ORIGINAL ARTICLE

Elevated serum RANTES chemokine levels in autoimmune Addison disease

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KEY WORDS

Addison disease, *CCL5*, chemokine, polymorphism, RANTES

ABSTRACT

INTRODUCTION Regulated on activation, normal T-cell expressed and secreted chemokine (RANTES), the product of the *CCL5* gene, is involved in trafficking immune cells into the inflammation site. It acts as coactivator of T cells and promotes polarization of the immune response towards the Th1 profile. In autoimmune Addison disease (AAD), the adrenal cortex is gradually destroyed by adrenal-specific immune cell infiltration. RANTES might be implicated in autoimmune adrenal failure through recruitment and activation of the immune cells. Furthermore, the promoter *CCL5* variant, rs2107538, seems to be associated with autoimmune endocrine conditions: diabetes and thyroid disease.

OBJECTIVES Our analysis was designed to evaluate the prevalence of rs2107538 and serum RANTES levels in AAD.

PATIENTS AND METHODS rs2107538 was genotyped using TaqMan technology in 239 individuals with AAD and 542 controls, while serum RANTES levels were evaluated by an enzyme-linked immunosorbent assay in 114 patients with AAD and 111 healthy age- and sex-matched individuals.

RESULTS No differences were found in rs2107538 genotype or allele frequencies between patients and controls ($P = 0.53$ and $P = 0.39$, respectively), and no association was detected with age at AAD onset ($P = 0.14$). Serum RANTES levels were elevated in patients with AAD compared with controls (mean [SD], 59.2 [30.3] ng/ml vs 45.5 [20.4] ng/ml, $P = 0.001$). Healthy carriers of various rs2107538 genotypes demonstrated differences in serum RANTES levels ($P = 0.02$), whereas AAD patients did not ($P = 0.26$). No correlation was found between circulating RANTES levels and age, AAD duration, serum autoantibodies, hydrocortisone dose, and body mass ($P > 0.05$).

CONCLUSIONS This study demonstrates for the first time elevated serum RANTES levels in AAD and confirms that rs2107538 may affect serum chemokine levels.

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INTRODUCTION Autoimmune Addison disease (AAD) is a rare endocrine condition characterized by primary adrenocortical insufficiency. Its etiology remains largely obscure. A complex interplay between genetic susceptibility and unknown environmental factors probably triggers autoimmune reaction against adrenocortical antigens and selective destruction of the steroid-producing cells.[1] Circulating autoantibodies to specific antigens such as 21-hydroxylase, are useful markers of the ongoing adrenal autoimmunity even before the onset of the clinical symptoms.[2,3] However, the causative mechanism of progressive gland devastation seems to be mainly cell-mediated.[4,5] Inflammatory infiltration by the mononuclear cells leads to gradual deterioration of the adrenal function and fibrous atrophy of the adrenal cortex.[6]

Regulated on activation, normal T-cell expressed and secreted (RANTES), also known as CC motif chemokine ligand 5 (CCL5), is a pro-inflammatory CC-chemokine (ie, with 2 adjacent conserved cysteine residues) encoded by the gene on chromosome 17q12 and produced by many hematopoietic cells. Its primary role consists in trafficking T-helper cells, monocytes, and

eosinophils into the inflammation site (chemotaxis).[7] Additionally, RANTES may act as an antigen-independent, calcium-dependent coactivator of T cells in vitro, leading to increased interleukin (IL)-2 synthesis, upregulated IL-2 receptor expression, and enhanced T-cell clone proliferation.[8,9] Administration of RANTES together with viral antigens in experimental animal models resulted in polarization of the specific immune response towards the Th1 profile.[10,11] On the contrary, RANTES-deficient mice displayed reduced antigen-specific T-cell proliferation and decreased production of Th1 cytokines (interferon [IFN] γ and IL-2).[12] Th1-type immune response is predominant in AAD, as supported by the prevalence of immunoglobin G1 subclass autoantibody against adrenal antigens and by serum chemokine profile.[13,14]

