

Eotaxin-3 in Churg–Strauss syndrome: a clinical and immunogenetic study

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Abstract

Objectives. To determine the potential of eotaxin-3 as a diagnostic marker for active disease and genetic susceptibility factor for Churg–Strauss syndrome (CSS).

Methods. A total of 37 patients with active, relapsed or inactive CSS, 123 healthy controls and 138 disease controls were studied. Clinical data were collected and serum levels of eotaxin-3 were determined. *Ex vivo* stability of eotaxin-3 in serum samples was tested. Furthermore, the association of single nucleotide polymorphisms (SNPs) in the *eotaxin-3* gene with CSS was determined in 161 CSS patients and 124 healthy controls.

Results. Serum eotaxin-3 was highly elevated in active CSS patients. Neither eosinophilic diseases nor other small-vessel vasculitides were associated with high serum eotaxin-3 levels. Receiver operating characteristic curve analysis determined a sensitivity and specificity of 87.5 and 98.6% at a cut-off level of 80 pg/ml. None of the tested SNPs within the *eotaxin-3* gene influenced the susceptibility to develop CSS.

Conclusions. Serum eotaxin-3 is a sensitive and specific marker for the diagnosis of active CSS suitable for routine clinical practice. Previously described SNPs in the *eotaxin-3* gene do not predict the risk of developing CSS.

Key words: Churg–Strauss syndrome, CCL26, Eotaxin-3, Diagnosis, Biomarker.

Introduction

Churg–Strauss syndrome (CSS) is a systemic necrotizing small-vessel vasculitis associated with blood and tissue eosinophilia and granulomas occurring predominantly in

middle-aged individuals [1, 2]. Diagnosis of CSS is difficult as there are neither diagnostic criteria nor a specific biomarker available. Although the clinical picture may be highly suggestive, a number of differential diagnoses have to be considered. Many patients undergo biopsy procedures to confirm the suspected diagnosis of CSS but the most frequent finding is tissue eosinophilia, which is non-specific and may occur in many other conditions. Necrotizing vasculitis is only found in about one-third of CSS patients, whereas granulomas are hardly found at all [3]. Peripheral blood and tissue eosinophilia and elevated serum immunoglobulin E levels (IgE) are often observed in CSS but they are not specific [4, 5]. Moreover, ANCA are found in only one-third of CSS patients [3, 6].

Recently, we demonstrated the involvement of the eotactic chemokine eotaxin-3 (CCL26) in CSS [7]. In particular, we showed that eotaxin-3 levels are high in patients with active CSS and that they correlate with disease activity, eosinophil counts and IgE levels; at a tissue level, strong expression of eotaxin-3 was found in

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endothelial and inflammatory cells in the diseased sites. Several findings provide additional support for a potential role of eotaxin-3 in CSS. First, eotaxin-3 induces chemotaxis and activation of eosinophilic granulocytes *in vitro* [8]. Second, eotaxin-3 mRNA increases drastically in mildly asthmatic patients after antigen challenge [9]. Last, several studies suggest the association of single nucleotide polymorphisms (SNPs) in the *eotaxin-3* gene with asthma, allergic rhinitis and eosinophilic oesophagitis [10–13]. In this study, we investigated the reliability of serum eotaxin-3 as a diagnostic marker for CSS, and explored whether SNPs within the *eotaxin-3* gene confer susceptibility to the development of CSS.

