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Genetic Variation on the *BAT1-NFKBIL1-LTA* Region of Major Histocompatibility Complex Class III Associates with Periodontitis

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Periodontitis is a chronic inflammatory disease with a multifactorial etiology. We investigated whether human major histocompatibility complex (MHC) polymorphisms (6p21.3) are associated with periodontal parameters. Parogene 1 population samples ($n = 169$) were analyzed with 13,245 single nucleotide polymorphisms (SNPs) of the MHC region. Eighteen selected SNPs ($P \leq 0.001$) were replicated in Parogene 2 population samples ($n = 339$) and the Health 2000 Survey ($n = 1,420$). All subjects had a detailed clinical and radiographic oral health examination. Serum lymphotoxin-α (LTA) concentrations were measured in the Parogene populations, and the protein was detected in inflamed periodontal tissue. In the Parogene 1 population, 10 SNPs were associated with periodontal parameters. The strongest associations emerged from the parameters bleeding on probing (BOP) and a probing pocket depth (PPD) of ≥6 mm with the genes *BAT1*, *NFKBIL1*, and *LTA*. Six SNPs, rs11796, rs3130059, rs2239527, rs2071591, rs909253, and rs1041981 (r^2, ≥0.92), constituted a risk haplotype. In the Parogene 1 population, the haplotype had the strongest association with the parameter BOP, a PPD of ≥6 mm, and severe periodontitis with odds ratios (95% confidence intervals) of 2.63 (2.21 to 3.20), 2.90 (2.37 to 3.52), and 3.10 (1.63 to 5.98), respectively. These results were replicated in the other two populations. High serum LTA concentrations in the Parogene population were associated with the periodontitis risk alleles of the *LTA* SNPs (rs909253 and rs1041981) of the haplotype. In addition, the protein was expressed in inflamed gingival connective tissue. We identified a novel *BAT1-NFKBIL1-LTA* haplotype as a significant contributor to the risk of periodontitis. The genetic polymorphisms in the MHC class III region may be functionally important in periodontitis susceptibility.

Periodontitis is a common chronic infectious disease affecting especially the middle-aged and elderly. It is characterized by gingival bleeding, the formation of deepened periodontal pockets, and slowly progressing inflammatory destruction of periodontal ligament and alveolar bone. Consequently, the teeth become mobile and are finally lost if the disease is left untreated. Over 47% of the U.S. adult population suffers from periodontitis (1), and according to a national-population-based health examination survey, 64% of Finnish adults have deepened periodontal pockets (2).

Periodontitis is considered a complex, multifactorial disease initiating with unbalanced oral microbiota resulting in the predominance of periodontopathic bacteria. Other risk factors for periodontitis include smoking, alcohol consumption, diabetes, obesity, osteoporosis, and stress (3). These risk factors are modifiable, but other risk indicators such as age, gender, race, and genetic factors are unchangeable. On the basis of previous studies, genetics may form the basis for susceptibility to periodontitis (4), although study results are diffuse. Case-control association studies have suggested that polymorphism in the genes for interleukin-1 (IL-1), IL-6, IL-10, vitamin D receptor, CD14, matrix metalloproteinase 1, and Toll-like receptor 4 may play a role in chronic periodontitis (3, 4). Two recent genome-wide association studies (GWAS) detected no significant association with periodontal disease (5, 6), but another found evidence suggestive of an association of six loci with different stages of chronic periodontitis. One of these loci is located in the human major histocompatibility complex (MHC) region (6p21.3) (5), which showed a concordant effect estimate for "high periodontal pathogen colonization" traits in another GWAS (7). Earlier, smaller case-control studies have shown that the MHC region is associated with periodontitis (8–10). HLA-DP, HLA-DQ, and HLA-DR molecules are expressed by antigen-presenting cells in oral gingival and pocket epithelium (9), and Palikhe et al. showed a cytokine lymphotoxin-α (LTA) variant (LTA+496C) emerging as a risk gene for alveolar bone loss (ABL). This prompted us to study whether the genetic polymorphisms in the human MHC region are associated with periodontal parameters and periodontitis. In the original study, we analyzed 13,245 single nucleotide polymor-

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phisms (SNPs) in the MHC region, and the results were replicated in two different populations.

MATERIALS AND METHODS

Subjects and recruitment. (i) Parogene 1 and 2 populations. The COROGENE study is based on 5,294 patients who underwent coronary angiography for any reason in Helsinki University Central Hospital, Helsinki, Finland, between June 2006 and March 2008. The cohort profile has been described earlier in detail (11). The Parogene study is a subsample of the COROGENE study. A random sample of 567 patients was invited to undergo an extensive oral examination. Eventually, only 508 (89%) were examined because of no-shows, and the population has been described earlier (12). Coronary angiography showed that 123 (24.3%) of the patients had no significant coronary artery disease (CAD) (<50% stenosis), 184 (36.4%) had stable CAD (≥50% stenosis), and 169 (33.4%) had acute coronary syndrome (ACS). Thirty-two patients had the diagnosis "ACS-like, but no significant CAD." All of the subjects enrolled in the study gave informed consent, and the research plan was approved by the local ethics committee. In the present study, the ACS patients made up the Parogene 1 population ($n = 169$) and the rest of the original Parogene patients formed the Parogene 2 population ($n = 339$).

(ii) Health 2000 Survey. This study is part of the nationwide comprehensive Health 2000 Survey in Finland, which used a stratified two-stage cluster sample of 8,028 citizens aged 30 years and older (2, 13, 14). The sampling frame was regionally stratified according to Finland's five university hospital regions. Data collection was carried out by means of structured health interviews, blood sample collection, and clinical oral health examinations. Permission for the study was given by the ethics committees of the University Hospital Region of Helsinki and Surroundings and the National Public Health Institute (KTL). Informed consent was obtained from each survey participant. The present sample is a subcohort of the Health 2000 Survey originally selected for a case-control GWAS of metabolic syndrome. The study setting was previously described in detail by Kristiansson et al. (15). In short, the patients had nondiabetic metabolic syndrome as defined by the International Diabetes Federation and the controls were age- and sex-matched healthy individuals from the same regions of Finland. The study was based on a subpopulation with an age limitation of ≥45 years ($n = 1,420$).

