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Localized aggressive periodontitis is linked to human chromosome 1q25

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Abstract Localized aggressive periodontitis (LAP; previously known as localized juvenile periodontitis) is one of the rapidly progressive periodontal diseases. Certain forms of familial LAP show a simple Mendelian pattern of transmission. However, no gene mutation has been identified to be responsible for the LAP phenotype. As an initial step to identify a gene mutation associated with LAP, we have performed genetic linkage analysis with four multigenerational families exhibiting the LAP phenotype. Affected individuals in the families were identified based on clinical and laboratory criteria in an attempt to define a homogeneous phenotype, since the clinical presentation of LAP may represent a manifestation of a heterogeneous group of diseases. The LAP phenotype is linked to a DNA marker, D1S492, with LOD score 3.48, $\theta=0.00$. The haplotype analysis of the chromosome interval associated with D1S492 indicates that a LAP locus is located between D1S196 and D1S533 on chromosome 1, covering about 26 million DNA basepairs. We have also examined the DNA sequence of *prostaglandin-endoperoxide synthase 2* (*PTGS2* or *cyclooxygenase 2*, *COX2*) since prostaglandin 2 (PGE₂), the product of *COX2*, is upregulated in LAP patients and *COX2* is located between D1S196 and D1S533. No mutation in *COX2* was identified in the patients.

Introduction

Localized aggressive periodontitis (LAP) is estimated to affect 0.1%–3% of adolescent children depending on the specific form of disease and the population studied (Loe and Brown 1991; Papapanou 1999). The aggressive periodontal diseases are clinically heterogeneous and include forms of disease clinically indistinguishable from other forms of periodontitis, and several forms of disease with a uniquely localized clinical presentation with significant destruction at a very early age (Caton et al. 1989; Albandar et al. 1997). These conditions show remarkable familial aggregation (Schenkein and Van Dyke 1994; Novak and Novak 1996). Reports of familial aggregation of LAP have been interpreted to support a major gene locus, with autosomal recessive and X-linked inheritance (Saxen and Nevanlinna 1984; Long et al. 1987). Whereas autosomal recessive forms of aggressive periodontitis appear in northern Europe, results of segregation analysis conducted on US populations are most consistent with autosomal dominant inheritance, although other forms of inheritance cannot be excluded (Boughman et al. 1986; Hart et al. 1993; Marazita et al. 1994).

Although there is heterogeneity within the aggressive periodontitis classification with regard to neutrophil abnormalities, it is clear that there is homogeneity within certain populations. For instance, in US-based studies, approximately 60%–70% of LAP patients exhibit a decreased neutrophil chemotaxis trait (Van Dyke et al. 1985, 1997; Daniel et al. 1993). This is far lower in northern Europe and Finland (Saxen 1980; Kinane et al. 1989). The finding is best illustrated in African-American study populations. Co-segregation of the neutrophil trait with clinical disease has been reported in certain African-American families (Van Dyke et al. 1983). In these families, the neutrophil abnormality relating to reduced chemotaxis has been well characterized by a number of laboratories. This includes decreased chemotaxis to a number of chemotactic factors associated with reduced receptor expression on the neutrophil surface for those chemotactic factors (Van Dyke et al. 1981), reduced calcium transport in LAP neutrophils (Agarwal et al. 1989), increased diacylglycerols levels in

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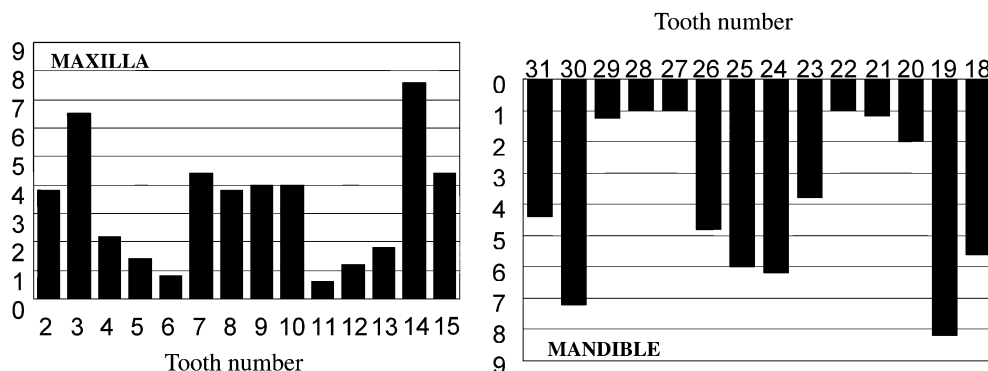


