RAPID COMMUNICATION

RANTES/CCL5 gene polymorphisms, serum concentrations, and incident type 2 diabetes: results from the MONICA/KORA Augsburg case-cohort study, 1984–2002

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Abstract

Objective: Regulated on activation, normal T-cell expressed and secreted (RANTES)/chemokine (C-C motif) ligand (CCL5) is expressed by adipocytes, and serum levels of RANTES are increased in obesity and type 2 diabetes. The aim of this study was to test the hypothesis that RANTES is involved in the pathogenesis of type 2 diabetes by analyzing the triangular association between CCL5 gene polymorphisms, systemic RANTES concentrations, and incident type 2 diabetes in a large prospective study.

Subjects and methods: The study is based on 502 individuals (293 men and 209 women) and 1632 individuals (859 men and 773 women) with and without incident type 2 diabetes from the population-based MONItoring of Trends and Determinants in Cardiovascular Disease (MONICA)/Cooperative Health Research in the Region of Augsburg (KORA) case-cohort study respectively (mean follow-up time $\pm$ s.d. 10.1 ± 4.9 years). CCL5 genotypes and RANTES serum concentrations were determined and associations between genotypes, haplotypes, serum levels, and incident type 2 diabetes were assessed.

Results: Minor alleles of four single nucleotide polymorphisms were associated with lower RANTES levels (P_{additive} between 1.2×10^{-9} and 3.1×10^{-8}), but neither genotypes, haplotypes, nor serum levels were associated with incident type 2 diabetes.

Conclusions: Our data suggest that RANTES/CCL5 gene variants and serum levels are not causally related with type 2 diabetes and that elevated RANTES levels in patients with type 2 diabetes may be a consequence of hyperglycemia. However, our findings cannot preclude a local role in adipose tissue where RANTES expression may contribute to leukocyte infiltration and a proinflammatory state.

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Introduction

Regulated on activation, normal T-cell expressed and secreted (RANTES)/CCL5 belongs to the family of CC chemokines that recruit and activate different subtypes of leukocytes such as T cells, monocytes, basophils, eosinophils, or mast cells (1). RANTES is expressed by adipocytes and has been hypothesized to mediate leukocyte infiltration of adipose tissue in obesity (2, 3). Circulating RANTES concentrations are elevated in obesity, impaired glucose tolerance (IGT), and type 2 diabetes (4, 5). In the Finnish Diabetes Prevention Study, high RANTES levels were associated with resistance to lifestyle intervention and higher incidence of type 2 diabetes in the intervention group (6). Thus, the aim of this study was to analyze whether RANTES may be involved in the pathogenesis of type 2 diabetes by investigating the triangular relationship between

CCL5 gene polymorphisms, RANTES serum levels, and incident type 2 diabetes in the population-based MONItoring of Trends and Determinants in Cardiovascular Disease (MONICA)/Cooperative Health Research in the Region of Augsburg (KORA) case-cohort study. This study is based on a Mendelian randomization approach. Any association between circulating RANTES concentrations and incident type 2 diabetes could be attributable to reverse causation or residual confounding factors. However, the additional analysis of the link between genotypes, serum levels, and type 2 diabetes incidence could reveal whether RANTES really plays a causal role in the development of type 2 diabetes because genotypes that are associated with high RANTES levels are randomly transmitted to carriers from their parents and potentially confounding factors should be distributed evenly across genotypes.

Subjects and methods

Subjects

The present study is based on the population-based MONICA/KORA case-cohort study. This prospective study (7–9) includes 2134 participants (293 men and 209 women with incident type 2 diabetes; 859 men and 773 women without incident type 2 diabetes). Participants with self-reported diabetes at baseline were excluded. The incidence of type 2 diabetes was assessed using a written follow-up questionnaire sent to all participants of the three baseline surveys in 1997/1998 and 2002/2003. Furthermore, all subjects who participated in S1 were invited to a follow-up examination in 1987/1988. Cases with self-reported incident diabetes were validated by a questionnaire mailed to the treating physician or medical chart review. Only subjects for whom the treating physician clearly reported a diagnosis of type 2 diabetes or for whom a diagnosis of type 2 diabetes was mentioned in the medical records, or who were taking antidiabetic medication were classified as cases for the present analysis. Time of incidence was generally based on the information from the treating physician if the physician reported that he/she had diagnosed the disease himself/herself. If no information could be obtained from the physician who had first diagnosed the disease, the date of diagnosis as reported by the study participant was used. Mean follow-up time ($\pm$ s.d.) was 10.1 ± 4.9 years. For analyses with incident type 2 diabetes as the endpoint, DNA was available from 2067 participants (1142 men and 925 women). For cross-sectional analyses in a random sample of the total study population, 1872 participants (1001 men and 871 women) with DNA samples and complete information on covariables were available. The study was approved by the local authorities, and all participants provided written informed consent.