The CC and CXC subfamilies of chemokines, which promote the initiation and maintenance of the inflammatory process, may play a role in autoimmunity, including autoaggressive reactions against the endocrine glands. Former reports revealed elevated serum levels of chemokines CXCL10, CXCL11, MIP-1α, and MIP-1β in patients with AAD, whereas RANTES has not been studied in this condition.[14-16] Increased serum RANTES levels were found in patients with other autoimmune endocrine disorders, such as type 1 diabetes, Graves disease, and Hashimoto thyroiditis.[17-19] Moreover, a correlation between RANTES mRNA levels in thyroid tissue and the degree of T-cell infiltration was reported.[20]

The sequence variants of the *CCL5* gene seem to be associated with autoimmune endocrine conditions. A haplotype comprising minor alleles of rs4251719, rs2306630, and rs2107538 was less frequently transmitted to individuals with type 1 diabetes, hence it is considered protective.[21] Likewise, the promoter variant rs2107538 (G-403A) appeared less frequent in patients with autoimmune thyroid disease, although the sample size was modest in that study.[22] There have been no reports on *CCL5* polymorphisms in AAD to date. This study was therefore designed to evaluate the likely implication of RANTES in primary autoimmune adrenal failure, by investigation of rs2107538, the most commonly reported functional *CCL5* variant, and assessment of serum levels of this chemokine in affected subjects.

PATIENTS AND METHODS The study comprised 239 individuals with AAD (174 [72.8%] women) and 542 healthy controls (372 [68.6%] women). Patients were recruited from the endocrine departments at the Poznan University of Medical Sciences (Poznań, Poland) and regional outpatient endocrine clinics. Adrenal failure was diagnosed on the basis of clinical signs and symptoms, and confirmed by low circulating cortisol levels accompanied by an elevated plasma adrenocorticotropic hormone (ACTH). In dubious cases, an intravenous stimulation test with 250 μg of tetracosactide ($ACTH_{1-24}$) was performed to confirm lack of responsiveness of the adrenal cortex. Autoimmune origin of the disease was evidenced with positive serum autoantibodies to 21-hydroxylase (21-OH) antibodies assessed using a radioimmunoassay (RSR Ltd, Cardiff, United Kingdom) or, in case of formerly diagnosed patients, anti–adrenal antibodies evaluated by an in-house solid-phase radioimmunoassay using microsomal fraction of human adrenals.[23] Concomitant autoimmune endocrine conditions were diagnosed on the basis of patients' medical records or through standard diagnostic procedures for autoimmune thyroid disease and type 1 diabetes. Individuals with monogenic autoimmune polyendocrine syndrome type 1, consisting of AAD combined with hypoparathyroidism and/or chronic candidiasis, were excluded from the study.

Healthy control patients were enrolled among healthy blood donors at the local blood transfusion center. Controls presented a negative history of autoimmunity and no signs or symptoms of autoimmune, chronic inflammatory, or neoplastic disease at the time of the study. Patients who were recruited for serum RANTES analysis had their anti–thyroid autoantibodies checked to exclude the most common endocrine autoimmunity. Radioimmunoassays (BRAHMS, Henningsdorf, Germany) were used to test for antibodies to thyroid peroxidase (aTPO) and thyroglobulin (aTg) on a scintillation gamma counter (CliniGamma 1272, LKB Wallac, Finland). Values of aTPO below 60 U/ml and aTg below 60 U/ml were considered negative, as recommended by the manufacturer.

The rs2107538 *CCL5* variant was genotyped in study participants. Genomic DNA was extracted from the peripheral blood with Gentra Puregene Blood Kit (Qiagen, Hilden, Germany). Genotyping was performed in Bio-Rad CFX96 Real-Time Detection System, using a validated commercial TaqMan SNP Genotyping assay (C_15874407_10), according to the manufacturer's instructions (Applied Biosystems; Thermo Fisher Scientific, Waltham, Massachusetts, United States). Genotypes were confirmed in 5% of samples by direct DNA sequencing with BigDye® Terminator Cycle Sequencing Ready Reaction Kit and 3730xl Genetic Analyzer (Thermo Fisher Scientific). A subset of 7% of randomly chosen samples was genotyped twice for accuracy.

Serum RANTES levels were determined in a subgroup of 114 patients with AAD (women, 68.4%) with complete clinical data (mean [SD] age, 43.9 [15.8] years) and 111 healthy carriers of various rs2107538 genotypes, matched for sex (women, 63.1%) and age (mean [SD] age, 43.0 [14.7] years). Circulating RANTES levels were evaluated using Human CCL5/RANTES Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota, United States). The intra-assay precision coefficient of variation (CV) was 1.7% to 2.9% and inter-assay CV was 6.4% to 6.7%.