Fig. 1 The cemento-enamel junction is defined as the joining point of the enamel of the tooth crown and the cementum that covers the radicular surface of the tooth. The distance between the tip of the alveolar crest and the enamel is a clinical and radiographic measure by which the levels of supporting alveolar bone are assessed. This distance, shown in *solid bars*, is 1–2 mm in healthy individuals without any periodontal disease. An increased distance suggests bone loss. This figure shows the average alveolar bone levels of each tooth in the LAP patients. As a characteristic of LAP, the bone destruction is localized around the incisor (numbers 7–10, 23–26) and first molar (numbers 3, 14, 19, 30) teeth. A significant amount of bone loss is also observed around second molar teeth (numbers 2, 15, 18, 31). On the other hand, no bone loss is observed around canines (numbers 6, 11, 22, 27) and premolar teeth (numbers 4, 5, 12, 13, 20, 21, 28, 29). We used this information for the estimation of bone loss in our clinical examination

stimulated LAP neutrophils, and decreased protein kinase C activity (Kurihara et al. 1993). The finding that these observations are not universal in the LAP populations reported in different geographic locations, or even within the same geographic population, strongly supports the hypothesis that there are several distinct forms of disease with the same clinical presentation. This aspect of the genetics of LAP has not been previously explored systematically.

We have performed genetic linkage analysis of candidate regions with four LAP multigenerational families. With regard to the complicated etiology of LAP, we selected the families based on clinical examination and laboratory tests to minimize the negative influence of non-allelic heterogeneity. The results from the linkage analysis showed that the DNA marker, D1S492, on chromosome 1 had a LOD score 3.48 with θ at 0.00. Haplotype analysis of the chromosome interval associated with D1S492 was performed, and the result from the analysis indicated that the *LAP* locus resided between D1S196 and D1S533. The DNA sequence of *prostaglandin-endoperoxide synthase 2* (*PTGS2*; formerly known as *cyclooxygenase 2*, *COX2*) in LAP patients was also examined since prostaglandin E2 (PGE2), the product of *COX2*, is upregulated in LAP patients, and *COX2* is located between D1S196 and D1S533. No mutation in *COX2* was found in the patients.

Materials and methods

Recruitment of LAP families for genetic linkage analysis

Although there is no direct genetic evidence that LAP is a heterogeneous disorder, there is published evidence of heterogeneity in

clinical presentation. We, therefore, collected LAP families with caution to minimize the potential negative influence of a heterogeneous etiology of the disorder on the genetic linkage analysis. All of the families were recruited from Boston University Medical and affiliated health centers, in accordance with the human subject protocol approved by Boston University Medical Center Institutional Review Board.

Clinical examination

At the beginning of each clinical visit, subjects were asked to fill out a questionnaire to obtain detailed medical and dental histories. A routine dental examination was performed including periodontal, dental, and radiological examinations. Subjects were diagnosed with LAP according to the clinical characteristics of the disease as defined by the American Academy of Periodontology, including attachment and bone loss limited to first molars and incisors. The bone pattern was characteristic of LAP as indicated in Fig. 1. Individuals with active infectious diseases such as hepatitis, AIDS, and tuberculosis or those being treated with any medication known to affect periodontal status were excluded from the genetic linkage study. Pregnant women were also excluded.

Laboratory evaluation of neutrophil function

One of the phenotypic characteristics of most LAP subjects of African-American origin is abnormal *in vitro* neutrophil function manifest as reduced chemotaxis and elevated superoxide production consistent with cell priming. To characterize our subjects further, chemotaxis and superoxide generation by peripheral blood neutrophils was analyzed in all subjects.