Measurement of RANTES serum concentrations

RANTES serum concentrations were measured with the Quantikine ELISA kit (R&D Systems, Wiesbaden, Germany). Mean intra- and inter-assay coefficient of variation (CV) values were 4.2 and 5.1% respectively, for recombinant controls, and 4.7 and 6.4% respectively, for test sera.

Genotyping

In order to obtain complete coverage of the *CCL5* gene, six single nucleotide polymorphisms (SNPs) were selected on the basis of data from the International HapMap project (<http://www.hapmap.org>) in May 2005 for a 9.2 kb locus of the *CCL5* gene (promoter: rs2107538 (–403G>A), rs2280788 (–28C>G);

intronic: rs2280789, rs4796120, rs3817655; 3'-flanking region: rs1065341) and covered the total common variation in the gene at the time of SNP selection. Genotyping was carried out with matrix-assisted laser desorption-ionization time of flight analysis of allele-dependent primer extension products, as reported previously (9). A subset of samples (13.3%) was genotyped in duplicate to assess genotyping quality. The mean discordance rate for the six SNPs was 0.44%.

Statistical analysis

Identification of haplotype blocks and statistical analysis were performed as described (9). The associations between RANTES levels or genotypes and incident type 2 diabetes were assessed using Cox proportional hazards analysis. As RANTES concentrations were log-normal distributed, the geometric mean adjusted for age, sex, and survey was computer stratified by *CCL5* genotypes. Pairwise linkage disequilibrium was measured using Lewontin's D' and the squared correlation coefficient r^2 (Fig. 1 of the online appendix which can be viewed online at <http://www.eje-online.org/supplemental/>).

In general, $P < 0.05$ was considered statistically significant. In our analysis of the associations between the six *CCL5* SNPs and serum levels or incident type 2 diabetes, we calculated the number of effectively independent tests from the correlation structure between the SNPs (10). Given five effective SNPs, a priori planned sex-stratified analyses and two outcomes (serum levels and type 2 diabetes), the significance level adjusted for multiple testing according to Bonferroni for each of the SNPs was 0.0025 in each analysis. All evaluations were performed with the statistical software package SAS (Version 8.02 for Unix, Version 9.1 for Windows, SAS Institute, Cary, NC, USA) and the statistical analysis software R, Version 2.4.1 (11).

Results

The baseline characteristics of the MONICA/KORA case-cohort study population have been reported before (9) (Table 1 of the online appendix which can be viewed online at <http://www.eje-online.org/supplemental/>). *CCL5* genotype frequencies for the randomly sampled subcohort are shown in Table 1. Genotyping success rates were 97.7, 98.3, 97.8, 98.2, 98.1, and 97.3% for rs2107538, rs2280788, rs2280789, rs4796120, rs3817655, and rs1065341 respectively. All SNPs were in Hardy-Weinberg equilibrium ($P > 0.2$) and lie in the same haplotype block ($D' > 0.98$ between consecutive SNPs; Fig. 1 of the online appendix which can be viewed online at <http://www.eje-online.org/supplemental/>). Rs3817655 and rs2107538 were highly correlated ($r^2 = 0.977$) and provided rather similar information. All following analyses were a priori

Table 1 Frequencies of *CCL5* genotypes in the randomly sampled subcohort ($n=1872$) and hazard ratios (95% CI) for incident type 2 diabetes comparing *CCL5* genotypes from Cox proportional hazards models ($n=2067$).