Methods

Patients

In this study, we investigated 37 CSS patients, 137 disease controls and 123 healthy controls. In particular, we studied the following groups: 15 active, untreated (newly diagnosed or relapsed) CSS patients, 22 CSS patients in remission, 24 patients with other ANCA-associated small-vessel vasculitides, 20 patients with idiopathic hypereosinophilic syndrome (HES), 19 patients with proven parasitic diseases (including the following infections: 3 trichinosis, 8 strongyloidosis, 8 schistosomiasis), 15 patients with eosinophilia of other causes (7 tropical eosinophilia, 2 drug-induced, 1 post-infectious, 3 eosinophilic pneumonia, 1 eosinophilic gastroenteritis, 1 mastocytosis), 40 patients with other rheumatic disorders (20 SLE, 20 SSc), 20 patients with ulcerative colitis (UC) and 123 healthy controls without a history of allergic disease. Some patients (eight active CSS and nine HES patients) included herein were part of a previous study [7]. SNP genotyping was performed on genomic DNA of a cohort of 161 CSS patients from the University Hospitals of Parma, Italy ($n=45$), Erlangen and Lübeck, Germany ($n=116$). DNAs from 124 normal healthy individuals from Parma, Italy ($n=42$) and Erlangen, Germany ($n=82$) were genotyped as controls. The study was approved by the local ethics committees of the University of Erlangen-Nuremberg, the University of Lübeck and the University of Parma. All patients gave informed consent.

Diagnostic assessment

Among the CSS patients, clinical data including treatment, laboratory studies, organ involvement and autoantibody status of active CSS patients are given in [supplementary table](#), available as [supplementary data](#) at *Rheumatology* Online in detail. All other patients with rheumatic diseases fulfilled the classification criteria established for each disease. The patients with parasitic diseases all had direct evidence of parasite burden either by stool microscopy or tissue biopsy and peripheral blood eosinophilia at the time of serum sampling. HES patients fulfilled the Chusid criteria for diagnosis of idiopathic HES [14].

Serum eotaxin-3 levels

Eotaxin-3 serum levels were determined by ELISA (R&D Systems, Minneapolis, MN, USA) as previously described [7]. To determine the stability of eotaxin-3 in serum samples, we chose six samples with intermediate to high serum levels [mean 99.3 (10.5) pg/ml]. Aliquots of serum samples were either frozen and thawed (F/T) three times, stored for 24 h at 4°C or at room temperature (RT) and measured together with the original sample (control). As shown in Fig. 1, storing the samples at 4°C up to 24 h [mean 102.2 (10.5) pg/ml, mean difference from control +3%, P =non-significant (NS)] or performing repeated freeze/thaw cycles [mean 100.9 (11.7) pg/ml, mean difference from control +1.6%, P =NS] did not have any significant effects on eotaxin-3 levels. Storing samples at RT for 24 h caused some minor but not significant drop in eotaxin-3 levels [mean 90.6 (9.0) pg/ml, mean difference from control –8.9%, P =NS].

SNP genotyping

A 2-kb fragment of the *eotaxin-3* gene (acc. no. NM_006072.4) was PCR amplified with the Phire Hot-Start DNA Polymerase (Biozym, Hess. Oldendorf, Germany) from genomic DNA of CSS patients and normal healthy controls (forward primer: 5'-gggtcaaaagtgcgtctgttccc aacc-3', reverse primer: 5'-taactctggaggaaacaccctctcc tccc-3'). Three regions spanning rs2240478, rs69965556 and rs2302009 were sequenced subsequently in forward and reverse direction from the purified eotaxin-3 PCR fragment on an ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing primers used were: 5'-aaacctgagaagggcctgat-3' and 5'-cag gcggtccgggaaggaac-3' for rs2240478; 5'-ggccacagagttc aaatcc-3' and 5'-gctcaccacaacctctgc-3' for rs69965556; 5'-gcaacaagttgtggcaagg-3' and 5'-gggaggaaacaccctc tcc-3' for rs2302009. Analysis of high-quality sequences was performed with the SeqScape Software (Applied Biosystems).