After obtaining informed consent, 30 ml peripheral venous blood was collected. A normal individual in each family served as a control for each laboratory analysis together with an unrelated healthy subject. The laboratory tests included the examination of levels of superoxide generation and chemotaxis to formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) by neutrophils. LAP patients in each family demonstrated increased levels of superoxide generation and decreased chemotaxis. In addition, the identification of *Actinobacillus actinomycetemcomitans*, the causative microorganism of LAP, by DNA-DNA hybridization checkerboard analysis was performed. If subjects who had been clinically diagnosed as having LAP did not meet the laboratory criteria (decrease chemotaxis, elevated O_2^-) and microbiological characteristics (*A.a.* infection), the families were not included in the genetic linkage study.

Isolation of neutrophils

Neutrophils were separated by discontinuous one-step Ficoll-Hypaque density gradient centrifugation (Kalmar et al. 1988). After centrifugation at 900 g for 20 min at room temperature, the neutrophils were isolated and washed in 4 ml phosphate-buffered saline (PBS). The samples were centrifuged at 300 g for 10 min at

room temperature, the supernatants were removed, and the contaminating erythrocytes were lysed with ice-cold NH_4Cl buffer for 10 min. The samples containing neutrophils were washed with PBS and centrifuged at 300 g for 10 min at room temperature. The purified cells were counted by using a hemocytometer, and the concentration was adjusted depending on the assay.

Superoxide production

Superoxide production (oxidative burst) of neutrophils was analyzed by using a superoxide-dismutase-inhibitable cytochrome C reduction assay (Tanigawa et al. 1993). The neutrophils were suspended in PBS at a concentration of $6 \times 10^6/\text{ml}$ in a well of a 96-well microplate. Each sample was run in triplicate with 5×10^5 neutrophils in each well.

Superoxide production was measured both at rest and after stimulation with fMLP (10^{-6}M). The plate was then incubated at 37°C for 5 min and read in the microplate reader at a wavelength of 550 nm. The amount of superoxide produced was expressed as nanomoles O_2^- per 5×10^5 neutrophils per minute.

Chemotaxis

Cells were suspended in Gey's buffer (GBSS, Life Technologies, Gaithersburg, Md.) supplemented with 2% bovine serum albumin at a concentration of 2.5×10^6 cells/ml. The cells suspension was placed in the upper compartment of a modified Boyden chamber separated by a 5- μm pore-size micropore filter (Nucleopore, Pleasanton, Calif.), whereas the lower compartment was loaded with the buffer solution containing fMLP (10 nM) or buffer alone. Cell migratory response was evaluated by counting the number of neutrophils that accumulated on the distal surface of the filter after a 2-h incubation in a 37°C , 5% CO_2 incubator. Three representative high-power microscopic fields ($\times 400$) were counted for each of the triplicate filters. Neutrophil chemotactic migration in response to fMLP was compared with random migration in the absence of chemotactic agent (Van Dyke et al. 1980).

Checkerboard analysis

Subgingival plaque samples were collected from the teeth and placed into sterile Eppendorf tubes containing 150 μl TE buffer (TE buffer = 10 mM TRIS HCl pH 7.4, 1 mM EDTA), and 100 μl 0.5 M NaOH was added. The samples were then blotted on nylon membranes. The membranes were hybridized with DNA probes including the following species: *Fusobacterium nucleatum ss. vincentii* (364), *Campylobacter concisus* (484), *Campylobacter rectus*

(371), *Bacteriodes forsythus* (338), *Porphyromonas gingivalis* (381), *Prevotella intermedia* (25611), *Prevotella nigrescens* (33563), *Capnocytophaga sputigena* (33562), *Streptococcus oralis* (SS11), *Actinomyces naeslundii* (43146), *Treponema denticola* (Td), *Campylobacter curva* (9584), *Eikenella corrodens* (23834), *Actinomyces israeli* (12102), and *Actinobacillus actinomycetemcomitans* (Y4). The hybridization was performed on a Miniblotter 45 apparatus (Immunetics, Cambridge, Mass.). The probes and hybridization buffer were placed in individual lanes of the Miniblotter. The apparatus was placed in a sealed plastic bag and incubated overnight at 42°C . After hybridization, the filters were washed and incubated with phosphatase-conjugated antibody against digoxigenin. Lumiphos 530 solution was applied to the filters, and the chemiluminescence signals were detected with X-ray film. The signals were compared with standards on the membrane (0: no signal detected; 1: $<10^5$; 2: $\sim 10^5$; 3: $>10^5$ and $<10^6$; 4: $\sim 10^6$; 5: $>10^6$ cells).

