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Interleukin-24, RANTES and CCR5 gene polymorphisms are not associated with chronic adult periodontitis

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Background and Objective: Cytokines, such as interleukin-10, and related genetic polymorphisms, have been implicated in the pathogenesis of chronic periodontitis. The aim of this study was to investigate a possible correlation between chronic periodontitis and genetic polymorphisms coding for two interleukin-10 related chemokines [interleukin-24 and regulated on activation, normal T cells expressed and secreted (RANTES)] as well as a RANTES receptor [CC chemokine receptor 5 (CCR5)].

Material and Methods: A single-blind, two-centre, case-controlled study was carried out with test patients from the Clinic of Periodontics, Göteborg University, and from the Department of Periodontology, Glasgow University, and control subjects from the undergraduate clinics of both schools. Blood samples were collected from 106 patients (56 women and 50 men, mean age 51.7 yr) with generalized, severe chronic periodontitis and from 69 periodontally healthy subjects (37 women and 32 men, mean age 53.3 yr). The polymerase chain reaction (PCR) was used to identify the genetic coding for interleukin-24, RANTES and CCR5. Genotype and allele frequencies were compared between the test and control groups using Fischer's exact test at the 5% level of significance.

Results: There were no statistically significant differences between patients with chronic periodontitis and control subjects, regarding genotype distribution or allele frequency, irrespective of smoking status, in the combined Glasgow and Gothenburg cohort or in the specific location cohorts. The allele frequencies for healthy and control subjects for RANTES gave a *p*-value of 0.80 (allele G was 58.8% in healthy subjects and and 54.4% in subjects with periodontitis), for interleukin-24 the *p*-value was 0.90 (allele T was 56.2% in healthy subjects and and 54.9% in subjects with periodontitis) and for CCR5 the *p*-value was 0.90 (the wild-type allele was 85% in healthy subjects and and 82.7% in subjects with periodontitis).

Conclusion: The interleukin-24, RANTES and CCR5 polymorphisms investigated are not associated with chronic periodontitis.

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Chronic periodontitis is characterized as a destructive inflammatory response to microbial challenge. Investigators have focused on the microorganisms involved and on the ensuing host response, in attempts to understand the aetiopathogenesis of this condition. About 10–15% of any population is regarded to be susceptible to severe

forms of chronic periodontitis, and thus investigators have attempted to ascertain whether or not there is a genetic predisposition. Evidence for a genetic variation associated with periodontal diseases has been most convincing with regard to the aggressive form of the disease (1) but has also been related to chronic periodontitis (2). Indeed, the authors of the study estimate that approximately half of the clinical variability of chronic periodontitis may be attributable to genetic factors.

In the study of the pathogenesis of periodontitis, it is thought that various cellular interactions in the affected tissues are modulated by cytokines. Chemokines are one particular group of structurally related cytokines. They exhibit the conservation of four cysteine residues that affect tertiary protein structure as a molecular hallmark. Approximately 40 chemokines and 16 chemokine receptors have been identified to date, and several studies have demonstrated the production of chemokines in the periodontal tissues (3,4). These cytokines, in turn, are influenced by environmental and genetic factors. Cytokine gene polymorphisms and immunoregulation in periodontal disease have been reviewed recently (5).

The present study investigates genetic polymorphisms in chemokines and their receptors related to interleukin-10; adhesion molecules and chemokine/chemokine–receptor interactions have been shown to regulate the production of interleukin-10 (6). Interleukin-10 is a cytokine produced by several cell types, including B- and T helper 2 lymphocytes, and is suggested to have an immunosuppressive effect as a result of the down-regulation of T helper 1 cell functions. Interleukin-10 also appears to enhance the proliferation of conventional (B-2) and autoreactive (B-1) B cells, as well as the production of autoantibodies. It has been suggested that both circulating and local B cells in the periodontitis prone are more likely to possess autoreactive properties than those with a low susceptibility (7). Genetic control of interleukin-10 has been shown to be linked with severe generalized chronic periodontitis (8), and the presence of polymorphisms in conjunction with informative dinucleotide repeats (microsatellites) in the interleukin-10 gene locus is associated with differential interleukin-10 production (9).