Statistical analysis

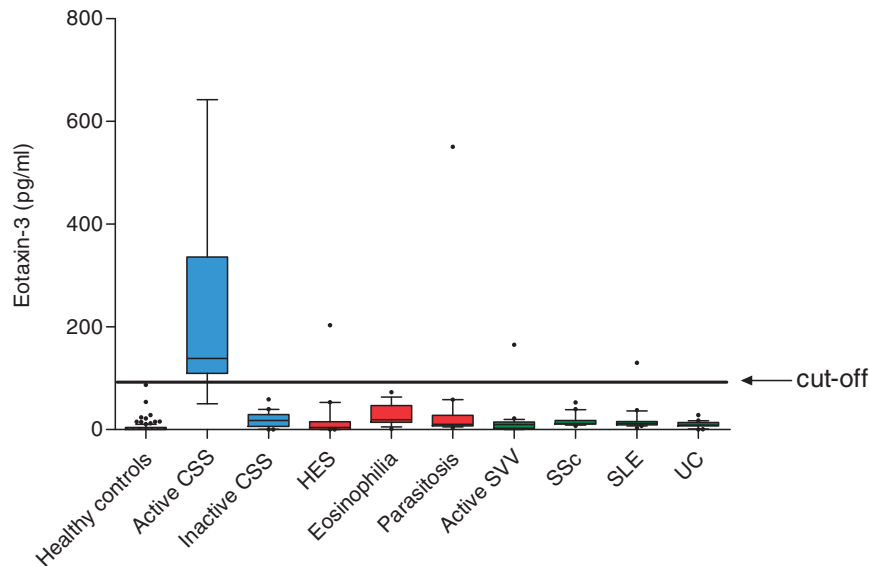
Data were entered into a database and analysed by SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Laboratory values are given as mean (s.d.). Nonparametric analyses were used to compare the two different groups of patients. Two-sided P -values 0.05 were considered significant throughout. ROC curves were used to calculate cut-off values for optimal sensitivity and specificity. Pearson's chi-squared values for genotype and allele frequencies and relative risk factors were calculated.

Results

Sensitivity and specificity of serum eotaxin-3 for CSS

A total of 123 healthy controls without any known allergic disease were tested for serum eotaxin-3 levels by ELISA. Values were generally at the lower detection limit [mean 4.5 (0.9) pg/ml, range 0–87 pg/ml] and even undetectable in 24 samples. To determine the diagnostic

Fig. 1 Eotaxin-3 as a diagnostic marker in CSS. Serum levels of eotaxin-3 in CSS patients and controls. Eotaxin-3 levels are highly elevated ($P < 0.0001$) in active CSS patients as compared with controls: HES, parasitic disease with eosinophilia, eosinophilia associated with other conditions, small-vessel vasculitides (SVV), SLE, SSc and UC. Values are given as mean (S.E.M.).



value of eotaxin-3 serum levels in CSS, 15 patients with careful clinical documentation were used. All patients were highly active at the time of serum sampling (see [supplementary table 1](#), available as [supplementary data](#) at *Rheumatology* Online). Mean eotaxin-3 levels were 219.9 (43) pg/ml ($P < 0.001$ vs healthy controls). In contrast to active disease, eotaxin-3 was low in quiescent disease [19.8 (3.2) pg/ml, $P < 0.05$ vs active CSS]. To determine specificity and sensitivity, serum samples from various other eosinophilic, vasculitic or rheumatic disorders were tested for eotaxin-3.

As shown in Fig. 1, eotaxin-3 levels were low in the majority of disease controls. Even disorders with highly elevated eosinophil counts such as parasitic diseases [45.2 (28.3) pg/ml, $P < 0.05$ vs active CSS] and HES [20.6 (10.3) pg/ml, $P < 0.05$ vs active CSS] revealed low eotaxin-3 levels. In 19 patients with proven parasitic diseases and eosinophilia, only one patient had significant eotaxin-3 levels. In one HES patient, eotaxin-3 levels were also elevated. When we reviewed the clinical data of this patient, allergic disease and highly elevated IgE levels were apparent, both of which are not typical of HES thus questioning the diagnosis. However, in the absence of a diagnostic biopsy or other suggestive parameters, a clinical diagnosis of HES was made initially. We also measured eotaxin-3 levels in patients with peripheral blood eosinophilia for various reasons, i.e. drug-induced eosinophilia or eosinophilic pneumonia. Interestingly, these patients also revealed low eotaxin-3 levels as compared with CSS patients [27 (5.4) pg/ml, $P < 0.05$ vs active CSS].