Genetic linkage analysis

In practice, 20 individuals from a large family or several small families are required to perform a linkage analysis to obtain a LOD score greater than 3. Although more than 20 individuals from the families were collected, a simulation study with the SLINK program was performed to estimate the maximum expected LOD score (ELOS; Ott 1989; Weeks et al. 1990).

A directed genomic screening approach was used to perform the genetic linkage analysis. In this approach, we did not randomly choose chromosome regions or evenly select regions across the genome for the analysis. Instead, the regions chosen were based on a chromosome interval containing functional candidate gene or genes for LAP. Fourteen autosomal chromosome intervals were selected (Table 1).

All of the DNA markers used were commercially available (Applied Biosystems, California). Genomic DNA was isolated from 3 ml peripheral blood from individuals by using a Puregene DNA Isolation Kit from Gentra Systems (Minneapolis, Minn.). For the polymerase chain reaction (PCR), 15 μl 1 \times PCR buffer containing 75 ng DNA, 5 μM each primer, and 200 nM dNTPs, and 1 U *Taq* polymerase were used. The PCR conditions were 95°C for 12 min, followed by 10 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s, followed by 20 cycles of 89°C for 15 s, 55°C for 15 s, and 72°C for 30 s. Finally, the PCR was extended at 72°C for 10 min. The PCR products were loaded on an ABI 3100 Genetic Analyzer to examine the size of the PCR products.

A direct linkage analysis method was used to identify a candidate chromosome interval for further testing. In this method, a DNA marker with the least recombinant fraction among all affected individuals was identified. A chromosomal region around this DNA marker was considered a region of interest for further haplotype

Table 1 Fourteen chromosome locations for the linkage analysis

Chromosome locations	DNA polymorphic markers
1q25	D1S413, D1S238
2q13-12	D2S160, D2S347, D2S112, D2S2333
2q35	D2S126, D2S206, D2S325, D2S117 D2S396, D2S38
3q27-28	D3S1580, D3S1262,
4q11-13	D4S405, D4S1592
6p21.3	D6S289, D6S422
7q11.23	D7S669, D7S502, D7S630, D7S657, D7S519
9q32-33	D9S287, D9S1682, D9S1826, D9S1776
11q14-21	D11S4175, D11S901, D11S925, D11S898, D11S908
12q13.3	D12S83, D12S85, D12S351
14q32.33	D14S985, D14S292, D14S65
17q22	D17S949, D17S1868, D17S787, D17S944
21q22.3	D21S266
22q13.1	D22S280, D22S423, D22S283

Table 2 Sequences of PCR primers

Forward primers	Reverse primers	Exons
tccgattttctcatttccgtgg	ttccaagtcacgtagcttctc	1
cgttttatccattctaagcaggt	gcgattaagatggaaggcaactt	2, 3
tggagttacattcaacctcagg	agcaatgcagcccgtcttatag	4
gtagatgacaagttgatgggtg	gtggataagttatttggatctatc	5
ttaatttagcaattcatggctatg	ttaccacatcttgcattactca	6,7
ttgcattccagttgcttgaag	gtggcagaaattctaaagtactg	8
attgtctccgctgggagtttca	gctccatctcgaagaaaacc	9
ggaacagagaagttggcagca	agtcttcacaagatgactctc	10
Two additional primers:		
atgatccacgctcttagttgaa	ttccaagtcacgtagcttctc	1.7 kb 5'-upstream
ggaacagagaagttggcagca	ggtaggagaatgcaagataatg	2.7 kb 3'-untranslated

analysis. If a DNA marker showed 30% recombination, a chromosome region 5 cM on each side of the marker would not be considered the region of interest for further linkage testing; DNA markers with 10-cM intervals across the genome were planned, if needed.

