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Linkage Disequilibrium of Interleukin-1 Genetic Polymorphisms With Early-Onset Periodontitis

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Background: Genetic polymorphisms at interleukin (IL)-1α and IL-1ß were recently suggested to be associated with severity of adult periodontitis. We evaluated whether these polymorphisms might also be associated with early-onset periodontitis (EOP) in 28 African American families and 7 Caucasian American families with 2 or more affected members.

Methods: Genomic DNA from peripheral blood was amplified, followed by restriction endonuclease digestion and acrylamide gel electrophoresis to distinguish alleles of different fragment sizes. Genetic epidemiological methods suitable for family data were used that are robust to false-positive findings due to mismatching of cases and controls or mixed subpopulations of different ethnic or geographic origin. The 2 major EOP subtypes, localized juvenile periodontitis (LJP), and generalized early-onset periodontitis (G-EOP, encompassing rapidly progressive periodontitis and generalized juvenile periodontitis), were analyzed both separately and together.

Results: We obtained highly significant evidence of linkage disequilibrium for both African American and Caucasian G-EOP subjects. A similar trend was noted for LJP. The IL-1 alleles associated with high risk of EOP had been suggested previously to be correlated with low risk for severe adult periodontitis. Disequilibrium with G-EOP was equally strong for smoking and non-smoking subjects. IL-1α and IL-1ß polymorphisms were in strong disequilibrium with each other in Caucasians, but not in African Americans. Haplotype analyses evaluating both polymorphisms simultaneously indicated that the IL-1ß variant is likely to be most important for EOP risk. Sibpair linkage analyses, by contrast, provided only marginal support for a gene of very major effect on EOP risk attributable to these IL-1 polymorphisms.

Conclusions: Recent theoretical analyses indicate that our findings are most consistent with an interpretation of EOP as a complex, oligogenic disorder, with IL-1 genetic variation contributing an important but not exclusive influence on disease risk. *J Periodontol 1999;70:418-430.*

KEY WORDS

Periodontitis, juvenile/etiology; periodontitis, early-onset/etiology; polymorphism; racial stocks; interleukin-1.

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Several studies indicate that genetic differences among individuals may play an important role in risk of EOP.[1-6] The familial nature of EOP has been described by clinical investigators for many years.[7,8] Genetic segregation analysis is a method whereby the observed transmission of a disease through families is compared with predictions based on alternative genetic and non-genetic models, and the support for the best fitting model is assessed by maximum likelihood statistical tests. No genetic markers are used in these studies. Segregation analyses of families containing one or more EOP cases in a number of studies support the hypothesis that EOP is a heritable trait.[9,11] Earlier support for X-linked dominant transmission has been refuted.[10,12,13] The 2 largest segregation analysis studies suggest that an autosomal dominant gene of major effect may influence differences in risk of EOP among individuals.[9, 11] However, the limitations of the segregation analysis approach need to be recognized. It has 2 major weaknesses. First, it cannot distinguish between unmeasured alternative causes of familial aggregation of disease which might mimic genetic effects (e.g., transmission of virulent biotypes of bacteria among close relatives). Second, it has

virtually no power to detect heterogeneity and complexity in genetic etiology among different families, such as dominant major genes in one subset of families, recessive major genes in other families, and multiple genes of lesser effect in others. Since we believe that such etiological complexity may quite possibly exist for EOP, the "one size fits all" limitation of segregation analysis is of considerable concern.