Clinical oral health examination. (i) Parogene 1 and 2 populations. Oral examination in the Parogene study has been described earlier (12). Briefly, periodontal probing pocket depths (PPDs) were registered from six sites around each tooth by manual probing, and bleeding from four sites around each tooth (bleeding on probing [BOP]) excluding wisdom teeth. The numbers of sites with a PPD of 4 to 5 mm and a PPD of ≥6 mm were registered. Digital panoramic radiographs of both dentate and edentulous subjects were made. ABL was calculated from radiographs by choosing from each dentate sextant the tooth with the most severe bone loss, which was categorized as follows: 0, no bone loss; 1, ABL in the cervical third of the root; 2, ABL in the middle third of the root; 3, ABL in the apical third of the root; 4, total bone support loss. The mean value of these six measurements was calculated for each patient. In addition, the numbers of angular bone defects and apical rarefactions were determined from radiographs.

(ii) Health 2000 Survey. Clinical oral health examinations were conducted by five calibrated dentists with a WHO periodontal probe (2). The dental health examination was based on the methodology of the Mini-Finland Survey (16) and on WHO guidelines (1997). PPD was measured at four sites around each tooth, excluding the wisdom teeth, and the deepest measurement was recorded by tooth as a PPD of 0, a PPD of 4 to 5 mm, or a PPD of ≥6 mm. Immediately after pocket measurements, BOP was recorded by sextants (bleeding or not bleeding). ABL was determined from panoramic radiographs by a specialist in oral radiology in each dentate sextant and categorized similarly as in the Parogene study. Numbers of teeth with angular bone defects were also determined from radiographs as follows: 0, no vertical bone pocket; 1, vertical bone pocket exceeding the middle third of the root; 2, vertical bone pocket exceeding the apical third of the root. The highest value of each tooth was recorded.

In all of the study populations, analyses were based on two different periodontitis definitions, advanced and severe periodontitis. Advanced periodontitis included ABL in the middle third of the root to total bone support loss and two or more sites with a PPD of 4 to 5 mm or one or more sites with a PPD of ≥6 mm. The reference group comprised subjects with no ABL or ABL only in the cervical third of the root but no PPD of ≥6 mm (healthy to mild periodontitis). Severe periodontitis included ABL in the middle third of the root to total bone support loss, a PPD of ≥6 mm at >3 sites, and a PPD of 4 to 5 mm at ≥10 sites. The reference group comprised subjects with no ABL, no PPD of ≥6 mm, and a PPD of 4 to 5 mm at <10 sites (healthy to gingivitis).

Genotyping. (i) Parogene 1 population. DNA was isolated by standard salt precipitation protocols. All subjects were genotyped for single-nucleotide polymorphisms (SNPs) with an Illumina 610K genotyping chip (HumanHap 610-Quad SNP array; Illumina, San Diego, CA) at the Wellcome Trust Sanger Institute (Hinxton, Cambridge, United Kingdom). Altogether, we analyzed 13,245 SNPs in the MHC region with locations from position 25749179 (rs932316) to position 33473618 (rs211457) of which 3,692 were genotyped SNPs. Quality control (QC) was performed according to Anderson et al. (17). Individuals with (i) a failed gender check, (ii) a low genotyping frequency (<95%), (iii) excess heterozygosity, (iv) a non-European background based on multidimensional scaling, or (v) excess relatedness were removed. Additionally, prior to genotype imputation, SNPs with (i) a low call rate (<95%), (ii) a low minor allele frequency, or (iii) a low Hardy-Weinberg disequilibrium P value ($<1 \times 10^{-6}$) were excluded. QC was performed with PLINK software (18). The cleaned data set was imputed with MACH 1.16 by using HapMap 2, release 22 CEU reference (http://hapmap.ncbi.nlm.nih.gov/downloads/phasing/2007-08_rel22/phased/). Imputation was performed in two steps. First, the error and recombination rates were estimated by using 200 random individuals, and second, by using the resulting maps, the individuals were sorted into four groups of ~1,000. The best-guess genotypes resulting from the imputation were added to the initial genotype data set with PLINK, and the merged data set was used in the analysis. From the univariate analysis of the Parogene 1 population, 18 SNPs associated with periodontal parameters ($P \leq 0.001$) were chosen for replication studies with the Parogene 2 and Health 2000 Survey populations.

(ii) Parogene 2 population. The Parogene 2 replication sample was genotyped with the Sequenom platform (iPlex MassARRAY, San Diego, CA) at the Institute for Molecular Medicine Finland (Helsinki, Finland). After validation, all 18 SNPs were genotyped and used in replication analyses.

(iii) Health 2000 Survey. DNA was isolated by using standard salt precipitation protocols. Like the Parogene 1 population samples, these samples were genotyped with the Illumina 610K genotyping chip (HumanHap 610-Quad SNP array; Illumina, San Diego, CA) at the Wellcome Trust Sanger Institute (Hinxton, Cambridge, United Kingdom). The imputed SNP genotype data were acquired with MACH 1.0.16 by using HapMap 3 as described earlier in detail (15), and the imputation success rate was >94%. The 18 SNPs selected for replication were analyzed.

Demographic data. Body mass index (BMI) data in the Parogene study were collected from medical records, and in the Health 2000 Survey, the BMI information was based on medical examinations. In the Parogene study, patients were considered to have diabetes (insulin dependent or not insulin dependent) if they had prescribed medication for it. In the Health 2000 Survey, the prevalence of diabetes and cardiovascular disease (CVD) was based on the self-reported information on the diseases diagnosed by a doctor. The CVD parameter was a combination of acute myocardial infarction, angina pectoris, and stroke. The smoking index (never, former, current) was based on the interview both in the Parogene study and in the Health 2000 Survey. All of the subjects who had quit smoking more than 1 month ago were considered former smokers. Current smokers included both occasional and daily smokers. The regional factor in the Health 2000

TABLE 1 Characteristics of the subjects in this study

Parameter	Parogene 1 ($n = 169$)	Parogene 2 ($n = 339$)	Health 2000 Survey ($n = 1,420$)	P value[d]
Age (yr)	62.9 (9.85)[a]	63.7 (8.76)	56.4 (8.09)	<0.001
BMI (kg/m^2)	28.2 (5.31)	27.7 (4.90)	27.6 (4.45)	0.219
No. of men	122 (72.2)	208 (61.4)	654 (46.1)	<0.001
No. with diabetes	39 (24.0)	80 (24.8)	55 (3.9)	<0.001
No. with CVD[b]			118 (8.3)	
No. with coronary artery blockage[c]				
0	·	155 (45.7)	·	
1	79 (46.7)	49 (14.5)	·	
2	44 (26.0)	57 (16.8)	·	
3	46 (27.2)	78 (23.0)	·	
No. who smoke(d):				<0.001
Currently	18 (10.7)	42 (12.4)	339 (23.9)	
Formerly	79 (46.7)	129 (38.1)	366 (25.8)	
Never	72 (42.6)	167 (49.3)	715 (50.3)	

[a] Values are means and standard deviations.
[b] CVD is a combination of acute myocardial infarction, angina pectoris, and stroke in the Health 2000 Survey.
[c] Number of coronary arteries with significant obstruction.
[d] P values were obtained by one-way analysis of variance or χ^2 test.