Regulated on activation, normal T cells expressed and secreted (RANTES) is one of the cytokine CC subfamilies (CCL5) (characterized by the number of amino acids separating the two cysteines). The investigation of the expression of RANTES and its receptor has generated substantial interest because it is a major co-receptor for human immunodeficiency virus cell entry (10). It displays significant chemotactic activity for eosinophils, monocytes and $CD45^+$ T cells. Data support a role for RANTES in both acute and chronic stages of inflammation. RANTES is specifically attractive to activated T cells displaying CC chemokine receptor 3 (CCR3) and CC chemokine receptor 5 (CCR5) and has been reported as a ligand for CCR5 (11). CCR5 down-regulation has been shown to be important in oral wound healing (12). RANTES has also been shown to interact with other cytokines, such as interleukin-10. Interleukin-10 is implicated in increasing the expression of CCR5 in monocyte subsets (13).

Melanoma differentiation-associated antigen 7 (*mda-7*) (interleukin-24) represents a differentiation, growth and apoptosis-associated gene with potential utility for the gene-based therapy of diverse human cancers (14). It has also been described as important in wound healing and inflammation in general by inducing the expression of other cytokines, such as tumor necrosis factor-α and interferon-γ (15). Interleukin-24 is one of a family of interleukin-10-related cytokines, which includes interleukin-19, -20, -22 [interleukin-10-related T-cell-derived inducible factor (interleukin-TIF)] and -26 (AK155) (16).

The aim of the present study was to investigate a possible correlation between chronic periodontitis and polymorphisms in the genes coding for two chemokines (interleukin-24 and RANTES) as well as a RANTES receptor (CCR5), all of which are associated with interleukin-10. A structurally homologous gene (*mda-7*) C/T single nucleotide polymorphism was investigated, as well as the −471 A/G single nucleotide polymorphism in RANTES and the Δ32/wt polymorphism in CCR5.

Material and methods

Subjects

Two groups of Caucasian subjects (groups I and II) were recruited ($n = 175$). Group I consisted of patients with generalized, severe chronic periodontitis (17) and exhibited bone loss of > 50% at all teeth. Prior to periodontal therapy, all patients had probing pocket depths of > 6 mm and bleeding on probing at > 80% of the proximal sites. Sets of intra-oral radiographs were obtained using a standardized parallel technique (18). In the radiographs, the distance between the cemento–enamel junction and the most coronal level of the bone crest was assessed at the mesial and distal aspects of each tooth. For details regarding the radiographic measurements, see Berglundh *et al.* (19). The patients were recruited from the Department of Periodontology, Glasgow Dental Hospital (Glasgow, UK) and from the Clinic of Periodontics, Göteborg (Sweden). The Glasgow Group I consisted of 35 patients (21 women and 14 men; age range: 40–66 yr, mean age 49.2 yr). The Göteborg Group I consisted of 71 patients (35 women and 36 men; age range 36–74 yr, mean age 54.2 yr).

Periodontally healthy subjects were also recruited (Group II). The subjects in this group demonstrated normal radiographic bone levels (i.e. a distance of < 3 mm between the cemento–enamel junction and bone crest at > 95% of the proximal tooth sites) in spite of the presence of plaque in all sextants. The subjects were recruited from the Periodontology and Restorative Departments at Glasgow Dental School as well as from Undergraduate Clinics in Göteborg. The Glasgow Group II consisted of 33 patients (16 women and 17 men; age range: 40–71 yr, mean age 55 yr). From Göteborg, 36 subjects

were recruited (21 women and 15 men; age range: 35–78 yr, mean age 51.6 yr). None of the subjects in either centre had a known systemic disorder that could have affected the periodontal condition. Smoking habits were recorded in both groups.

In total there were 59 smokers and 116 nonsmokers, with 48 smokers in Group I and 11 smokers in Group II.

Samples of peripheral blood were obtained by venepuncture from the antecubital fossa of each patient and were collected in EDTA tubes. The samples were coded and stored at −70°C until processing was initiated. Analysis for the relevant polymorphism was undertaken using polymerase chain reaction (PCR).