The problems of segregation analysis are overcome by the gene mapping approaches of linkage and association (linkage disequilibrium) analyses.[14,15] Linkage measures co-transmission of disease and alleles of a marker locus within a family (with different marker alleles "traveling" with the disease in different families). Linkage disequilibrium means that a specific allele at the linked locus is more commonly found in individuals affected by the disease across all or most families with one or more members affected. In contrast to segregation analyses, where only the pedigree structure and the disease phenotype are considered, gene mapping methods also use marker polymorphisms. The markers are used to track the transmission of segments of chromosomes through families, searching for correlations between the transmission of a specific chromosomal region and transmission of a disease. If adequately strong evidence of such a correlation is found, the conclusion that a disease susceptibility gene must lie within the chromosomal region is virtually certain. The alternative hypothesis of transmission of shared environmental factors such as diet, hygiene, or infectious agents is ruled out, since these factors cannot influence the meiotic segregation of chromosomal regions detected by the marker loci. For EOP, only very limited gene mapping data have been reported previously. An autosomal dominant form of juvenile periodontitis cosegregating with dentinogenesis imperfecta (DGI) in the Brandywine tri-racial isolate extended family has been described and mapped to the GC locus on chromosome 4.[16] Linkage studies of other DGI families (without EOP comorbidity) conclusively map DGI to this chromosomal region.[17] However, linkage studies of a set of families with EOP only (without DGI comorbidity) excluded this chromosomal region.[18]

Several putative genetic risk factors have been suggested for EOP, most of which are based upon data implicating aberrant host immunity or inflammatory reactions as likely pathogenic mechanisms. These include HLA antigens,[2,19-22] genetic variation influencing immunoglobulin production, in particular, production of IgG_2 antibodies,[23,24] inheritance of polymorphic forms of a receptor for IgG_2 (FcγRII or CD32),[25] genes related to aberrant polymorphonuclear leukocyte chemotactic function,[26-28] and elevated or depressed cytokine and prostanoid production.[29,30] In all cases, it is hypothesized that the host response to bacterial etiologic agents is aberrant or deficient, resulting in expression of periodontitis at a young age. This hypothesis is supported by the observation that a number of defined genetic abnormalities associated with depressed phagocytic function predispose patients to periodontitis during early childhood.[31-33] None of these studies have been consistently replicated, however, and most are based on small sample sizes and provide only weak statistical support for associations. The actual genetic architecture of EOP susceptibility remains undetermined to date.

The role of cytokines in the pathogenesis of periodontitis has been the focus of much research. Interleukin-1 (IL-1), in particular, has been implicated in disease pathogenesis for a number of reasons. IL-1 is a pro-inflammatory, multifunctional cytokine, which enables ingress of inflammatory cells into sites of infection, promotes bone resorption, stimulates eicosanoid (specifically, PGE_2) release by monocytes and fibroblasts, stimulates release of matrix metalloproteinases that degrade proteins of the extracellular matrix, and participates in many aspects of the immune response.[34] IL-1 levels in general are elevated in both tissues[35,36] and gingival crevicular fluid[37-40] from diseased, inflamed periodontal tissues compared to healthier sites, and elevated levels have been shown to be associated with active disease in animal models.[41] The predominant form of IL-1 in the periodontal tissues is IL-1ß, which is produced primarily by macrophages.[42,43]

A recent report suggested that genetic polymorphisms at the interleukin 1α and 1ß genes may be associated with severity of periodontitis in adult nonsmokers.[44] In a group of 134 Northern European patients with varying degrees of periodontal destruction a relationship was observed between severity of periodontitis and polymorphisms at the IL-ß gene (called IL-1ß+3953) and the IL-1α gene (called IL-1α-889). These 2 genes are located approximately 50 Kbp apart on chromosome 2q13. Non-smokers aged 40 to 60 carrying the "2" allele (in either homozygous or heterozygous states) at both loci were observed to have nearly 19 times the risk of developing severe periodontitis compared to subjects homozygous for the "1" allele at either or both of these loci. Although it is likely that patients with EOP, particularly LJP, may have a unique bacterial etiology in comparison to adult periodontitis,[45] there are only limited data suggesting that the pathogenic mechanisms leading to periodontal attachment loss may differ substantially. Because of the previous research implicating IL-1 in adult periodontitis, evaluation of the possible role of these IL-1α and IL-1ß genetic polymorphisms in early-onset disease is warranted.