TABLE 2 Frequencies of cases and controls according to periodontal parameters

Periodontal parameter[a]	Parogene 1 ($n = 169$)	Parogene 2 ($n = 339$)	Health 2000 Survey ($n = 1,420$)
No. of teeth			
<26	116 (68.6)[b]	211 (62.2)	927 (65.3)
≥26	53 (31.4)	128 (37.8)	493 (34.7)
% BOP			
>26	115 (68.0)	200 (59.0)	671 (78.3)
0–26	54 (32.0)	139 (41.0)	186 (21.7)
PPD of 4–5 mm			
≥1	134 (79.2)	298 (87.9)	860 (60.6)
0	35 (20.7)	41 (12.1)	560 (39.4)
PPD of ≥6 mm			
≥1	76 (45.0)	141 (41.6)	327 (23)
0	93 (55.0)	198 (58.4)	1093 (77)
ABL			
Yes	122 (77.2)	241 (75.8)	477 (61.1)
No	36 (22.8)	77 (24.2)	304 (38.9)
Angular bone defects			
≥1	64 (40.5)	132 (41.5)	169 (11.9)
0	94 (59.5)	186 (58.5)	1251 (88.1)
Advanced periodontitis			
Yes	89 (58.2)	163 (52.1)	216 (28.8)
No	64 (41.8)	150 (47.9)	534 (71.2)
Severe periodontitis			
Yes	27 (56.3)	38 (41.8)	16 (3.00)
No	21 (43.8)	53 (58.2)	515 (97.0)

[a] Advanced periodontitis included ABL in the middle third of the root to total bone support loss and ≥2 sites with a PPD of 4 to 5 mm or ≥1 site with a PPD of ≥6 mm. The reference group comprised subjects with no ABL or ABL only in the cervical third of the root but no PPD of ≥6 mm (healthy to mild periodontitis). Severe periodontitis included ABL in the middle third of the root to total bone support loss, a PPD of ≥6 mm at >3 sites and a PPD of 4 to 5 mm at ≥10 sites. The reference group comprised subjects with no ABL, no PPD of ≥6 mm, and a PPD of 4 to 5 mm at <10 sites (healthy to gingivitis).
[b] Values are numbers (percentages) of study subjects.

Survey used in the statistical analyses is based on the university hospital areas of Finland.

Serum LTA concentrations. The serum LTA concentrations of the Parogene 1 and 2 patients were quantified by enzyme-linked immunosorbent assay (BMS202; eBioscience Bender MedSystems GmbH). The detection limit of the assay was 7.0 pg/ml, and the interassay coefficient of variation was 7.6% ($n = 10$).

Gingival samples. Gingival tissue biopsy specimens were obtained from patients diagnosed with chronic periodontitis ($n = 2$) and from healthy subjects ($n = 2$). The subjects were generally healthy nonsmokers. Chronic periodontitis biopsy specimens were taken from individuals with indications of surgery at sites with a PPD of >6 mm. Samples of healthy gingival tissue were taken from individuals with indications of tooth crown lengthening surgery for either prosthodontic or endodontic purposes at sites with a PPD of <3 mm. Incisions of the inflamed tissues were made 1 to 2 mm subgingivally through the gingival crevice to the alveolar crest following the major tooth axis and fixed in 4% buffered paraformaldehyde for further histological and immunohistochemical procedures. The Ethics Committee of the Department of Conservative Dentistry, Faculty of Dentistry, University of Chile, Santiago, Chile, approved the research plan, and the subjects signed an informed-consent form.

Histological analysis and immunohistochemistry. Gingival samples were embedded in paraffin and cut into 5-μm sections by microtomy. The sections were immunostained for LTA with a polyclonal antibody (product no. HPA007729, 1:20 dilution; Sigma), detected by with goat-anti-rabbit IgG as the secondary antibody (Vector Laboratories), and visualized with 3-amino-9-ethylcarbazole as the substrate.

Statistical analysis. Power calculation was done according to the prevalence of a PPD of ≥6 mm. According to the Health 2000 Survey, 21% of Finnish adults have at least one tooth with a PPD of ≥6 mm (2). Assuming a 0.21 population prevalence, there was 80% power to detect a risk variant in 1,000 patients and 1,000 controls at $P < 0.001$ for an SNP with an allele frequency of at least 20% in a two-stage analysis given that 200 individuals will be genotyped in the first stage of the analysis. Hardy-Weinberg equilibrium (HWE) was tested for each SNP by comparing the observed and expected genotype frequencies in patients and controls (χ^2 test). SNPs showing a deviation from HWE with a P value of <0.01 or a minor allele frequency of <1% were excluded. For the analysis, the cohorts were divided into patients and controls according to the periodontal parameters. In the Parogene 1 population univariate analyses, 18 SNPs were associated with periodontal parameters ($P \leq 0.001$). These SNPs were selected for further analyses, and the significance threshold was set to $0.05/18 = 0.0028$ according to Bonferroni correction. Associations among gene polymorphisms, risk haplotypes, and periodontal parameters were analyzed with logistic regression models adjusted for age, sex, smoking (nonsmokers, former smokers, and current smokers), BMI, and diabetes. Significance was assessed by the Wald test. The Finnish population is genetically homogeneous; in our samples, Parogene 1 and Health 2000 Survey population stratification was minimal, as shown by quantile-quantile plots (see Fig. S1 in the supplemental material) produced by the R Base Package (version 2.14.2). Hence, covariates reflecting ancestry were not included in the model. In the analysis of the Health 2000 Survey, the diagnosis of metabolic syndrome (15) and CVD and the regional factor based on the university hospital areas of Finland were additionally taken into account in the logistic models. The association analyses in the Parogene 1 and 2 populations were performed with the PLINK software (18), and the Health 2000 Survey was analyzed with the ProbABEL package (19).