SNP	Allele ^a 1/2	n^a 11/12/22	Weighted frequency (%) ^a 11/12/22	Model ^b	Genotype ^a		
					11	12	22
rs2107538	C/T	1212/560/64	65.8/30.6/3.6	1	1 (ref)	0.88 (0.70–1.10)	0.91 (0.51–1.64)
				2	1 (ref)	0.83 (0.65–1.07)	0.88 (0.46–1.67)
				3	1 (ref)	0.84 (0.65–1.08)	0.95 (0.49–1.85)
rs2280788	C/G	1761/78/0	95.8/4.2/0	1	1 (ref)	0.67 (0.37–1.21)	N/A
				2	1 (ref)	0.77 (0.42–1.44)	N/A
				3	1 (ref)	0.88 (0.48–1.61)	N/A
rs2280789	T/C	1397/412/35	75.7/22.5/1.9	1	1 (ref)	0.94 (0.74–1.21)	0.46 (0.15–1.35)
				2	1 (ref)	0.97 (0.74–1.26)	0.44 (0.14–1.38)
				3	1 (ref)	1.01 (0.77–1.32)	0.45 (0.14–1.51)
rs4796120	A/G	1469/353/25	79.5/19.2/1.4	1	1 (ref)	1.04 (0.81–1.35)	0.40 (0.12–1.38)
				2	1 (ref)	1.05 (0.80–1.38)	0.36 (0.10–1.32)
				3	1 (ref)	1.07 (0.81–1.41)	0.38 (0.10–1.48)
rs3817655	T/A	1209/564/68	65.5/30.7/3.7	1	1 (ref)	0.89 (0.71–1.11)	0.89 (0.50–1.58)
				2	1 (ref)	0.85 (0.66–1.09)	0.86 (0.46–1.61)
				3	1 (ref)	0.86 (0.67–1.10)	0.94 (0.49–1.80)
rs1065341	A/G	1,634/196/9	88.8/10.7/0.5	1	1 (ref)	1.01 (0.72–1.42)	0.52 (0.13–2.05)
				2	1 (ref)	0.87 (0.58–1.29)	0.53 (0.15–1.89)
				3	1 (ref)	0.85 (0.57–1.26)	0.54 (0.14–2.09)

N/A, not applicable (no homozygous carriers of the minor allele); ref, reference genotype.

^aAllele 1 denotes the major allele and allele 2 denotes the minor allele.^bModel 1, adjusted for age, sex, and survey; Model 2, Model 1 + BMI; Model 3, Model 2 + smoking status, alcohol intake, physical activity, systolic blood pressure, and ratio of total cholesterol:HDL cholesterol.

planned and performed separately for men and women, but as no sex differences were detected, combined results are given.

First, we investigated whether *CCL5* genotypes have an impact on RANTES levels and found that the minor alleles of four SNPs were associated with lower serum levels (P_{additive} between 1.2×10^{-9} and 3.1×10^{-8} ; Fig. 1A). The *CCL5* haplotype TCCGAA that contained these four minor alleles was also associated with significantly lower serum levels of RANTES compared with most frequent haplotype CCTATA ($P = 4.9 \times 10^{-9}$; see also Table 3 of the online appendix which can be viewed online at <http://www.eje-online.org/supplemental/>). In the age-, sex-, and survey-adjusted analyses, RANTES levels for one or two copies of the TCCGAA haplotype were 24 445 and 20 561 pg/ml respectively, compared with 29 063 pg/ml for two copies of the CCTATA haplotype (calculated for a 60-year-old male participant in survey 1).

Second, we analyzed the association between RANTES levels and the risk for type 2 diabetes. In the unadjusted analysis, RANTES levels at baseline were slightly higher in cases than in non-cases (weighted geometric means 25 420 and 23 807 pg/ml respectively; $P = 0.029$, see also Table 1 of the online appendix which can be viewed online at <http://www.eje-online.org/supplemental/>). Figure 1B shows hazard ratios (HRs) for incident type 2 diabetes across quartiles of RANTES levels from Cox proportional hazards models with different degrees of adjustment for the potential confounders. When compared with the lowest RANTES quartile, higher RANTES concentrations were not significantly associated with increased risk to develop type 2 diabetes during

follow-up. In addition, we assessed the association between continuous RANTES levels and the incidence of type 2 diabetes. A 1-SD increase of RANTES was associated with incident type 2 diabetes only in the age-, sex-, and survey-adjusted model (HR (95% confidence interval (CI)) 1.12 (1.02–1.24); $P = 0.024$), but was lost when including body mass index (BMI) (HR (95% CI) 1.03 (0.91–1.16); $P = 0.689$) into the model. Furthermore, adjustment for other potential confounders had almost no impact on the BMI-adjusted HR (data not shown).