Statistical analysis was performed using GraphPad Prism 6.0c (GraphPad Software, San

TABLE 1 Clinical and serological characteristics of patients with autoimmune Addison disease

Variable	Mean (SD)	Median (IQR)
Age, y	43.9 (15.7)	42.0 (32.0–55.0)
AAD duration, y	8.1 (9.8)	3.0 (1.0–15.3)
Body mass, kg	69.0 (14.2)	67.0 (59.0–76.0)
HC dose, mg/d	24.9 (4.4)	22.5 (22.5–30.0)
21-OH antibodies, U/ml	179 (570)	8.5 (2.1–59.7)
aTPO, U/ml	371 (667)	141 (19–388)
aTg, U/ml	309 (735)	67 (19–218)

Abbreviations: AAD, autoimmune Addison disease; aTg, antibodies to thyroglobulin; aTPO, antibodies to thyreoperoxidase; 21-OH, 21-hydroxylase; HC, hydrocortisone; IQR, interquartile range

TABLE 2 Distribution of alleles and genotypes of the *CCL5* gene variant patients with autoimmune Addison disease compared controls

rs2107538	AAD, % (n = 239)	Controls, % (n = 542)	*P* value
GG	161 (67.4)	355 (65.5)	0.53
GA	70 (29.3)	159 (29.3)	
AA	8 (3.3)	28 (5.2)	
G	392 (82.0)	869 (80.2)	0.39
A	86 (18.0)	215 (19.8)	

Data are presented as mean (SD).

Abbreviations: see TABLE 1

Diego, California, United States). Data normality was verified with the Shapiro–Wilk test. Normally distributed results were compared using the *t* test or 1-way analysis of variance in the case of the genotype-stratified data, whereas those with nonnormal distribution were analyzed either by the nonparametric Mann–Whitney test or the Kruskal–Wallis test. Categorical variables were compared using the χ^2 test. Two-tailed *P* values of less than 0.05 were considered significant. Assuming an odds ratio of 1.5 at a significance level of 0.05, the power of this study to detect an effect was 87.9% as calculated with the PS Power and Sample Size calculator v.2.1.30 (Vanderbilt University, Nashville, Tennessee, United States). The Hardy–Weinberg equilibrium of the genotyped single nucleotide polymorphisms was tested using an online calculator devised at Tufts University (Medford, Massachusetts, United States).

All procedures were conducted in accordance with the ethical standards of the Declaration of Helsinki. The local ethics committee at the Poznan University of Medical Sciences approved the study (decision 457/14). Informed consent was obtained from all study participants.

RESULTS The mean (SD) duration of AAD in the studied cohort was 8.1 (9.8) years (range, 0–46 years); 82.8% of the patients presented with autoimmune polyendocrine syndrome (type 2 or 4), whereas only 17.2% suffered from isolated AAD. Precise data were available for 212 patients: autoimmune thyroid disease was found in 166 individuals (78.3%; Hashimoto thyroiditis in 132 and Graves disease in 34), 22 patients (10.4%) suffered from type 1 diabetes, and 15 (7.1%) presented with vitiligo. Clinical and serological characteristics of the cohort are presented in TABLE 1.

All rs2107538 genotypes remained in the Hardy–Weinberg equilibrium ($P = 0.91$ for AAD and $P = 0.07$ for controls). No differences were found in genotype or allele frequencies between patients with AAD and controls ($P = 0.53$ and $P = 0.39$, respectively; TABLE 2). Likewise, no association was found between age at AAD onset and the rs2107538 variant ($P = 0.14$).