TABLE 3 The most important MHC class III SNPs associated with periodontal parameters in Parogene 1

Location	SNP	Position	Periodontal parameter with strongest association	*P* value[a]	Parameter(s) also significantly associated
Intergenic	rs3130695	31319029	PPD of ≥6 mm	0.0045	
Intergenic	rs3130408	31321770	No. of teeth	0.004	
Intergenic	rs4084090	31326814	No. of teeth	0.004	
Intergenic	rs3130433	31327617	No. of teeth	0.004	
Intergenic	rs4416711	31329018	No. of teeth	0.004	
BAT1 intron	rs11796	31609191	PPD of ≥6 mm	**0.0009**	PPD of 4–5 mm, % BOP
BAT1 intron	rs3130059	31617263	PPD of ≥6 mm	**0.0009**	PPD of 4–5 mm, % BOP
BAT1 5′ UTR[b]	rs2239527	31617758	% BOP	**0.00051**	PPD of 4–5 mm, PPD of ≥6 mm
NFKBIL1 intron	rs2071591	31623778	% BOP	**0.00051**	PPD of 4–5 mm, PPD of ≥6 mm
NFKBIL1 intron	rs2255798	31629281	ABL	**0.0028**	PPD of ≥6 mm
LTA promoter	rs2857708	31641585	ABL	**0.0028**	PPD of ≥6 mm
LTA promoter	rs2857706	31645585	No. of teeth	0.006	
LTA promoter	rs2009658	31646223	No. of teeth	0.006	
LTA intron	rs909253	31648292	% BOP	**0.00078**	PPD of 4–5 mm, PPD of ≥6 mm
LTA coding exon	rs1041981	31648763	% BOP	**0.00078**	PPD of 4–5 mm, PPD of ≥6 mm
LST1 3′ UTR	rs1052248	31664560	No. of teeth	**0.0018**	
NCR3 promoter	rs2736189	31672707	ABL	**0.0023**	
NCR3 promoter	rs2844480	31672800	ABL	0.003	PPD of ≥6 mm

[a] *P* value obtained by Wald statistic of the logistic regression model adjusting for age, sex, smoking, BMI, and diabetes. The bold *P* values are considered significant ($P < 0.0028$) after Bonferroni correction for multiple testing.
[b] UTR, untranslated region.

Linkage disequilibrium analysis was conducted with the Haploview software (version 3.32) (20). Haplotype reconstruction was performed with FAMHAP (version 08/2008) (21) and PHASE (version 2.1) (22). According to the Parogene 1 population results, the risk haplotype associated with periodontal parameters was constructed from SNPs with r^2 values of >0.9. Conditional regression analysis of the six associated SNPs was performed in order to identify independent genetic markers. This analysis could not separate the effect of a single SNP because of the very high linkage disequilibrium. As a conclusion, we chose to analyze the associated haplotypes further.

Correlation analysis of serum LTA concentrations was performed by using the two-tailed Spearman correlation. Significances of differences between LTA concentrations and LTA allotypes were analyzed by the Mann-Whitney U test of two independent samples and the chi-square test. These statistical analyses were executed with the IBM SPSS Statistics 20 Statistical Package for the Social Sciences.

RESULTS

Characteristics of the subjects and division of different periodontal parameters in three populations are presented in Tables 1 and 2. The subjects in the Health 2000 Survey were younger and included more women than the Parogene 1 and 2 populations. They also smoked more frequently but suffered less from diabetes. In terms of oral health status, they had fewer deepened periodontal pockets and angular bone defects and milder ABL.

The Parogene 1 population established 18 single SNPs associated with periodontal parameters. A list of these SNPs with MHC class III locations, the positions of the SNPs, and associations among different periodontal parameters is presented in Table 3. After adjustment for covariates, 10 single SNPs were considered to be significantly associated with periodontal parameters. The strongest associations emerged from the parameters BOP and a PPD of ≥6 mm with the genes *LTA*, *NFKBIL1* (encodes the nuclear factor of κ light chain gene enhancer in inhibitor-like 1 B cells), and *BAT1* (encodes HLA-B-associated transcript 1, also known as *DDX39B* [DEAD Asp-Glu-Ala-Asp box polypeptide 39B]) (Fig. 1).

The degree of pairwise linkage disequilibrium was tested in 18 candidate SNPs associated by using r^2 statistics. Six SNPs, rs11796, rs3130059, rs2239527, rs2071591, rs909253, and rs1041981, with r^2 values of ≥0.92 constituted the risk haplotype, AGCGAC, respectively (Fig. 2). The other possible haplotypes were not significantly associated with periodontal parameters (data not shown).

The risk haplotype was associated with the periodontal parameters BOP, a PPD of 4 to 5 mm, and a PPD of ≥6 mm but not with the number of teeth, ABL, angular bone defects, or the number of apical rarefactions. In the Parogene 1 population, the risk haplotype had the strongest association with a PPD of ≥6 mm with an odds ratio (OR) of 2.90 (95% confidence interval, 2.37 to 3.52, $P = 0.00042$) (Table 4). In both replication populations, the risk haplotype was significantly associated with the parameters BOP and a PPD of ≥6 mm. The other 12 single SNPs studied were not associated with any of the periodontal parameters either in the Parogene 2 or the Health 2000 Survey population.

In addition to the periodontal parameters, we analyzed the association of the risk haplotype with two different definitions for periodontal disease: advanced and severe periodontitis. In the Parogene 1 population, advanced periodontitis was significantly associated with the risk haplotype, displaying an OR of 1.69 (0.73 to 2.62, $P = 0.041$) compared to healthy to mild periodontitis, but the result was not replicated in the other two populations (Fig. 3). However, the risk haplotype was significantly associated with severe periodontitis in all of the study populations compared to