DNA extraction

Genomic DNA was prepared from whole-blood samples from the cohort of Glasgow and Göteborg patients and from healthy unrelated subjects. Genomic DNA was extracted using the MasterPure™ DNA Purification kit (Cambio Ltd, Cambridge, UK), according to the manufacturer's instructions. The CCR5 Δ32/wt, interleukin-24 C/T and RANTES A/G polymorphisms were determined as described below.

PCR

Each PCR required a different pair of primers, as detailed in Table 1, and optimized thermocycling conditions, as detailed below. Primers were from MWG Biotech (Ebersberg, Germany) and the thermocycler used a Biometra Uno Thermoblock (Biometra, Gottigen, Germany). The DNA polymerase was BIOTAQ™ (Bioline, London, UK) with the supplied 50 mM $MgCl_2$ optimized buffer. The dNTPs were used at a concentration of 0.2 mM in the reaction. Each reaction mixture was overlaid with mineral oil. After the PCR reaction, all samples were mixed with 5 μl of loading buffer [0.25% Orange G (Sigma, Gillingham, UK) and 30% (w/v) glycerol]. Aliquots of the PCR products were then electrophoresed on a 2% agarose gel (Bioline) and visualized by ethidium bromide staining.

The CCR5, *mda-7* and RANTES PCR reaction volumes were 13 μl in 0.2 ml thin-walled tubes (Biometra), containing the following components. The CCR5 reaction volume contained 1 μl of DNA (50 ng), 3.4 μM forward allele-specific primer (wild-type or Δ32), 2 μM reverse constant primer, 0.1 μM forward control primer, 0.1 μM reverse control primer, 200 μM dNTPs, 2 mM $MgCl_2$ and 0.5 U enzyme in ×1 buffer. The *mda-7* reaction volume contained 1 μM allele-specific primer (allele C) or 3 μM allele-specific primer (allele T), 1 μM constant primer, 0.1 μM forward control primer, 0.1 μM reverse control primer, 200 μM dNTPs, 2 mM $MgCl_2$ and 0.5 U enzyme in ×1 buffer. The RANTES reaction volume contained 1 μl of DNA (50 ng), 2 μM allele-specific primer (allele A) or 1 μM allele-specific primer (allele G), 1 μM constant primer, 0.1 μM forward control primer, 0.1 μM reverse control primer, 200 μM dNTPs, 3 mM $MgCl_2$ and 0.5 U enzyme in ×1 buffer. An initial denaturation at 96°C for 1 min was followed by five cycles at 96°C for 25 s (denaturation) for CCR5 and RANTES, but four cycles for *mda-7*, at 70°C for 45 s (annealing), at 72°C for 45 s (extension), then 21 cycles at 96°C for 25 s (denaturation), at 65°C for 50 s (annealing) for CCR5 and *mda-7*, but 66°C for RANTES, at 72°C for 45 s (extension) and finally four cycles at 96°C for 25 s (denaturation), at 55°C for 1 min (annealing) and 72°C for 2 min (extension).

Table 1. Primers used in the polymerase chain reaction (PCR)

Primers		
CCR5 (wt)	Forward	5′-CTC TCA TTT TCC ATA CAG TCA G-3′
CCR5 (Δ32)	Forward	5′-CTG CAG CTC TCA TTT TCC ATA CAT TA-3′
CCR5	Reverse	5′-GCC TCA CAG CCC TGT GCC-3′
Interleukin-24 (T)	Forward	5′-CTT CAA CTG TTC TAT TGT GGT A-3′
Interleukin-24 (C)	Forward	5′-CTT CAA CTG TTC TAT TGT GGT G-3′
Interleukin-24	Reverse	5′-CCT GGG AGC TAG GCT GTG-3′
RANTES	Forward	5′-TCC ATG GAT GAG GGA AAG GAG G-3′
RANTES	Forward	5′-TCC ATG GAT GAG GGA AAG GAG A-3′
RANTES	Reverse	5′-ACA TCC TTA GTT TTA CCT TCC AGG-3′
Primers (control)		
HLA-DRB1 gene		5′-TGC CAA GTG GAG CAC CCA A-3′
HLA-DRB1 gene		5′-GCA TCT TGC TCT GTG CAG AT-3′

CCR5, CC chemokine receptor 5; RANTES, regulated on activation, normal T cells expressed and secreted.