The present study was designed to capitalize on a new method of genetic epidemiology known as the transmission disequilibrium test (TDT).[46,47] This method employs a family-based experimental design to

avoid potential pitfalls due to mismatching of case and control groups or admixture of subpopulations consisting of different racial or ethnic groups. In addition, theoretical analyses indicate that this approach may be much more powerful than traditional linkage-based approaches for detecting alleles of relatively small effect such those conferring a 2-fold or 4-fold increase in disease risk.[48,49] We characterized these IL-1α and IL-1ß polymorphisms for DNA samples from our collection of EOP families, and used the TDT to evaluate these data. We considered the effect of smoking, as well as the relationship between the IL-1α and IL-1ß polymorphisms to each other, to attempt to determine which of these 2 genetic variants may be more strongly associated with the disease.

MATERIALS AND METHODS

Multiplex EOP Families

Probands for this study were referred to the Clinical Research Center for Periodontal Disease from School of Dentistry Clinics at Virginia Commonwealth University or by practicing clinicians in the Richmond, Virginia area and surrounding counties. Subsequently, family members were identified and recruited. Our study sample included 28 African American families with 107 EOP-affected subjects (94 with DNA available for study) and 99 unaffected subjects (85 with DNA available), and 7 Caucasian families with 34 EOP-affected subjects (31 with DNA available) and 45 unaffected subjects (36 with DNA available). These families were chosen for participation based upon the finding of multiple cases of EOP within the first, second, or third degree relatives of the proband (multiplex families). Tentative family pedigrees were established based upon information from family members; such pedigrees were modified if necessary following verification following DNA analyses (see below). Each subject received a complete periodontal evaluation which included assessments of probing depth, attachment loss, plaque index,[50] gingival index,[51] bleeding upon probing,[52] and suppuration.[53] Measurements were performed at 4 sites per tooth (mesiobuccal, midbuccal, distobuccal, and midpalatal). At the time of the examination, a blood sample was taken from some of the subjects and processed for DNA and establishment of EBV-immortalized cell lines.

EOP probands were initially diagnosed with either a localized (LJP) or generalized (G-EOP) form of disease as described below. All available family members were clinically assessed and assigned a periodontal diagnosis of LJP, G-EOP, adult periodontitis (AP), or periodontal health (HP). Family members under the age of 12 were not included in this study unless they exhibited a form of EOP. The diagnostic criteria for non-proband family members were of necessity slightly different than for probands in order to both accurately identify EOP probands (thus applying very stringent diagnostic criteria) and to subsequently allow diagnosis of all other individuals within that family as accurately as possible. This strategy of using more stringent criteria for the proband is motivated, in part, by our desire to limit our recruitment to families where at least one member had unequivocal symptoms of EOP. Thus, we set slightly higher thresholds for our EOP probands. Furthermore, studies of other genetically complex diseases have found that in some cases by selecting more severely affected subjects, a sample of families may be enriched for genetically-based etiology. Once the family is selected by identification of an eligible EOP proband, however, we believe that application of more standard diagnostic criteria to the other family members is the optimal way to proceed. In cases where family members with periodontitis were beyond the arbitrary age limits imposed for the EOP diagnostic categories, diagnoses of EOP were based upon historical information as well as radiographic evidence if available. In such cases, the clinician performing the examination utilized available data to assign a clinical diagnosis of adult periodontitis with a "% likelihood" that the patient was previously affected with EOP. For this analysis, only cases in which the clinician assigned >50% likelihood of previous EOP (which required compelling historical or radiographic data) were included in this category. For example, this included access to old radiographs (when the subject was younger than age 35) demonstrating 4 mm or greater bone loss on multiple teeth in patterns consistent with the EOP definitions noted below; or clinical evidence of severe attachment loss with a definitive history of periodontitis diagnosed (in the past, as reported by the subject) by a dentist. AP and edentulous subjects assessed as having a >0% but ≤50% likelihood of previous EOP were conservatively classified as "diagnosis unknown" for all statistical analyses. Patients were categorized according to the following criteria:

Healthy periodontium (HP). Subjects of any age with no evidence of attachment loss (AL) at more than one site, or pockets greater than 3 mm; i.e., who have no detectable periodontitis.