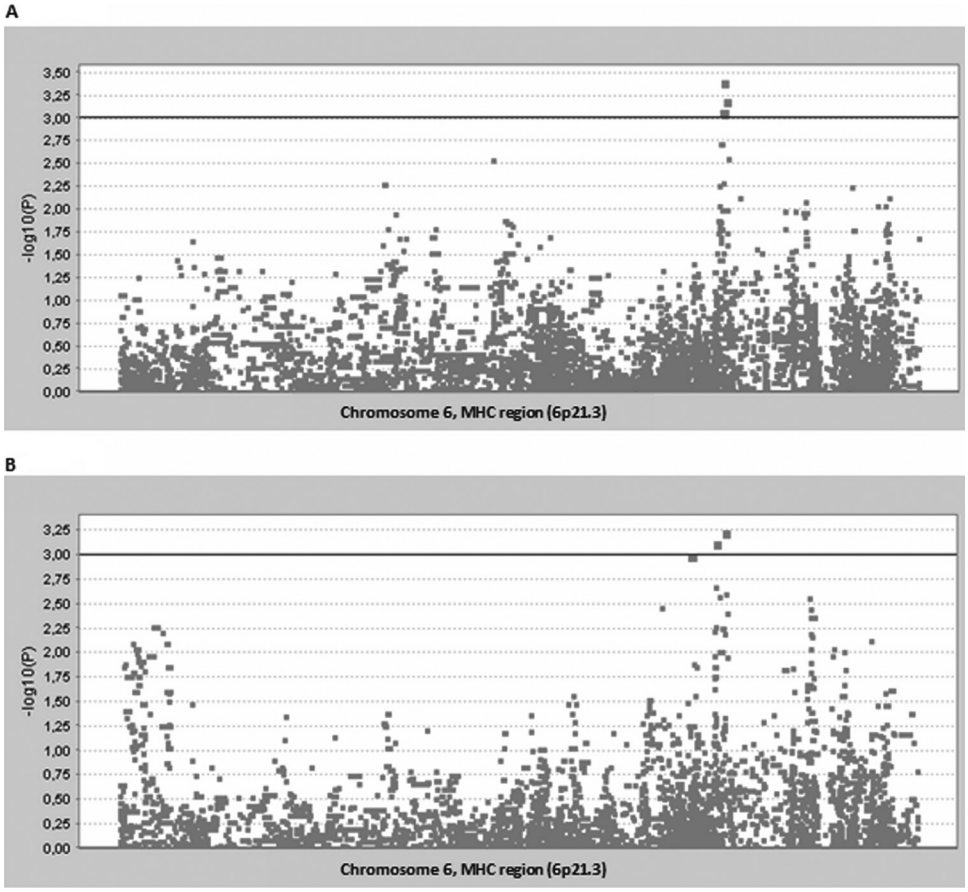

FIG 1 Plots of MHC region genetic variation associated with clinical periodontal parameters in the Parogene 1 population. The −log *P* values are for the association of each SNP with BOP (A) and a PPD of ≥6 mm (B). The horizontal line indicates a *P* value of <0.001 for the logistic regression model.

healthy-to-gingivitis subjects, with ORs of 3.10 (1.63 to 5.98, $P = 0.00019$), 3.25 (1.78 to 6.01, $P = 0.0009$), and 2.45 (1.0 to 6.01, $P = 0.04$) in the Parogene 1, Parogene 2, and Health 2000 Survey populations, respectively (Fig. 3).

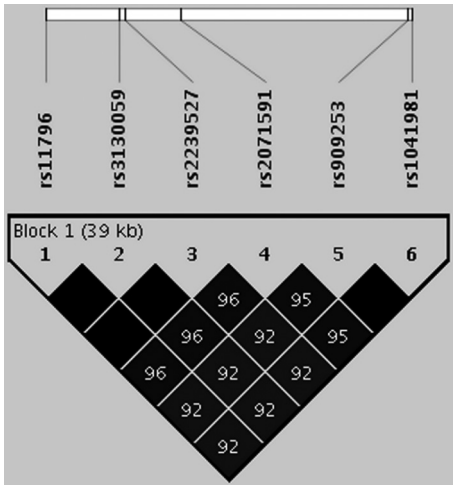

FIG 2 Pairwise linkage disequilibrium values of MHC class III SNPs in Parogene 1 population patients. Linkage disequilibrium ($r^2 = 1.0$) between markers is indicated by black boxes and lower r^2 values (×100) are shown in the corresponding boxes. The SNPs are located in the *BAT1-NFKBIL1-LTA* region.

Five of the original 18 SNPs selected according to the Parogene 1 population to be studied further were located in the gene for LTA, and two of them belonged to the constructed haplotype. Therefore, serum LTA concentrations were measured in the Parogene 1 and 2 populations. Combined results from these two populations showed that 71% of the serum samples had an LTA concentration above the detection limit, and these frequencies did not differ between alleles of the LTA SNPs. There were no significant differences in LTA concentrations between the Parogene 1 and 2 populations (median values, 32.7 [interquartile range {IQR}, 66.8] pg/ml versus 36.2 [IQR, 79.5] pg/ml, $P = 0.318$) or any of the periodontal parameters. However, higher LTA concentrations were associated with the periodontal risk alleles of LTA SNPs of the haplotype (rs909253 and rs1041981), and the results were significant when the homozygous subjects were compared (Table 5). The serum LTA concentration correlated positively with the number of teeth ($r = 0.142$, $P = 0.008$) and with a PPD of 4 to 5 mm ($r = 0.108$, $P = 0.048$).

To show that LTA is expressed in gingival tissue in periodontitis, immunohistochemical staining of four samples (from two subjects with periodontitis and two who were periodontally healthy) was performed. The inflamed connective tissue of the other periodontal patient stained positive for LTA. Immunostaining was located on the surface of cells with lymphocyte morphology and in the interstitium (Fig. 4). No staining was seen in the other samples.

TABLE 4 Associations between periodontal parameters and the SNPs comprising the risk haplotype in the Parogene 1 and 2 and Health 2000 Survey populations