Once a chromosome region of interest was identified, haplotype analysis of this region was performed in all families. Two-point LOD scores were calculated by using the MLINK option of the LINKAGE package (version 5.10) and FASTLINK (version 4.0P) under the assumption of 0.001 disease-gene frequency and 95% penetrance.

Examination of DNA sequence of prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2, COX2)

The DNA sequence of COX2 from LAP patients and normal individuals was directly examined. PCR fragments were generated, and primers for PCR were designed according to the published DNA sequence of COX2 (Table 2).

For the PCR, 50 µl 1×PCR reaction buffer containing 200 ng genomic DNA, 1 µM of each primer, and 200 nM dNTPs, and 5 U Taq polymerase were used. The PCR conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 40 s, 60°C for 45 s, and 72°C for 60 s. Finally, the PCR was extended at 72°C for 10 min. The PCR products were purified by gel extraction kit (Qiagen). The purified PCR products were then sequenced on an ABI 3100 Genetic Analyzer.

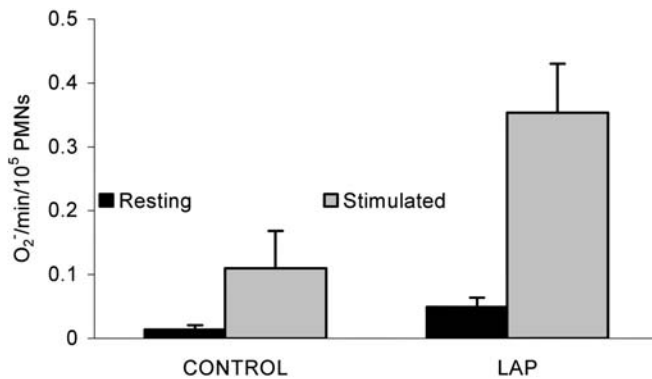


Fig. 2 Superoxide generation in LAP and age-, race-, and gender-matched healthy controls. There was a statistically significant increase of superoxide generation of both resting and fMLP (1 µM)-stimulated neutrophils from LAP patients compared with controls subjects (**P*<0.05, ANOVA)

Results

Recruitment of LAP families for genetic linkage analysis

Eight African-American families with LAP phenotype were recruited for the genetic linkage analysis.

Clinical examination and laboratory evaluation of neutrophil function

Superoxide production

Affected individuals exhibited significantly higher superoxide generation than non-affected relatives and normal controls (Fig. 2).

Chemotaxis

Neutrophil chemotactic migration in response to fMLP was dramatically reduced in LAP. Random migration was equivalent between groups (Fig. 3).

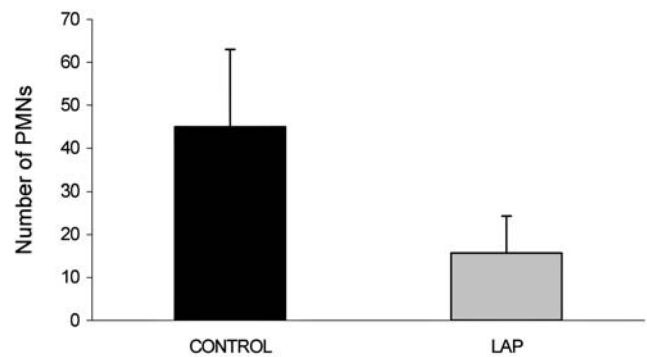


Fig. 3 Chemotactic migration of polymorphonuclear neutrophils (PMNs) from LAP and age-, race-, and gender-matched healthy controls (*n*=6). Chemotaxis was measured by subtracting random migration of neutrophils from the migration of neutrophils toward a chemotactic agent (fMLP, 10 nM). LAP neutrophils show a statistically significant reduction in chemotaxis compared with neutrophils from healthy donors (**P*<0.05, ANOVA)