Serum RANTES levels were higher in patients with AAD compared with controls (mean [SD], 59.2 [30.3] ng/ml vs 45.5 [20.4] ng/ml, $P = 0.001$) (FIGURE 1). Healthy carriers of various rs2107538 genotypes demonstrated differences in the RANTES concentration (mean [SD], 49.8 [22.5] in GG homozygotes, 45.11 [18.9] in heterozygotes, and 34.3 [12.0] in AA carriers, $P = 0.02$). On the contrary, despite a similar trend, differences found in genotype-stratified RANTES analysis in patients with AAD were not significant (mean [SD], 62.9 [31.9] ng/ml in GG homozygotes, 52.7 [26.1] ng/ml in heterozygotes, and 43.3 [22.6] ng/ml in AA carriers, $P = 0.26$). Serum RANTES levels were similar in patients with isolated AAD and in those with polyglandular

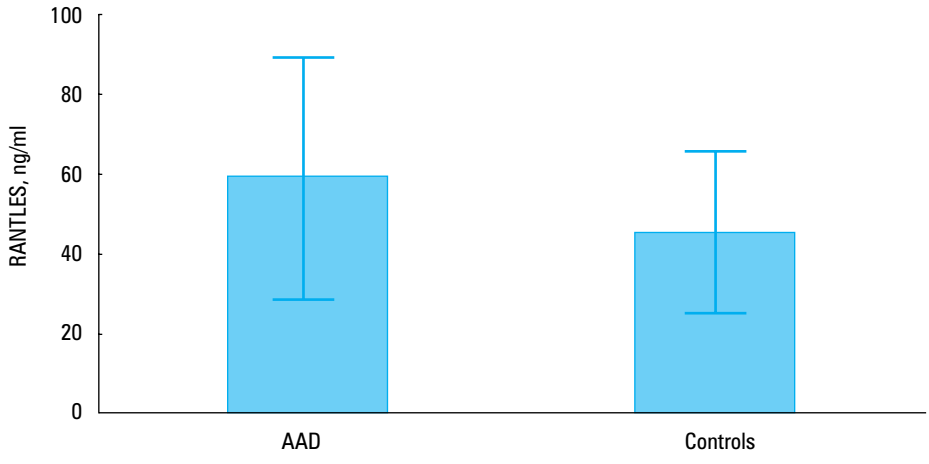

FIGURE 1 Mean (SD) serum RANTES levels in patients with autoimmune Addison disease (AAD) and healthy controls

autoimmunity (mean [SD], 59.9 [25.4] ng/ml vs 59.0 [31.3] ng/ml, $P = 0.66$]. No correlation was found between circulating RANTES levels and age in patients ($P = 0.52$) and controls ($P = 0.39$). The RANTES level was not correlated with AAD duration ($P = 0.74$) and body mass ($P = 0.77$). A borderline negative correlation noted between serum RANTES levels and hydrocortisone dose ($r = –0.184$, $P = 0.051$) was lost when adjusted for body mass ($P = 0.12$). Finally, none of the studied serum autoantibodies (21-OH antibodies, aTg, or aTPO) revealed correlations with the RANTES level ($P = 0.67$, $P = 0.70$, and $P = 0.31$, respectively).

DISCUSSION The rationale for the current study was based on the assumption that RANTES might be implicated in autoimmune destruction of the adrenal cortex through its role in recruitment and activation of the immune cells. Elevated serum RANTES levels were formerly shown in both forms of autoimmune thyroid disease, and the *CCL5* gene expression appeared proportional to thyroid infiltration by T cells in Graves disease.[18-20] Local gene expression is difficult to analyze in autoimmune adrenal failure due to limited accessibility of the human adrenal tissue in vivo. Therefore, we investigated serum RANTES levels in a large cohort of patients with AAD and found that it was significantly elevated compared with healthy individuals. However, no relationship between circulating chemokine levels and disease duration was found. We had expected that newly diagnosed patients, with still abundant adrenocortical antigens and an active phase of the autoaggressive reaction, would display a more vigorous attraction of the immune cells. Correlations of circulating RANTES levels with disease activity were reported for systemic autoimmune disorders, such as rheumatoid and juvenile idiopathic arthritis, and multiple sclerosis.[24-26] Nonetheless, these conditions usually present a relapsing-remitting pattern and disease activity is easily scalable based on clinical scores. On the contrary, AAD is characterized by progressive deterioration of the adrenal function, and spontaneous remissions are only rarely described, usually at the initial stages of the disease.[27] Nevertheless, in a post-hoc comparison, circulating RANTES levels in patients within the first year from AAD diagnosis and in those with long-lasting disease were similar (mean [SD], 56.9 [33.2] ng/ml vs 59.3 [28.7] ng/ml, $P = 0.69$). Accordingly, our study did not reveal a correlation between RANTES and circulating autoantibody levels, which usually peaks at clinical onset when the gland destruction and ensuing autoantigen presentation are the most pronounced. To our knowledge, there are no other literature data regarding RANTES and adrenal specific antibodies. However, a correlation between circulating aTPO and CCL5 mRNA was observed in thyroid autoimmunity.[20] Quite surprisingly, a negative correlation between serum RANTES and autoantibodies against thyroid-stimulating hormone receptor was described in patients with Graves disease.[28]