Adult periodontitis (AP). Subjects of age >25 years; with AL 2 mm or greater in any extent or severity pattern on more than one tooth. In subjects less than 35 years old, AL must have appeared to have been consistent with plaque levels, other local contributing etiologic factors, and age, with less severe attachment loss than in LJP or G-EOP, or with an indication of adult onset. Further, the distribution (extent) of the disease was such as to not suggest localization to first molars and incisors, nor related to teeth affected by trauma, endodontic disorders, or other determinable local etiology other than periodontitis.

Localized juvenile periodontitis (LJP). For probands, subjects with disease of onset from puberty

up to age 30 with at least 4 mm AL on at least 2 permanent first molars and incisors (at least one molar must have been affected) and no more than 2 teeth which were not first molars or incisors that were affected by 5 mm AL or more. A subject with generalized AL of 4 mm would not qualify as an LJP proband and would be excluded from our study (but no such cases have been encountered to date). For family members of probands, severity of AL may have been less than 4 mm but with a minimum of 2 mm at affected molars and incisors.

Generalized early-onset (rapidly progressive, generalized juvenile) periodontitis (G-EOP). For probands, subjects with disease onset up to age 35 with at least 8 teeth affected (5 mm AL or more), at least 3 of which were not first molars and incisors. For family members of probands, attachment loss at multiple sites must have been 3 or 4 mm; however, AL must have indicated that the pattern is more excessive than would be likely for adult periodontitis.

Our study sample included one family with 5 generations, 5 families with 4 generations, 16 families with 3 generations, and 13 families with only 2 generations. Our largest full sibship contained 8 EOP-affected offspring (with DNA available for typing), and the rest of the data set included one sibship with 5 affected, 3 sibships with 4 affected, 6 sibships with 3 affected, 21 sibships with only a pair of affected siblings, and 32 sibships with only a single affected offspring. For these EOP-affected subjects, a total of 43 had both parents genotyped, 51 with one parent typed and 23 with neither parent available for genotyping. Because of the low heterozygosity levels of the IL-1 markers studied, only a limited portion of these subjects were informative for transmission disequilibrium tests, as reflected in the results presented below. Approximately one quarter of the sibships contained one or more affected or unaffected half siblings in addition to these affected full sibs, and extensive statistical analyses with highly polymorphic loci (data not shown) have confirmed the validity of the pedigree structures used in these analyses.

Laboratory Assays

DNA was extracted from peripheral blood following hypertonic sucrose cell lysis using proteinase K digestion and alcohol precipitation methods.[54] In some cases, immortalized cell lines were established by EBV transformation of subjects' lymphocytes, and these were used for DNA extractions. DNAs were amplified via the polymerase chain reaction in 2 separate reactions using oligonucleotide primers designed to amplify either portions of the IL-1α gene surrounding the IL-1α-889 polymorphic site[55] or the IL-1β gene surrounding the IL-1ß+3953 polymorphic site.[44] PCR conditions consisted of 60 ng of template genomic DNA, 200 µ*M* of dNTPs, 2 m*M* $MgCl_2$, 1X concentration of the reaction buffer supplied by the manufacturer with 0.3 U of Amplitaq‡ thermostable DNA polymerase and 3 ρM of each primer in a volume of 15 µL. Amplifications were performed for 10 cycles of 94°C denaturing for 15 seconds, 55°C annealing for 15 seconds, and extension at 72°C for 30 seconds; 20 additional cycles with the same parameters except denaturing at 89°C (to retain polymerase activity); followed by a final extension period at 72°C for 10 minutes. PCR products were diluted 1:20 with water and 2 µL of each diluted product were incubated overnight in a total volume of 10 µL with the buffers supplied by the enzyme manufacturers at 37°C with 10 U of *Nco* I for the IL-1α-889 fragment and at 65°C with 20 U of *Taq* I for IL-1ß+3953. Enzymes were supplied by New England Biolabs (Beverly, Massachusetts). One of the oligonucleotide primers for each PCR reaction had been synthesized using a FAM phosphoramadite, so the PCR products could be analyzed using fluorescence-based detection. The restriction endonuclease digestion products were sized by denaturing PAGE on automated DNA analyzers§ with appropriate software as described by Ziegle et al.[56] For the IL-1α-889 polymorphism the digestion product of 83 bp was designated allele 1, while the 99 bp fragment (lacking the polymorphic restriction site) was designated allele 2. For the IL-1ß+3953 polymorphism, the digestion product of 85 bp was designated allele 1, while the 182 bp fragment (lacking the polymorphic restriction site) was designated allele 2. These allele designations are consistent with those used previously by Kornman et al.[44] in their study of adult periodontitis.