Finally, we measured serum eotaxin-3 levels in active patients with small-vessel vasculitides and other

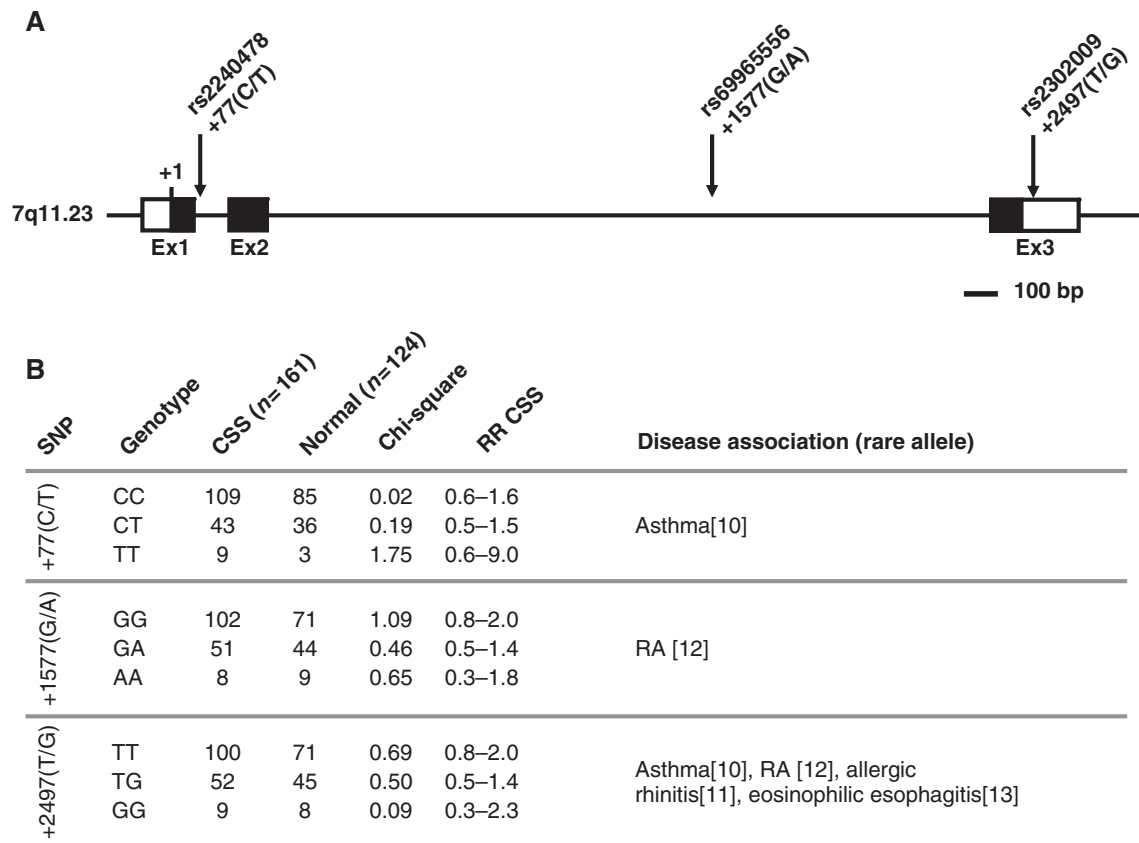
rheumatic disorders (SLE, SSc and UC). Occasionally, single patients in these disease control groups revealed elevated eotaxin-3 levels, while the vast majority showed low levels (all groups $P < 0.05$ vs active CSS). Optimal cut-off value for serum eotaxin-3 was determined by ROC curve analysis. At a cut-off value of 80 pg/ml, sensitivity and specificity were 87.5% (95% CI 61, 98%) and 98.6% (95% CI 95.9, 99.4%), respectively. The area under the ROC curve was 0.9887. Thus, serum eotaxin-3 appears to be a sensitive and highly specific marker for active CSS when compared with a broad spectrum of eosinophilic, rheumatic and vasculitic disorders.

