>A) and DN and renal insufficiency in the type 2 diabetic population.[8,11–13] *CCR5*_rs1799987 is a putatively functional variant, located in the

promoter region, which influences expression of the CCR5 protein.[19] As *CCR5*_rs1799987 is the most frequently investigated SNP in previous publications, a sample size calculation was performed for this variant. Our case–control collection is of sufficient size to give approximately 80% power to detect as statistically significant ($P<0.05$) an OR of 1.4 for the A allele of *CCR5 CCR5*_rs1799987, and an OR of 1.6 for any locus of which the minor allele frequency exceeds 10%. Risk haplotypes of *CCR5*_rs1799987 and *CCR5*_rs333 have been associated with DN in Caucasian type 1 diabetic male patients only;[10] this was not supported by our study (data not shown). Some other contradictory findings have also been reported.[14,15]

The published association studies on *CCL5* and *CCR5* performed to date have focused on one or two selected variants in the gene, which were often chosen on the basis of function. A meta-analysis was performed to include data from these reports,[8–14] which investigated three loci relevant to our study: *CCL5*_rs2107538 (pooled OR 0.99; 95% CI 0.80–1.23; $P=0.94$), *CCR5*_rs1799987 (pooled OR 0.96; 95% CI 0.78–1.17; $P=0.66$) and *CCR5*_rs333 (pooled OR 0.96; 95% CI 0.78–1.19; $P=0.73$). It should be noted that studies investigating *CCR5*_rs1799987 exhibit heterogeneity even after the exclusion of the data reported by Ahluwalia *et al.*,[13] which showed very marked deviations from Hardy–Weinberg equilibrium. This suggests that, for this analysis, the results from the different studies were not consistent. Further details are available in Supplementary Figure 2. Collectively, no significant association has been revealed between these variants and DN.

The importance of screening for variants is highlighted by the identification of three novel variants in this study with MAF > 10%, one of which was selected by Stata as an htSNP (Table 1). Furthermore, comparison with HapMap (http://hapmap.ncbi.nlm.nih.gov/ accessed 23 November 2009) revealed that genotype data were available for only 40% of the *CCL5* variants and 6% of the *CCR5* variants investigated in this study (Table 1). This reinforces the value of resequencing for a comprehensive study of all common variations in the *CCR5* gene.

This study has investigated common *CCL5* and *CCR5* variants in the Irish type 1 diabetic population and did not reveal a statistically significant association with DN. Despite evidence of association between individually selected variants and DN in primarily Japanese and Indian type 2 diabetic populations, this could not be confirmed in our Caucasian type 1 diabetic collection. This may reflect the divergent genetic background of different ethnic and disease groups.

Our results indicate that common variants in *CCL5* and *CCR5* do not strongly influence genetic susceptibility to DN in Caucasian individuals with type 1 diabetes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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