Statistical analysis

Data were analysed by comparing the presence of a particular genotype, the presence or absence of a particular allele on a per-subject basis, as well as the smoking status. The chi-squared test was used for genotype comparisons and the Fisher's exact test was used to evaluate differences in allele distribution between groups. *p*-values of < 0.05 were considered as significant.

Results

Genotype frequencies

The results for the Glasgow, Göteborg and combined cohorts of patients and controls are reported in Table 2. AG (the heterogeneous state) was the most common genotype recorded for RANTES, whilst the most common genotype for interleukin-24 and CCR5 was CT (also heterogeneous) and wt/wt, respectively. There were no statistically significant differences evident for any genotype or allele frequency and disease status for any group analyzed. The genotype frequencies were in agreement with Hardy–Weinberg equilibrium ($p > 0.1$ for all analyses). Discrepancies in the numbers of samples investigated by PCR with those recruited are accounted for by the fact that there were some samples that displayed a band pattern during PCR that could not determine a specific genotype and so could not be included in the statistical analysis.

Table 2. Distribution of single nucleotide polymorphisms status for Glasgow and Göteborg separately and combined

		Combined			Glasgow			Göteborg		
		H	ChP	*p*-values	H	ChP	*p*-values	H	ChP	*p*-values
RANTES										
Genotypes	AA	1	1		0	1		1	0	
	AG	48	81		21	31		27	50	
	GG	12	9	0.98	9	3	0.98	3	6	0.75
Allele frequency	A	51	83		21	33		29	50	
	G	73	99	0.80	39	39	0.80	33	62	0.85
Interleukin-24										
Genotypes	CC	6	8		4	4		2	4	
	CT	45	76	0.90	17	23	0.90	28	53	0.90
	TT	14	18		10	8		4	10	
Allele frequency	C	57	92		25	31		32	61	
	T	73	112	0.90	37	39	0.90	36	73	0.90
CCR5										
Genotypes	ΔΔ	0	2		0	0		0	2	
	ΔWT	15	23	0.80	8	11	0.80	7	12	0.80
	WT/WT	35	53		17	20		18	33	
Allele frequency	Δ	15	27		8	3		7	16	
	WT	85	129	0.90	42	21	0.90	43	78	0.60

CCR5, CC chemokine receptor 5; ChP, chronic periodontitis; H, healthy; RANTES, regulated on activation, normal T cells expressed and secreted.
No significant differences were found between chronic periodontitis and healthy control subjects when studying genotype frequency or allele frequency. Almost identical distributions were found in the Glasgow and Göteborg subjects.

Smoking data

The reported gene frequencies comparing smokers and nonsmokers are reported in Table 3. The allele frequencies, for healthy and control subjects, for RANTES gave a *p*-value of 0.80 (allele G was 58.8% in healthy individuals and 54.4% in subjects with periodontitis), for interleukin-24 gave a *p*-value of 0.90 (allele T was 56.2% in healthy individuals and 54.9% in subjects with periodontitis) and for CCR5 gave a *p*-value of 0.90 for the frequency comparison between healthy and control subjects (the wild-type allele was 85% in healthy individuals and 82.7% in subjects with periodontitis). There were no statistically significant differences evident for any genotype or allele frequency and smoking or disease status for any group analyzed. The genotype frequencies were in agreement with Hardy–Weinberg equilibrium ($p > 0.1$ for all analyses).

Discrepancies in the numbers of samples investigated by PCR with those recruited are accounted for by the fact that there were some samples which displayed a band pattern during PCR that could not determine a specific genotype and so could not be included in the statistical analysis.

Presence or absence of allele

Fisher exact test *p*-values, comparing the presence or absence of a given allele, revealed no significant differences in the Glasgow, Göteborg or combined data (Table 4).