Association of eotaxin-3 SNPs with CSS

The minor genotypes of three SNPs (+77 C/T, +1577 G/A, +2497 T/G) within the *eotaxin-3* gene have previously been associated with asthma, RA, allergic rhinitis and eosinophilic oesophagitis (Fig. 2). Because of the striking specificity of high serum levels of eotaxin-3 in CSS patients, we investigated whether these SNP genotypes are also enriched in CSS patients. To this end, we genotyped the SNPs indicated in Fig. 2 in a large cohort of 161 CSS patients and 124 normal healthy controls. Chi-squared values for the association of each SNP genotype with CSS ranged from 0.02 to 1.75 corresponding to P -values of 0.89–0.19. Therefore, no single SNP genotype was significantly associated with CSS in our cohort.

Additionally, we performed an analysis of eotaxin-3 SNP haplotypes in CSS patients. Sequencing data from 109 CSS patients and 80 normal healthy individuals from our cohort allowed for the unambiguous identification of eotaxin-3 haplotypes. However, no haplotype showed significant association with CSS (data not shown).

Fig. 2 Association of SNPs in the *eotaxin-3* gene with CSS. **(A)** Schematic representation of the intron-exon structure of the human *eotaxin-3* gene with protein-coding regions depicted as black boxes. White boxes represent 5'- and 3'-UTRs. The positions of the three SNPs analysed are indicated by arrows. **(B)** Genotype frequencies of the three SNPs shown in **(A)** were determined by direct sequencing of 161 CSS patients and 124 normal healthy controls. Pearson's chi-squared values and relative risk factors (95% CI) are given for each genotype. Previously described disease associations of the rare allele of each SNP are indicated in the right column.



Moreover, we could also not identify certain CSS subgroups (e.g. ANCA-positive vs negative) associated with *eotaxin-3* SNPs (data not shown). Taken together, these results indicate that the genotypes of the three SNPs analysed do not constitute risk factors for the development of CSS.

Discussion

Differential diagnosis of CSS is broad, as there are many other disorders associated with hypereosinophilia and organ damage. For instance, a relevant differential diagnosis for CSS is parasitic disease, as its treatment is entirely different. Schistosomiasis, trichinosis and other helminthic infections can mimic conditions suspicious for CSS [5]. We had the unique opportunity to test sera from patients with proven parasitic infections associated with peripheral eosinophilia. Interestingly, all but one sample revealed low levels of *eotaxin-3*, suggesting a high discriminative capacity for *eotaxin-3*. This also applied to other conditions such as acute eosinophilic pneumonia,

drug-induced eosinophilia and tropical eosinophilia. These findings extend our previous observations to practically all relevant differential diagnoses for CSS [7]. Moreover, the herein demonstrated *ex vivo* stability of *eotaxin-3* facilitates its use as a diagnostic marker.

Recently, association studies in CSS patients have been performed. We demonstrated a significant association of HLA-DRB1*07 and the linked *HLA-DRB4* gene with the disease [15]. Moreover, HLA-DRB4-positive patients were more frequent in CSS subgroups with more severe disease phenotypes. These findings have been confirmed in an independent German CSS population and indicate that specific HLA alleles play a role in the pathophysiology of CSS [16]. Another study provides evidence of a specific IL-10 promoter haplotype being significantly more frequent in CSS patients than in normal controls [17]. Taken together, these data suggest the presence of genetic risk factors contributing to CSS.