To determine which of our subjects were smokers at the time of examination, we analyzed serum levels of cotinine, a stable primary metabolite of nicotine. Serum cotinine was measured by double antibody radioimmunoassay.‖ The distribution of serum levels of cotinine in the subject population was bimodal, with a large group of subjects with levels <50 ng/ml. These distributions were used to empirically establish conservative cutoff values for non-smokers as ≤25 ng/ml and ≥75ng/ml for smokers. Individuals with intermediate levels of cotinine were classified as smoking status unknown and excluded from analyses involving smoking.

Statistical Analyses

We evaluated African American families and Caucasian American families both separately and combined, and also evaluated LJP and G-EOP subtypes of EOP both separately and combined for all analyses. Linkage disequilibrium between EOP and IL-1 polymorphisms was

‡ Perkin-Elmer, Norwalk, CT.
§ Applied Biosystems Model 373, Perkin-Elmer.
‖ Double Antibody Nicotine Metabolite, Diagnostic Products Corporation, Los Angeles, CA.

assessed using the transmission disequilibrium test (TDT).[46,47] The TDT counts numbers of transmissions of allele 1 versus allele 2 from heterozygous parents to affected offspring. Under the null hypothesis that the polymorphic marker has no effect on disease risk, the two alleles are expected to be transmitted with equal frequency. Deviation from the 1:1 expected ratio of transmission indicates that the marker is in linkage disequilibrium with the disease. This means that the marker polymorphism itself, or another variable DNA site in very close proximity to the marker influences risk of the disease. The computer programs SIB-PAIR Version 0.92[57] URL: (http://www.qimr.edu.au/davidd/sibpair.html), TRANSMIT (D. Clayton, unpublished), and TDTEX of S.A.G.E. Release 3.1 were used to perform these analyses. Most tests were performed using SIB-PAIR, except as otherwise indicated, because this program uses permutation methods to robustly estimate *P* values for small sample sizes. It is possible that biased transmission to affected offspring might be unrelated to disease risk, due either to an artifact of the molecular assay (such as partial digestion by restriction enzymes) or meiotic drive (where, for example, an embryonic lethal gene in the vicinity of the marker might cause one marker allele to be more common in all viable offspring). We addressed these confounding alternatives by also performing the TDT for unaffected offspring, who would be expected to exhibit the same levels of biased transmission as affected offspring if the bias was due to these alternative, non-disease related mechanisms.

IL-1 genotype frequencies were compared in the affected versus the unaffected members of our multiplex families using the SAS System for Windows Release 6.11 (SAS Institute, Inc.). Other miscellaneous data manipulations and statistical tests such as Fisher's exact test used for these analyses were also performed using this program. The SIB-PAIR[57] program was used to assess the statistical significance of differences in marker alleles between affected and unaffected subjects, and between the racial groups using our entire collection of family members. It is necessary to account for the correlations between subjects due to familial relationships when performing such tests. SIB-PAIR does this by an empirical permutation approach known as gene dropping. Founding members and spouses marrying into pedigrees are randomly assigned genotypes with probabilities equal to the overall frequencies in the entire set of families under study. For these computer simulation analyses we used allele frequencies estimated separately from the members of the families for each racial group (except for analyses combining the races, where we used estimates based on families of both races). Our allele frequency estimates are consistent with those reported elsewhere.[44] Furthermore, since the key comparison in these tests of marker association is between affected and unaffected members of the families, slight differences in assumed allele frequencies are not expected to bias the results. The algorithm then uses codominant Mendelian transmission ratios to assign genotypes to offspring of the founders, ignoring subjects' disease status (or subjects' race for comparison of racial groups). Only individuals who actually have DNA and a marker genotype in the real data set are then assigned genotypes in the simulated set. For each simulated data set a contingency Pearson (χ^2 statistic is calculated and recorded. This same statistic is also calculated for the real data set. The process is repeated a sufficient number of times for the entire set of pedigrees to obtain a precise estimate of the distribution of the statistic under the null hypothesis for the simulated values. The empirical *P* value is then determined by counting the percentage of times the simulations exceed the actual observed statistic for the marker.