Population, periodontal parameter, and SNP location	SNP	Position	Genotyped	Haplotype alleles		Risk allele frequency		SNP association		Haplotype association	
				Protective	Risk	Cases	Controls	*P* value[a]	Odds ratio (95% CI)	*P* value[a]	Odds ratio (95% CI)
Parogene 1											
% BOP											
BAT1 intron	rs11796	31609191	No	T	A	0.68	0.46	**0.00083**	2.50 (2.36–2.65)	**0.00056**	2.59 (1.99–3.12)
BAT1 intron	rs3130059	31617263	No	C	G	0.68	0.46	**0.00083**	2.50 (2.36–2.65)		
BAT1 5′ UTR[b]	rs2239527	31617758	No	G	C	0.68	0.44	**0.00039**	2.65 (2.21–3.19)		
NFKBIL1 intron	rs2071591	31623778	Yes	A	G	0.68	0.44	**0.00039**	2.65 (2.21–3.18)		
LTA intron	rs909253	31648292	No	G	A	0.68	0.46	**0.00063**	2.56 (2.14–3.07)		
LTA coding exon	rs1041981	31648763	Yes	A	C	0.68	0.46	**0.00063**	2.56 (2.14–3.07)		
PPD of 4–5 mm											
BAT1 intron	rs11796	31609191	No	T	A	0.67	0.57	0.027	1.49 (1.24–1.78)	**0.029**	1.42 (1.02–1.63)
BAT1 intron	rs3130059	31617263	No	C	G	0.67	0.57	0.027	1.49 (1.24–1.78)		
BAT1 5′ UTR	rs2239527	31617758	No	G	C	0.66	0.57	0.037	1.46 (1.22–1.75)		
NFKBIL1 intron	rs2071591	31623778	Yes	A	G	0.66	0.58	0.037	1.43 (1.19–1.71)		
LTA intron	rs909253	31648292	No	G	A	0.66	0.60	0.028	1.32 (1.10–1.58)		
LTA coding exon	rs1041981	31648763	Yes	A	C	0.66	0.60	0.028	1.32 (1.10–1.58)		
PPD of ≥6 mm											
BAT1 intron	rs11796	31609191	No	T	A	0.68	0.44	**0.00082**	2.70 (2.25–3.23)	**0.00042**	2.76 (2.27–3.36)
BAT1 intron	rs3130059	31617263	No	C	G	0.68	0.44	**0.00082**	2.70 (2.25–3.23)		
BAT1 5′ UTR	rs2239527	31617758	No	G	C	0.68	0.42	**0.00045**	2.92 (2.43 −3.51)		
NFKBIL1 intron	rs2071591	31623778	Yes	A	G	0.68	0.42	**0.00045**	2.92 (2.43 −3.51)		
LTA intron	rs909253	31648292	No	G	A	0.69	0.44	**0.00071**	2.82 (2.35–3.39)		
LTA coding exon	rs1041981	31648763	Yes	A	C	0.69	0.44	**0.00071**	2.82 (2.35–3.39)		
Parogene 2											
% BOP											
BAT1 intron	rs11796	31609191	Yes	T	A	0.68	0.61	0.011	1.36 (1.13–1.64)	**0.0097**	1.35 (1.10–1.72)
BAT1 intron	rs3130059	31617263	Yes	C	G	0.68	0.61	0.011	1.36 (1.13–1.64)		
BAT1 5′ UTR	rs2239527	31617758	Yes	G	C	0.68	0.61	0.011	1.36 (1.13–1.64)		
NFKBIL1 intron	rs2071591	31623778	Yes	A	G	0.69	0.62	0.0032	1.38 (1.14–1.66)		
LTA intron	rs909253	31648292	Yes	G	A	0.69	0.59	**0.0012**	1.53 (1.27–1.84)		
LTA coding exon	rs1041981	31648763	Yes	A	C	0.69	0.59	**0.0012**	1.53 (1.27–1.84)		
PPD of ≥6 mm											
BAT1 intron	rs11796	31609191	Yes	T	A	0.69	0.61	0.013	1.41 (1.17–1.70)	**0.0099**	1.49 (1.18–2.01)
BAT1 intron	rs3130059	31617263	Yes	C	G	0.69	0.61	0.013	1.41 (1.17–1.70)		
BAT1 5′ UTR	rs2239527	31617758	Yes	G	C	0.69	0.61	0.013	1.41 (1.17–1.70)		
NFKBIL1 intron	rs2071591	31623778	Yes	A	G	0.70	0.58	**0.0027**	1.64 (1.36–1.97)		
LTA intron	rs909253	31648292	Yes	G	A	0.70	0.59	**0.0010**	1.59 (1.32–1.91)		
LTA coding exon	rs1041981	31648763	Yes	A	C	0.70	0.59	**0.0010**	1.59 (1.32–1.91)		
Health 2000 Survey											
% BOP											
BAT1 intron	rs11796	31609191	No	T	A	0.69	0.62	0.023	1.33 (1.04–1.71)	**0.0105**	1.38 (1.05–1.79)
BAT1 intron	rs3130059	31617263	Yes	C	G	0.69	0.62	0.023	1.33 (1.04–1.71)		
BAT1 5′ UTR	rs2239527	31617758	Yes	G	C	0.69	0.62	0.023	1.34 (1.04–1.71)		
NFKBIL1 intron	rs2071591	31623778	No	A	G	0.68	0.61	0.012	1.37 (1.07–1.76)		
LTA intron	rs909253	31648292	No	G	A	0.69	0.62	0.01	1.38 (1.08–1.77)		
LTA coding exon	rs1041981	31648763	No	A	C	0.69	0.62	0.01	1.38 (1.08–1.77)		
PPD of ≥6 mm											
BAT1 intron	rs11796	31609191	No	T	A	0.71	0.66	0.013	1.29 (1.06–1.57)	**0.007**	1.31 (1.06–1.61)
BAT1 intron	rs3130059	31617263	Yes	C	G	0.71	0.66	0.013	1.29 (1.06–1.57)		
BAT1 5′ UTR	rs2239527	31617758	Yes	G	C	0.71	0.66	0.012	1.29 (1.06–1.57)		
NFKBIL1 intron	rs2071591	31623778	No	A	G	0.70	0.65	0.009	1.30 (1.07–1.59)		
LTA intron	rs909253	31648292	No	G	A	0.71	0.66	0.014	1.28 (1.05–1.56)		
LTA coding exon	rs1041981	31648763	No	A	C	0.71	0.66	0.015	1.28 (1.05–1.56)		

[a] *P* value obtained by Wald statistic of the logistic regression model adjusting for age, sex, smoking, BMI, and diabetes in the Parogene populations and for age, sex, smoking, diabetes, regional factor, BMI, CVD, and metabolic syndrome in the Health 2000 Survey population. *P* values considered significant are in boldface.
[b] UTR, untranslated region.

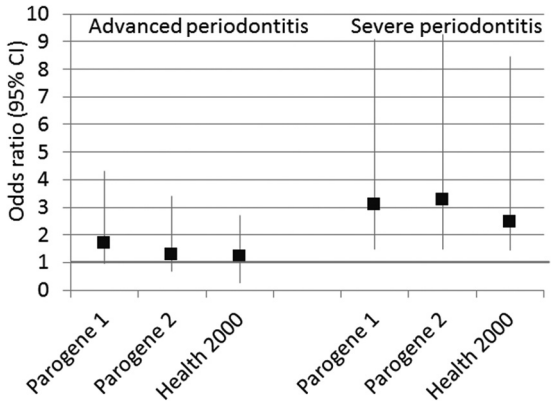

FIG 3 Association of the risk haplotype with two different definitions of periodontal disease, advanced and severe periodontitis. Advanced periodontitis included ABL in the middle third of the root to total bone support loss and ≥2 sites with a PPD of 4 to 5 mm or one or more sites with a PPD of ≥6 mm. The reference group comprised subjects with no ABL or ABL only in the cervical third of the root but no PPD of ≥6 mm (healthy to mild periodontitis). Severe periodontitis included ABL in the middle third of the root to total bone support loss, a PPD of ≥6 mm at >3 sites, and a PPD of 4 to 5 mm at ≥10 sites. The reference group comprised subjects with no ABL, no PPD of ≥6 mm, and a PPD of 4 to 5 mm at <10 sites (healthy to gingivitis). The logistic regression model was adjusted for age, sex, smoking, BMI, and diabetes in the Parogene population and for age, sex, smoking, diabetes, regional factor, BMI, CVD, and metabolic syndrome in the Health 2000 Survey population. CI, confidence interval.