Increased mRNA stability mediated by SNPs might be a possible explanation for the up-regulation of *eotaxin-3* in CSS patients. Interestingly, several studies provide a

potential link between the minor genotypes of SNPs within the *eotaxin-3* gene and allergic and rheumatoid diseases other than CSS. Chae *et al.* [12] established a potential involvement of SNPs +1577G/A and +2497T/G in RA. The same group also demonstrated the association of the SNPs +77C/T and +2497T/G with atopic and non-atopic asthma and SNP +2497 T/G with allergic rhinitis [10, 11]. For other patient cohorts, conclusions from these studies have to be drawn carefully, however, because they were performed in local Korean populations.

Blanchard *et al.* [13] demonstrated an association of SNP +2497T/G with eotaxin-3 expression in American patients suffering from eosinophilic oesophagitis. Because CSS patients frequently have a history of asthma and are characterized by eosinophilia and systemic inflammation, these results would be consistent with an association of eotaxin-3 SNPs with CSS. Our genetic analysis of 161 CSS patients, however, failed to reveal a significant association of any of the three previously described SNPs +77C/T, +1577G/A and +2497T/G with CSS. Therefore, our data do not support the potential use of these SNPs for risk assessment or diagnosis of CSS, at least in European patients. Consequently, further studies will be required to provide an explanation for the observed specific up-regulation of serum eotaxin-3 in CSS patients.

In conclusion, we provide evidence that previously described SNPs within the *eotaxin-3* gene are not significantly associated with CSS. Notably, we demonstrate, however, that serum eotaxin-3 is a reliable diagnostic marker for active CSS.

Rheumatology key messages

- Serum eotaxin-3 levels are a reliable diagnostic marker for active CSS.
- Previously described SNPs in the *eotaxin-3* gene are not associated with CSS.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Supplementary Table. Clinical features of active CSS patients

Patient	Clinical activity	Organ involvement	CRP (mg/dl)	Eo (n/μl)	ANCA	IgE (kU/l)	Eotaxin-3 (pg/ml)
1	Active	A,E,L,P,S,G	11,9	2546	cANCA/PR3	43,7	99,3
	Relapse	A,E,L,P,S,G	7,9	1036	cANCA/PR3	ND	151,7
2	Active	A,E,P,H	3,0	4736	Negative	535	165,2
3	Active	A,E,P,H,B	6,4	4350	Negative	91	461
	Relapse	A,E,P,H,B	4	4320	Negative	21	643
4	Relapse	A,E,L,P,H	0,7	1099	Negative	360	138,8
5	Active	A,E,L,P	0,8	785	pANCA/MPO	9	120
6	Active	A,E,P,S	5,5	5200	pANCA/MPO	197	150,5
7	Active	A,E,L,P	1,6	2034	Negative	96	63
8	Active	A,E,L	7,3	49280	Negative	2295	429
9	Active	A,E,P,S	1,0	3686	Negative	3527	128,9
10	Active	A,E,S,G	13	7650	Negative	769	51
11	Active	A,E,L,P,H,K,S	14,4	896	pANCA/MPO	1384	348,8
12	Active	A,E,L,P,S,H	1,1	9000	Negative	380	260
13	Active	A,E,L,H	1,6	5140	Negative	1692	227
14	Active	A,E,L,K,P,S	0,3	924	pANCA/MPO	127	246,1
15	Relapse	A,E,P,K,H	1,6	4800	Negative	182	243

CRP - C-reactive protein, Eo – Eosinophils, ANCA – Anti-neutrophil cytoplasmic antibodies, PR3 – proteinase 3, MPO – myeloperoxidase, IgE – Immunoglobulin E, A – asthma, E – sinusitis, L – lung, P – peripheral neuropathy, S – skin, G – gastrointestinal tract, H – heart, K – kidney, B - CN