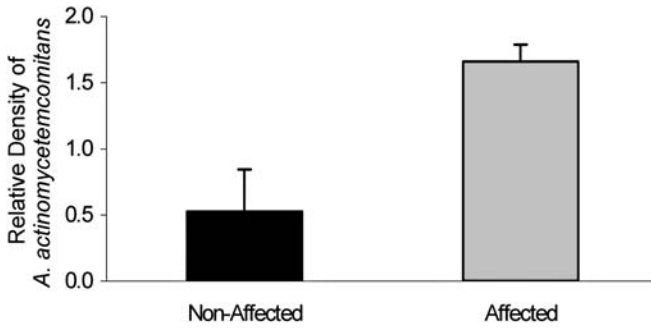


Fig. 4 Presence of *A. actinomycetemcomitans* in LAP subjects. *A. actinomycetemcomitans* is significantly higher in plaque samples obtained around the affected teeth with periodontal tissue destruction compared with non-affected sites ($P < 0.05$)

Checkerboard analysis

LAP affected individuals harbored significantly more *A. actinomycetemcomitans* (Fig. 4).

Based on clinical and laboratory evaluations, four families (A–D) were suitable for genetic linkage analysis. Two families did not meet our laboratory criteria, and the other two families were too small to be informative for genetic linkage analysis.

Genetic linkage analysis

There were more than 20 individuals in families A, B, C, and D, as required for linkage analysis. To verify further that a LOD score greater than 3 from those families was possible, a simulation study with the SLINK program was performed to obtain a maximum expected LOD scores (ELOD) of 3.75 with θ at 0.00 based on 1,000 replicates.

The initial result from our direct linkage analysis showed that one specific DNA marker, D1S238, was shared by all affected individuals but not by normal individuals in all four families. Another DNA marker, D1S413 (10 cM telomeric side of D1S238), was also shared by all of the affected individuals with the exception of one recombination in an affected individual. The chromosome region around D1S238 was chosen as the candidate region to contain *LAP*. Therefore, haplotype analysis was performed on this chromosome region (Fig. 5).

The result from the haplotype analysis showed that a number of recombinant events had occurred among sev-

Fig. 5 Haplotype analysis of four families (arrows probands). The results from the haplotype analysis shows that several recombinant events occurred within the families (in C.II-3 and D.I-1), which positioned a *LAP* locus between D1S196 and D1S533. The length of this chromosome interval is about 26 million DNA basepairs