Since the IL-1α and IL-1ß markers are located so close together on the chromosome it is possible that the alleles at their respective marker polymorphisms are correlated. This means, for example, if a chromosome contains a 1 allele at the IL-1α marker, it may be highly likely to contain a 1 allele at the IL-1ß marker. This is important information that is necessary to distinguish whether IL-1α, IL-1ß or both polymorphisms are responsible for influencing EOP risk. First, we confirmed the expectation that these 2 IL-1 polymorphic markers are located very close to each other by performing logarithm of the odds (LOD) score linkage analysis using the computer program MLINK.[58] Next, we used maximum likelihood methods implemented in the program 3LOCUS.PAS[59] to evaluate linkage disequilibrium between the 2 marker polymorphisms in the African American and Caucasian American subjects. These analyses were performed separately for affected and unaffected subjects to avoid any confounding between disequilibrium with the disease and disequilibrium between the 2 markers. This method requires collections of unrelated individuals, so we first identified all members of our families who were biologically unrelated (i.e., founders and spouses who married into the family).

A simple SAS program and manual analyses were used to identify haplotypes of the IL-1α and IL-1ß markers. A haplotype refers to a combination of the alleles at the 2 markers contained on a single chromosome. A chromosome might have a 1 allele at the IL-1α marker and a 1 allele at the adjacent IL-1ß marker position, or any of the other 3 possible combinations (1 and 2, 2 and 1, or 2 and 2, respectively). We first used SAS to convert all unambiguous 2 locus genotypes to their respective haplotypes. Then we manually inspected the pedigree structures to infer the phase, where possible, of individuals heterozygous at both

marker loci. We then repeated our TDT analyses using the 2 locus combined haplotypes to assess the relative importance of the IL-1α versus the IL-1ß markers for EOP disease risk.

To assess evidence of linkage to EOP for each of the IL-1 markers separately, as well as for their combined haplotypes, we conducted 2 sibpair tests.[15,59] Affected sibpair tests and concordant/discordant sibpair regression tests were performed using the computer programs SIB-PAIR Version 0.92[57] and SIBPAL of S.A.G.E. Release 3.1. The affected sibpair test focuses on affected siblings only. This is done because of the concern that some disease gene carriers may not express the disease (incomplete penetrance) which means that unaffected individuals may or may not be gene carriers. By contrast, affected siblings are assumed to both be disease gene carriers even in the presence of incomplete penetrance, so a linkage test limited to affected sibling pairs only may be more powerful. The affected sibpair test evaluates whether, for the entire set of families under study, pairs of affected siblings share more than 50% of marker alleles identical by descent (IBD; i.e., commonly inherited from the same parent). Under the null hypothesis that the marker has no influence on disease risk, siblings will share 50% of their alleles IBD. However, if a disease gene that the affected pairs of siblings share is located near a polymorphic marker locus, this increases allele sharing above the null hypothesis 50% value. The concordant/discordant sibpair regression test is an extension of this strategy to include unaffected siblings. It may be especially applicable for diseases with some non-genetic causes (phenocopies) and/or where several different, unlinked disease-predisposing genes cause the same clinical phenotype. In other words, sometimes one affected sibling may have the disease due to a particular gene while the other affected sibling may have gotten the disease via non-genetic factors or due to some other, unlinked gene. Under these circumstances, pairs of affected siblings may be no more reliable for inferring disease gene carrier status than pairs of unaffected siblings, so investigators would be wise to utilize information from all kinds of siblings. The regression test evaluates whether allele sharing is above 50% IBD at a putative disease-linked marker for pairs of siblings which are the same with respect to their disease status (i.e., pairs of affected siblings and pairs of unaffected siblings). These are called concordant pairs and their level of IBD allele sharing is contrasted to the allele sharing for pairs of siblings where one is affected and the other unaffected (discordant pairs). Discordant sibling pairs are expected to share less than 50% of their alleles IBD at a marker locus close to a disease gene. The significance test evaluates whether the slope of mean IBD sharing for concordant versus discordant sibling pairs is negative.