Discussion

The aim of the present study was to investigate a possible correlation between chronic periodontitis and polymorphisms in the genes coding for two chemokines (interleukin-24 and RANTES), as well as a RANTES receptor (CCR5), all of which are associated with the cytokine interleukin-10, which is thought to be involved in the aetiology and pathogenesis of chronic periodontitis.

Interleukin-10 and tumor necrosis factor microsatellites have been previously investigated (20) in relation to periodontitis. Microsatellite markers are stable, polymorphic, easily analyzed and occur at regular intervals throughout the genome, making them especially suitable for genetic analysis. In the present study, single nucleotide polymorphisms were analysed. Single nucleotide polymorphisms have similar properties to, but are more frequent than, microsatellite markers. In addition, single nucleotide polymorphism markers can be near to or in the locus of interest, some located within the gene itself (cSNP) and can directly influence protein structure or expression, giving insights into disease mechanisms.

It should be remembered, when studying genetic markers, that these are only a guide to a possible suspected gene in a particular condition, but can be helpful in concentrating efforts to the appropriate genes for more detailed analyses.

The present study investigated the chemokine RANTES and its receptor CCR5. RANTES seems to be found specifically in the periodontal tissues of periodontitis patients compared with controls (21,22), and its concentration has been shown to be decreased in the crevicular fluid after periodontal therapy (23). RANTES-producing cells have been demonstrated in the gingivae of patients with marginal periodontitis, but not in healthy controls. In addition in this study, CCR5-positive cells (T helper 1) were also present in the gingivae of the periodontitis patients studied (24). A further study has shown a higher expression of CCR5 and lower expression of interleukin-10 in aggressive periodontitis patients, but a higher level of interleukin-10 in chronic periodontitis patients. The authors state that chemokines, such as RANTES, in addition to the classical cytokines, may be involved in the pathogenesis of periodontal disease, driving the migration and maintenance of several cell types, such as polymorphonuclear leukocytes, dendritic cells, natural killer cells, macrophages and subsets of lymphocytes in the gingival tissues (25). Genetic variation in the CCR5 Δ32 mutation has been studied previously, with frequencies similar to those of the present study being reported, as well as similarity in the lack of difference between chronic

Table 3. Distribution of single nucleotide polymorphisms by smoking and periodontitis status for Glasgow and Göteborg and combined

			Combined			Glasgow			Göteborg		
	Smoking		H	ChP	*p*-values	H	ChP	*p*-values	H	ChP	*p*-values
RANTES											
Genotypes	S	AA	0	0		0	0		0	0	
		AG	7	37	0.98	2	9	0.975	5	28	0.75
		GG	3	7		2	1		1	6	
Genotypes	NS	AA	1	1		0	1		1	0	
		AG	41	44	0.98	19	22	0.975	22	22	0.50
		GG	9	2		7	2		2	0	
Allele frequency	S	A	8	37	0.75	2	9	0.75	5	28	0.80
		G	14	51		6	11		7	40	
Allele frequency	NS	A	43	46		19	24		24	22	
		G	59	48	0.80	33	26	0.80	26	22	0.85
Interleukin-24											
Genotypes	S	CC	2	4		0	1		2	3	
		CT	6	32		2	6		4	26	
		TT	2	11	0.90	2	3	0.90	0	8	0.90
Genotypes	NS	CC	4	4		4	3		0	1	
		CT	39	44	0.92	15	17	0.92	24	27	0.92
		TT	12	7		8	5		4	2	
Allele frequency	S	C	10	40		2	8		8	32	
		T	10	54	0.90	6	12	0.90	4	42	0.92
Allele frequency	NS	C	47	52		23	23		24	29	
		T	63	58	0.90	31	27	0.90	32	31	0.90
CCR5											
Genotypes	S	ΔΔ	0	2		0	0		0	2	
		ΔWT	2	10	0.85	0	3	0.85	2	7	0.80
		WT/WT	6	27		2	6		4	21	
Genotypes	NS	ΔΔ	0	0		0	0		0	0	
		ΔWT	13	13	0.80	8	8	0.80	5	5	0.80
		WT/WT	29	26		15	14		14	12	
Allele frequency	S	Δ	2	14		0	3		2	11	
		WT	14	64	0.90	4	5	0.98	10	49	0.60
Allele frequency	NS	Δ	13	13		8	0		5	5	
		WT	71	65	0.80	38	16	0.80	33	29	0.60