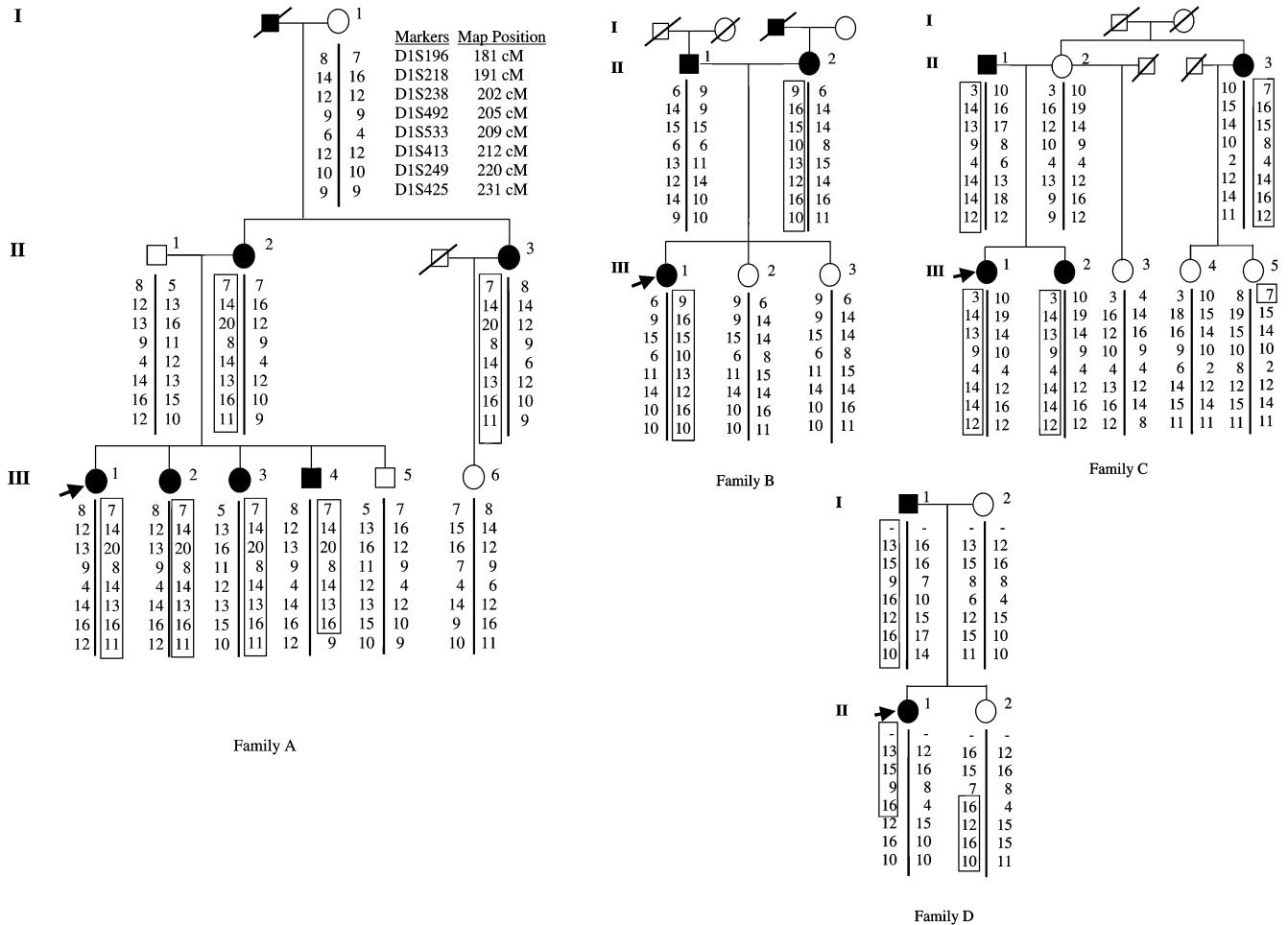


Table 3 Two-point LOD scores calculated by MLINK

Marker	Map position	LOD scores at recombination fractions (θ)						
		0.00	0.01	0.05	0.10	0.20	0.30	0.40
D1S196	181 cM	-0.05	-0.34	-0.17	-0.10	-0.02	-0.01	0.00
D1S218	191 cM	2.59	2.56	2.42	2.17	1.66	1.00	0.46
D1S238	202 cM	2.93	2.87	2.61	2.30	1.67	1.07	0.69
D1S492	205 cM	3.48	3.41	3.10	2.72	1.93	1.20	0.56
D1S533	209 cM	1.19	1.34	1.59	1.61	1.36	0.94	0.48
D1S413	212 cM	2.47	2.45	2.30	2.10	1.56	1.02	0.51
D1S249	220 cM	-0.15	0.06	0.53	0.74	0.80	0.61	0.33
D1S425	231 cM	0.28	0.46	0.82	0.95	0.90	0.65	0.33

eral individuals. The recombinant events in C.II-3 and in D.I-1 positioned the *LAP* locus between D1S196 and D1S533. The physical distance of this chromosome interval is 26,316,987 bp. To confirm the linkage result from the haplotype analysis, two-point LOD scores for each marker were calculated (Table 3) by using the MLINK option of the LINKAGE package (version 5.10) and FASTLINK (version 4.0P) under the assumption of 0.001 disease-gene frequency and 95% penetrance. The pedigrees were not sensitive to estimates of marker allele frequencies.

Examination of DNA sequence of prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2, *COX 2*)

The entire coding region and a 1.7-kb 5'-upstream and 2.7-kb 3'-untranslated region of *COX2* were sequenced. No DNA mutation of *COX2* in LAP patients was identified.