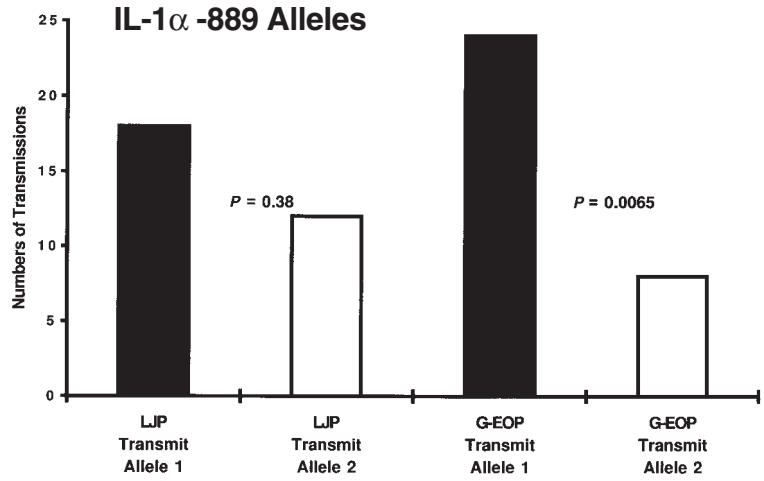

Figure 1.
Transmission disequilibrium test results showing that allele 1 of the IL-1α -889 marker is transmitted significantly more often than allele 2 to G-EOP affected individuals.

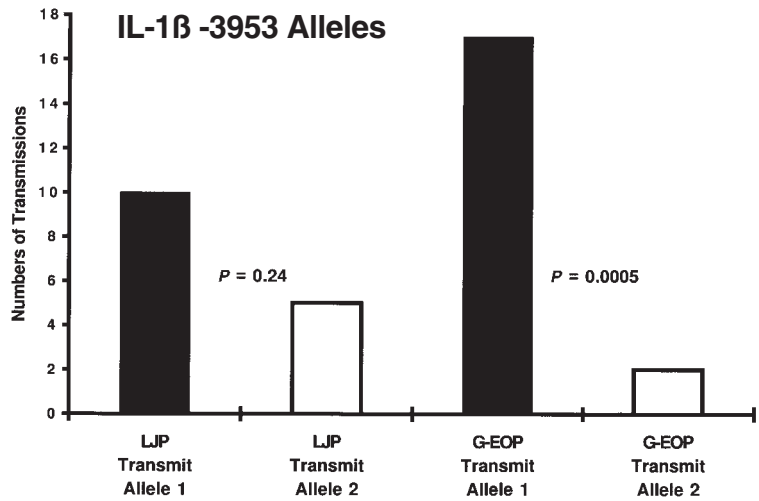

Figure 2.
Transmission disequilibrium test results showing that allele 1 of the IL-1ß+3953 marker is transmitted significantly more often than allele 2 to G-EOP affected individuals.

RESULTS

Allele 1 of the IL-1α-889 marker was transmitted significantly more often ($P = 0.0065$) than allele 2 to G-EOP affected individuals when we used the SIB-PAIR program to empirically estimate P values (Fig. 1). A similar trend was also found for LJP subjects, but was not statistically significant. Evidence of disequilibrium for allele 1 of the IL-1ß+3953 marker was even stronger for the G-EOP cases (Fig. 2; $P = 0.0005$), with 17 transmissions of the allele 1 and only 2 transmissions of allele 2 (versus a null hypothesis expectation of a 1:1

Table 1.