DISCUSSION

We found a novel haplotype located on the MHC class III region showing an association with a risk of periodontitis. The haplotype was associated with BOP, a PPD of ≥6 mm, and severe periodontitis, and the associations were replicated with consistency in two different study populations. This haplotype, comprising regions of the *BAT1*, *NFKBIL1*, and *LTA* genes, was common in our study populations, with the lowest frequency of 42% in controls and the highest of 81% in patients. First, we showed the association in Parogene populations 1 and 2 with symptomatic patients who underwent coronary angiography. We then replicated the results in the Health 2000 Survey population comprising adults from the Finnish population-based random sample. Second, we showed that the serum LTA concentration was associated with two LTA SNPs in the risk haplotype in homozygous patients. Third, we showed that LTA is expressed on lymphocytes and in the interstitium of inflamed periodontal connective tissue.

Periodontitis initiates as dysbiosis of oral biofilm, leading to a host response, which gradually induces the degradation of connective tissue and bone support. The consequence is chronic inflammation characterized by gingival bleeding, formation of deepened periodontal pockets, and destruction of periodontal ligaments and alveolar bone. The end stage of the disease is tooth loss. The chronic and aggressive forms of periodontitis are clinical types of periodontal disease and have some significant differences, including (i) the age of onset, (ii) the rates of progression, (iii) the patterns of destruction, (iv) the clinical signs of inflammation, and (v) the relative abundance of plaque and calculus (23). The role of genetics in aggressive periodontitis affecting mainly younger people and in chronic periodontitis, the most common form of the disease in the middle-aged and elderly, has been investigated in family and twin studies. Evidence of familial aggregation of aggressive periodontitis has been reported in various publications (24, 25), supporting a genetic background of the disease. A recent GWAS identified the *GLT6D1* locus as an important factor in susceptibility to aggressive periodontitis (26). In addition, the results of a study of adult twins showed that chronic periodontitis may have approximately 50% heritability (27). Although aggressive periodontitis is quite rare, affecting around 2 to 4% of the population under 30 years of age (28), the clinical symptoms of the disease are parallel in various forms of the disease. In this study, we focused on the clinical signs of the disease, including the number of teeth, BOP, PPDs, ABL, and angular bone defects, and their association with genetic polymorphisms in the MHC class III region. Interestingly, the SNPs constituting the risk haplotype had the strongest associations with the periodontal parameters describing the current periodontal inflammation and disease activity, while the rest of the significant SNPs were most clearly associated with the parameters describing past periodontal disease, namely, ABL and missing teeth. As the current consensus on the definition of periodontitis is based on a combination of pocket formation and alveolar bone level, we analyzed the association of the risk haplotype with different classifications. The haplotype was not associated with the risk of advanced periodontitis compared to healthy to mild periodontitis. When the groups of "the periodontitis extremes" were compared, the risk haplotype was significantly associated with severe periodontitis compared to healthy-to-gingivitis subjects. The results indicate that the complex biological mechanisms underlying the clinical phenotype are not yet fully understood. Diagnostics would possibly benefit from broader information about clinical, microbial, and host-response characteristics, as suggested, for example, by Offenbacher et al. (29).

LTA (formerly tumor necrosis factor beta) is a cytokine with wide range of proinflammatory activities (30). It is expressed by lymphocytes (T, B, and natural killer cells) (31) and may exist in cell-bound and soluble forms (32). *LTA* gene polymorphism has also previously been associated with susceptibility to periodontitis (10, 33–35). In our study, five significant SNPs in the original study were located in the *LTA* gene and two of them were part of the risk haplotype. One of them, rs1041981, is exonic with a threonine-to-asparagine change (36) causing increased expression of adhesion molecules, such as VCAM-1 and E-selectin (37). The other SNP (rs909253) has been associated with periodontal disease (34, 35), as well as with myocardial infarction (37), rheumatoid arthritis (38), and asthma (39). In our study, these two SNPs were associated with current periodontitis and the homozygous subjects had higher serum LTA concentrations. We showed for the first time that LTA was expressed in inflammatory infiltrates from diseased gingiva on the surface of lymphocyte-like cells and the interstitium corresponding to the two previously described forms (32). Our results indicate that *LTA* polymorphisms participate in the regulation of inflammatory processes of periodontitis. No associations between serum LTA concentrations and any periodontal parameters were found suggesting, that saliva could have been a more suitable sample for LTA concentration measurements. Unfortunately, saliva samples were not available in this study.

A number of reports have focused on the association between a specific gene polymorphism and periodontitis, and haplotype analysis is a promising approach (4, 40), as our findings also demonstrate. The risk haplotype showed very high linkage disequilibrium (r^2 value of ~1) within the selected SNPs, and conditional

TABLE 5 Serum LTA concentrations in Parogene 1 and 2 populations in relation to LTA genotypes and alleles