CCR5, CC chemokine receptor 5; ChP, chronic periodontitis; H, healthy; NS, nonsmoker; RANTES, regulated on activation, normal T cells expressed and secreted; S, smoker.
There were no statistically significant differences recorded between the control (Group I) and test (Group II) groups ($p > 0.05$) when the results were adjusted for smoking status.

Table 4. Exact p-values from Fisher exact tests

	Glasgow	Göteborg	Combined
RANTES			
Any G vs. no G	$p = 1.000$	$p = 0.3448$	$p = 1.000$
Any A vs. no A	$p = 0.0529$	$p = 0.7128$	$p = 0.2355$
Interleukin-24			
Any C vs. no C	$p = 0.4209$	$p = 1.000$	$p = 0.4259$
Any T vs. no T	$p = 1.000$	$p = 1.000$	$p = 0.7781$
CCR5			
Any Δ32 vs. no Δ 32	$p = 1.000$	$p = 1.000$	$p = 1.000$
Any WT vs. no WT	(no patients with no WT)	$p = 0.5403$	$p = 0.5106$

CCR5, CC chemokine receptor 5; RANTES, regulated on activation, normal T cells expressed and secreted.

periodontitis patients and healthy controls (26).

Neither interleukin-24 (*mda-7*) itself, nor its genetic polymorphisms, have been specifically investigated in chronic periodontitis patients; however, radiation hybrid mapping has assigned the *mda-7* gene to human chromosome 1q, at 1q 32.2–1q41, an area containing a cluster of genes associated with the interleukin-10 family of cytokines. The *mda-7* gene product (interleukin-24) seems to provide antagonistic functions to interleukin-10 (27). It has been suggested that the homology of interleukin-24 to interleukin-10 may mean that polymorphic variants in interleukin-10 may affect interleukin-24 regulation (9). Also, genetic associations with certain pathologies may be reflecting unknown genes in linkage with the interleukin-10 genes rather than being a direct effect of structural variation in the interleukin-10 gene locus itself. Candidate genes (such as *mda-7*) that are sufficiently close to the

interleukin-10 locus may be involved in linkage disequilibrium (28) and thus may be important in the genetic variation of individuals in their inflammatory response in certain conditions, such as chronic periodontitis.

In the present study, it was assumed that the genetic make-up of patients in Glasgow is similar to that of patients in Göteborg. This would seem reasonable in light of the fact that a global survey of genetic variation in CCR5 and RANTES showed 'Europeans' to be a homogenous genetic group (10).

Our results suggest that the CCR5 Δ32 deletion is not common in populations and this study does not support an important role of CCR5 Δ32 in the pathogenesis of chronic periodontitis. Similarly, AG and CT seem to be the commonest genotype for RANTES and interleukin-24, respectively, in both cohorts of patients.

For all comparisons between groups, there is no evidence of a statistically significant association between the presence or absence of a given allele of the given gene and group for any of the three genes. In addition, the actual genotype did not correlate with the presence or absence of periodontal disease (Table 2). However, for RANTES/Glasgow patients, the *p*-value is borderline significant, based on the small numbers, and not quite statistically significant at the 5% significance level (Table 4). This association takes the form of being a greater percentage of control patients with GG (29%) compared with test patients (9%), and similarly a greater percentage of test patients with AG (89%) compared with control patients (71%).

When the results are re-interpreted to take into account the smoking status of the participants, there is still no evidence for an association between genotype and group, for each of the three genes in both groups (Table 3).

The results of the present study would infer that there is unlikely to be an association between the polymorphisms in the genes coding for RANTES, CCR5 or interleukin-24, investigated in this study, and chronic periodontitis.

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