Discussion

In this paper, a *LAP* locus was assigned to chromosome 1q25 in four African-American families. The families were chosen based on clinical periodontal presentation and specific host response and microbiologic parameters. Although there is no direct genetic evidence that LAP is a heterogeneous disorder, previous clinical studies have indicated a simple Mendelian pattern of transmission in certain numbers of LAP families with the wide-range of clinical phenotype. This suggests that LAP can be caused by either different mutations in different genes (non-allelic heterogeneity) or different mutations in the same gene (allelic heterogeneity). To minimize the negative influence of non-allelic heterogeneity on our genetic linkage analysis, we performed laboratory tests to screen LAP families before genetic linkage analysis.

Although it is evident that LAP is a rare genetic disorder, few genetic linkage analyses have been performed to identify a locus associated with the disease. In 1986, Boughman et al. conducted a study involving a large five-generation kindred family including more than 70 individuals. This family had two distinct dental disorders, type III dentinogenesis imperfecta (DGI-III) and aggressive peri-

odontitis (juvenile periodontitis, JP). The pedigree showed that the two disorders did not completely co-segregate. Subsequently, genetic linkage analysis showed that JP and DGI-III mapped to two different loci on the same region of chromosome 4 within less than 7 cM. However, two main questions remained to be addressed in this linkage study. The two dental disorder loci showed a higher recombinant frequency ($5/15=0.33$) than expected (less than 0.07) if the loci were located within less than 7 cM on an autosomal chromosome, unless they were in a chromosomal interval with a very high number of "recombinant hot spots". Another issue was that the genetic linkage analysis was carried out with a protein polymorphic marker. Protein polymorphic markers for genetic linkage analysis are often not as sensitive and specific as DNA markers. Therefore, it would be more informative if the linkage data could be confirmed by using DNA polymorphic markers. Another genetic linkage study was performed with affected sib-pair (ASP) analysis and included 29 African-American families and 15 Caucasian families (Wang et al. 1996); 385 DNA polymorphic markers covering the entire human genome were used for the ASP analysis. Two chromosome loci, viz., a locus around HLA region on chromosome 6 and a locus on chromosome 9, were suggested to be the loci of interest for aggressive periodontitis. One of the advantages of the ASP approach is that genetic linkage analysis can be performed, without knowledge of the mode of transmission of a genetic disorder, by evaluation of the distribution pattern of a specific marker among ASPs. A marker presenting in ASPs with a higher frequency than expected indicates the possibility of linkage between the marker and the disease phenotype. However, it has to be assumed that this marker is identical by descent (IBD), since a marker IBD is not distinguishable from a marker identical by state in ASP analysis. In addition, non-allelic heterogeneity in human genetic disorders reduces dramatically the power of ASP analysis. Obviously, the loci of interest for LAP on chromosomes 4, 6, and 9 need to be confirmed by further genetic linkage analyses.

Our linkage study positioned a *LAP* locus between D1S196 and D1S533. The data is the first report of a chromosome interval that contains a *LAP* locus. The fact that all families in the genetic linkage study have different haplotypes suggests that the LAP phenotype in the families is probably attributable to multiple mutations in a same

gene. Based on the chromosome interval that contains the *LAP* locus, it should be possible to identify the DNA polymorphic markers through genome databases. DNA markers will ultimately be useful for clinical genetic consultations and the diagnosis of certain forms of LAP.

Initially, *COX2* was considered a primary candidate gene for LAP, because previous studies had shown that prostaglandin E2, the product of *COX2*, was over-expressed in LAP patient neutrophils (Shapira et al. 1996). Thus, the chromosome region containing *COX2* was selected as a candidate locus for genetic linkage analysis. Once this chromosome region was linked to the LAP phenotype, DNA sequences of *COX2* in LAP patients including a 1.7-kb 5'-upstream region (assumed to be the promoter region), the entire coding region, and a 2.7-kb 3'-untranslated region were examined. No mutation was detected in *COX2* in LAP patients. Although *COX2* cannot be completely ruled out as the primary defect of LAP, simple DNA alterations in *COX2*, such as a point mutation, insertion, or small deletion, do not appear to be responsible for the disease.

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