SNP	Median LTA concn[a] in pg/ml, IQR (*n*) and *P* value		
	Parogene 1	Parogene 2	Total
rs909253			
AA	32.4, 13.1–68.8 (39)	38.2, 16.6–96.6 (95)	36.3, 14.9–89.3 (134)
AG	25.9, 11.6–69.5 (51)	29.5, 13.9–72.0 (81)	28.9, 13.3–70.7 (132)
GG	27.4, 15.3–164 (13)	30.2, 15.4–158 (37)	28.4,15.8–150 (50)
A allele (AA + AG)[b]	29.3, 12.9–69.0 (90)	34.9, 15.2–86.8 (176)	33.6, 14.5–80.0 (266)
G allele (AG + GG)	27.0, 13.3–67.5 (64)	29.9, 14.4–89.0 (118)	28.9, 13.9–80.0 (182)
P value (AA vs GG)[c]	0.001	0.184	0.007
rs1041981			
CC	32.4, 13.1–68.8 (39)	37.8, 16.6–95.2 (96)	35.9, 14.9–88.8 (135)
CA	25.9, 11.6–69.5 (51)	29.5, 13.9–72.0 (81)	28.8, 13.3–70.7 (132)
AA	26.6, 13.8–273 (11)	30.2, 15.4–158 (37)	28.1, 15.0–165 (48)
C allele (CC + CA)[b]	29.3, 12.9–69.0 (90)	33.9, 15.2–86.3 (177)	33.4, 14.5–79.6 (267)
A allele (CA + AA)	26.3, 12.7–71.3 (62)	29.9, 14.4–89.0 (118)	28.9, 13.8–80.6 (180)
P value (CC vs AA)[c]	0.001	0.184	0.009
rs2009658			
CC	38.1, 17.7–86.5 (69)	30.6, 15.2–107 (144)	33.0, 16.6–96.8 (213)
GC	15.8, 10.2–38.1 (31)	44.0, 16.6–83.0 (58)	32.4, 14.2–74.3 (89)
GG	51.6 (2)	44.4, 12.5–87.4 (11)	44.4, 12.1–91.3 (13)
C allele (CC + GC)	28.4, 13.3–69.3 (100)	33.5, 16.0–96.0 (202)	32.7, 14.9–86.7 (302)
G allele (GC + GG)	15.8, 10.2–39.6 (33)	44.4, 15.6–86.3 (69)	33.4, 13.4–80.0 (102)
P value (CC vs GG)[c]	0.591	0.389	0.323
rs2844482			
GG	38.1, 17.7–86.5 (69)	30.6, 15.2–107 (144)	33.0, 16.6–96.8 (213)
GA	16.2, 10.4–36.8 (32)	44.0, 16.6–83.0 (58)	32.4, 14.4–70.0 (90)
AA	51.6 (2)	44.4, 12.5–87.4 (11)	44.4, 12.1–91.3 (13)
G allele (GG + GA)	27.4, 13.4–65.4 (101)	33.5, 16.0–96.0 (202)	32.4, 14.9–86.3 (302)
A allele (GA + AA)	16.2, 10.2–38.8 (34)	44.4, 15.6–86.3 (69)	33.1, 13.4–74.9 (103)
P value (GG vs AA)[c]	0.591	0.389	0.323
rs2857708			
GG	36.3, 16.0–79.5 (74)	31.3, 15.0–97.7 (150)	32.7, 15.2–87.3 (244)
GA	18.8, 11.6–40.3 (28)	53.0, 16.6–88.8 (55)	33.1, 14.9–81.0 (83)
AA	8.1 (1)	39.1, 12.7–136 (8)	33.8, 10.6–119 (9)
G allele (GG + GA)	28.4, 13.6–69.0 (102)	33.9, 15.8–93.4 (205)	33.0, 14.9–86.6 (307)
A allele (GA + AA)	18.8, 11.3–39.6 (29)	44.4, 16.3–88.8 (63)	33.4, 14.5–81.0 (92)
P value (GG vs AA)[c]	0.089	0.343	0.171

[a] Only detectable LTA concentrations are shown.
[b] Risk allele in the haplotype.
[c] *P* value obtained by Mann-Whitney U test.

regression analysis could not separate the effect of a single SNP. Although similar haplotype analyses have not previously been reported for periodontitis, the *BAT1-NFKBIL1-LTA* region has drawn attention in studies concerning other inflammatory diseases. Ozaki et al. showed significant associations between myocardial infarction and a haplotype made up of five SNPS in *LTA*, *NFKBIL1*, and *BAT1* in Japanese patients. The recent GWAS of CAD in Japanese patients also found a prominent association with 6p21, encompassing the *LTA* gene (41). On the contrary, Koch et al. revealed the presence of a haplotype of the *BAT1-NFKBIL1-LTA* region with a protective effect against myocardial infarction in Europeans (42).

This study has some limitations. The Parogene 1 and 2 population samples are quite homogeneous considering the characteristics and oral status of the patients. They are strongly related to each other, since together they make up the cohort and are subgrouped according to coronary angiography results. Therefore, we wanted to replicate our results in a larger, independent population, the Health 2000 Survey population. The subjects in the Parogene 1 and 2 populations were middle-aged or older; therefore, the analysis in the Health 2000 Survey was based on a subpopulation aged ≥45 years ($n = 1{,}420$). Since our studies were not designed as case-control studies according to periodontal disease, the numbers of affected and unaffected subjects in the periodontitis classifications differed between the populations. It is possible that the use of disease data as continuous variables would have been useful in addition to a dichotomous approach. In neither of the populations was the cement-enamel junction of each tooth recorded; therefore, we defined the periodontal diagnosis according to the PPD and ABL parameters. Further, the genotyping was different in the Parogene 1 and 2 and Health 2000 Survey populations, which may have affected the LTA concentration results,

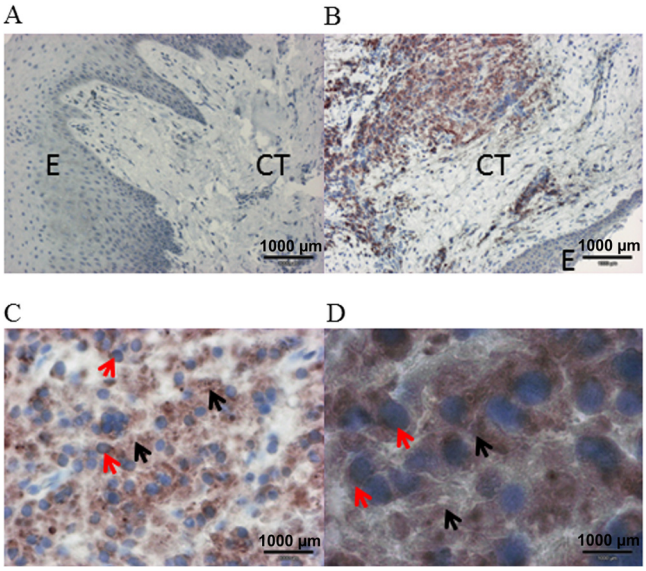

FIG 4 Immunohistochemical staining of gingival tissue samples for LTA. Gingival samples were obtained from two periodontitis patients and two periodontally healthy persons. Samples were embedded in paraffin and cut into 5-μm sections by microtomy. Immunohistochemical staining was performed with a polyclonal antibody against LTA. (A) Healthy subject; magnification, ×10. (B) Periodontitis patient; magnification, ×10. (C) Periodontitis patient; magnification, ×40. (D) Periodontitis patient; magnification, ×100. E, epithelium; CT, connective tissue. Arrows indicate LTA staining on the lymphocyte-like cell surface (red) and in the interstitium (black).

where a significant association was observed only in the Parogene 1 population patients. The Parogene study patients were examined in 2007 and 2008, the Health 2000 Survey patients were examined in 2000 and 2001, and gingival biopsy sampling was not part of the study protocols. The findings from the genetic and serum analyses prompted us to examine if LTA is expressed in periodontitis-affected tissue. Fortunately, via our collaboration with a Chilean research group, these samples were available. However, the number of gingival samples in the immunohistochemical analyses was limited and the conclusions about the association of LTA with periodontitis are tentative.

In conclusion, our study reveals a novel periodontitis-associated haplotype comprising six SNPs in the *BAT1-NFKBIL1-LTA* region. The variation on the MHC region, which is essential in both innate and adaptive immunity, may be important in periodontitis susceptibility.

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We have no competing interests to declare.

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