Chemokines could be important agents attracting specific autoreactive T cells to the adrenal cortex.[5] Two previously analyzed IFN-induced chemokines, CXCL9 and CXCL10, were also significantly elevated in patients with AAD.[14-16,29] However, peripheral blood mononuclear cells stimulated with IFN revealed lower CXCL10/CXCL9 synthesis compared with healthy controls, irrespective of the medication used, disease duration, or comorbidities.[29] The authors concluded that elevated serum CXCL10 levels in patients with AAD might be due to the local production of this chemokine in affected adrenal cortex, under the influence of the IFN-rich, inflammatory environment. Indeed, stimulation of the human adrenocortical cells in vitro with IFN-γ induces high CXCL10 secretion.[15] Hence excess circulating chemokines observed in organ-specific autoimmune conditions might be at least partially of the local origin. This hypothesis remains in line with the experimental findings in cultured thyrocytes stimulated with immunoglobulins from patients with Graves disease, which upregulate the production of T-cell attracting agents, RANTES, and IL-16.[30] However, some authors argue that infiltrating lymphocytes are themselves the main source of RANTES in autoimmune thyroid disease.[20] The same issues remain to be analyzed in AAD.

We also hypothesized that exogenous steroid replacement in AAD might affect circulating chemokine levels. However, a borderline negative correlation between serum RANTES and daily hydrocortisone dose was lost after adjustment for body mass. In fact, former studies with the use of much higher immunosuppressive steroid doses did not confirm decreasing RANTES levels in response to corticosteroid, regardless of considerable improvement of the clinical symptoms.[28,31]

Despite former reports indicating that the *CCL5* variant, rs2107538, might be associated with other autoimmune endocrine conditions, our study failed to demonstrate its association with AAD.[21,22] A unique analysis in an Italian AAD cohort concerned the RANTES receptor gene (*CCR5*)—its variant characterized by a 32-base pair deletion within the coding region did not appear associated with adrenal autoimmunity.[32] However, both AAD cohorts, Italian and ours, were relatively small and hence underpowered to detect a minor effect. Moreover, another variant in linkage disequilibrium with rs2107538 might prove to be a genuine causative variant. Nonetheless, our study supports a plausible functional aspect of rs2107538, which may affect the *CCL5* expression. Healthy wild-type homozygotes presented significantly higher circulating RANTES levels compared with carriers of the variant allele, although this observation was not replicated in patients with AAD, where this correlation could be somewhat obscured by an ongoing autoimmune process. Decreased

serum RANTES in rs2107538 minor allele carriers was previously reported in several studies including patients with type 2 diabetes, those with coronary artery disease, and healthy individuals.[21,33,34] The rs4251719*A-rs2306630*A--rs2107538*A *CCL5* haplotype associated with low RANTES production protects from type 1 diabetes, an autoimmune disorder which shares several genetic and pathophysiologic features with AAD.[21,35]

To summarize, this study demonstrates for the first time elevated serum RANTES levels in patients with AAD and confirms previous reports about the functional role of the rs2107538 *CCL5* variant, which may alter circulating RANTES levels. Further studies, especially in asymptomatic individuals at high risk of AAD, such as adrenal autoantibody-positive first--degree relatives, are warranted to evaluate if this chemokine might be a useful biomarker or even a therapeutic target at early stages of autoimmune adrenal insufficiency.

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CONTRIBUTION STATEMENT MF and PF conceived the idea of the study. MF and MŻ designed the study protocol. MF, MŻ, BB, HK, EN, and MR were involved in data collection and laboratory analyses. MF, MŻ, and PF analyzed and interpreted the data. MF and EN drafted the manuscript. MR critically revised the manuscript. All authors edited and approved the final version of the manuscript.

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