Third, all SNPs and haplotypes calculated from them were tested for association with incident type 2 diabetes. None of the SNPs or haplotypes were significantly associated with diabetes risk (Table 1; Tables 2 and 3 of the online appendix which can be viewed online at <http://www.eje-online.org/supplemental/>).

Discussion

This is the first population-based study to investigate the triangular relationship between *CCL5* gene variants, circulating RANTES levels, and incident type 2 diabetes. Several *CCL5* alleles were strongly related to decreased RANTES levels, but neither *CCL5* genotypes nor RANTES concentrations were associated with incident type 2 diabetes. Our data corroborate and extend findings from smaller studies that suggested an association between *CCL5* genotypes and circulating RANTES levels (12, 13). In addition, our data are in line with previous findings in the Finnish Diabetes Prevention Study. High RANTES levels were not associated with elevated risk for type 2 diabetes in the control

Figure 1 (A) Serum concentrations of RANTES (geometric means) stratified by *CCL5* genotype in the randomly drawn subcohort ($n=1872$). Open bars, hatched bars, and black bars indicate homozygous carriers of the major allele, heterozygotes, and homozygous carriers of the minor allele respectively. NS, not significant; $*P_{\text{additive}} \leq 3.1 \times 10^{-8}$. (B) Hazard ratios (95% CI) of developing type 2 diabetes comparing quartiles of RANTES serum concentrations. Model 1, adjusted for age, sex, and survey; Model 2, adjusted for age, sex, survey, and BMI; Model 3, adjusted for age, sex, survey, BMI, systolic blood pressure, ratio of total cholesterol:HDL cholesterol, physical activity (active/inactive), alcohol intake ($0 > 0$ to $39.9 \geq 40$ g/d for men and $0 > 0$ to $19.9 \geq 20$ g/d for women), smoking status (current, former, never smoker), and parental type 2 diabetes (positive, unknown, negative). If not indicated otherwise, variables were included in continuous form. Ref., reference quartile.

group which may be comparable with our group of study participants (6). However, high RANTES levels were predictive of incident type 2 diabetes in the intervention group which indicates that for as yet unknown reasons individuals with lower RANTES levels could be more responsive to intensive lifestyle intervention than individuals with higher RANTES levels (6).

Strengths of the study include the use of the MONICA/KORA cohort with a large number of cases and non-cases, long follow-up, and detailed information on immune markers and disease risk. Our study had a

power to detect an association with a HR of 1.5 of RANTES serum levels with incident type 2 diabetes of 98% assuming an exposition frequency of 25% as calculated *a priori*. We chose a HR of 1.5 for the power calculation because we found previously in the same study population that HRs for the comparison of extreme quartiles or tertiles of other immune mediators such as C-reactive protein, interleukin-6, interleukin-18, monocyte chemoattractant protein-1, and macrophage migration inhibitory factor were often ≈ 1.5 or higher (7–9, 16).

However, statistical power was lower for the genetic analyses. Assuming HRs of 1.3, our study had a power of 78, 68, and 47% to detect an association between *CCL5* genotypes with diabetes risk in a dominant model with a frequency of 30, 20, and 10% respectively of the genotype with at least one minor allele (*post hoc* power calculation). Thus, we cannot exclude that we missed weaker associations between *CCL5* genotypes and incident type 2 diabetes in this study.

Taken together, our findings indicate that elevated RANTES levels in IGT and type 2 diabetes as reported before (4, 5) may represent a consequence rather than cause of hyperglycemia. However, our data do not preclude a local pathogenetic role in adipose tissue where RANTES expression may contribute to leukocyte chemotaxis and infiltration. Furthermore, it remains to be seen whether RANTES may be more relevant for the risk of diabetic complications than for type 2 diabetes itself, as previous studies demonstrated associations of *CCL5* variants with diabetic neuropathy (14) and cardiovascular disease (13, 15).

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