Transmission Disequilibrium by Smoking Status

Diagnosis*	Smoking Status†	IL-1α -889		IL-1ß+3953	
		T 1‡	T 2	T 1	T 2
LJP	N	9	5	8	0
	Y	3	3	1	1
	Y+N+U	18	12	10	5
G-EOP	N	11	4	9	1
	Y	7	2	5	0
	Y+N+U	24	8	17	2

* LJP, juvenile periodontitis = localized early-onset periodontitis; G-EOP, rapidly progressive, generalized juvenile periodontitis.
† Non-smoker (N) defined as ≤ 25 ng/ml serum cotinine; smoker (Y) defined as ≥ 75 ng/ml serum cotinine; individuals with intermediate cotinine levels classified as unknown (U) smoking status.
‡ Numbers of transmissions from heterozygous parents of the 1 (T1) or the 2 (T2) allele to affected offspring.

ratio of transmissions of each allele). Allele 1 at the IL-1ß marker was also transmitted more often than allele 2 for LJP cases but this asymmetry was not statistically significant. These findings were confirmed by re-analyses using 2 other computer programs implementing similar but not identical algorithms designed to assess transmission disequilibrium. The TDTEX program is highly conservative in that it excludes all cases where genotype information is available for only one parent. For our data, this yields a ratio of 12 transmissions of allele 1 and 1 transmission of allele 2 at the IL-1ß+3953 marker for G-EOP cases ($P = 0.003$) and weaker support for transmission disequilibrium at the IL-1α-889 polymorphism ($P = 0.024$). A third algorithm using maximum likelihood methods to estimate the probability distribution of genotypes for missing parents was implemented using the program TRANSMIT. This analysis provided strong rejection of the null hypothesis of transmission equilibrium for G-EOP cases for the IL-1ß+3953 polymorphism ($\chi^2 = 12.3$, 1 df, $P < 0.001$), with borderline statistical significance for the IL-1α-889 maker ($\chi^2 = 4.7$, 1 df, $P < 0.05$) and no evidence of disequilibrium for LJP cases. To be sure that the biased transmission observed is related to periodontal disease and not an artifact of the molecular assay or due to meiotic drive associated with this portion of chromosome 2, we evaluated transmission disequilibrium in unaffected offspring. We found no evidence whatsoever of biased transmission for any of the tests focused on unaffected offspring. For example, for the IL-1ß+3953 marker we observed 13 transmissions of allele 1 and 10 transmissions of allele 2 ($P = 0.51$) using the SIB-PAIR program, 8 transmissions of allele 1 and 10 transmissions of allele 2 using the TDTEX program ($P > 0.05$), and a $\chi^2 = 0.02$, 1 df, $P > 0.05$ using the TRANSMIT program.

Disequilibrium between G-EOP and the IL-1ß+3953 polymorphism was of approximately equal magnitude in smokers and non-smokers (Table 1). For non-smoking LJP cases, the difference between the 8 transmissions of allele 1 versus 0 transmissions of allele 2 is statistically significant ($P = 0.0085$).

Since individuals exist in the population as genotypes and not as individual alleles, we also performed a disequilibrium analysis at the genotype level. For presentation purposes, we focus on the (1,1) homozygous genotype because this is by far the most common (frequencies reported below). Most of the other individuals are of the (1,2) genotype, while the (2,2) homozygote is quite rare (< 3%). We extended the allelic TDT to the genotype level by evaluating how often the (1,1) IL-1ß+3953 genotype is transmitted (i.e., the 1 allele is transmitted by both parents) versus transmission of alternative genotypes (i.e., transmission of a 2 allele by either or both parents). Under the null hypothesis, the frequency of transmission of this (1,1) genotype should be transmitted equally often to EOP affected versus EOP unaffected subjects. In fact, we found that the (1,1) IL-1ß+3953 genotype is transmitted to G-EOP affected subjects 92% of the time versus a frequency of transmission of only 40% to unaffected subjects (Fig. 3). This contrast is statistically significant (Fisher's exact test, 2-tailed, $P = 0.014$). Because (1,1) homozygotes differ from (1,2) heterozygotes in transmission frequency, we can conclude that the 1 allele is not dominant over the 2 allele. However, since (2,2) homozygotes occur so rarely, we cannot determine whether this genotype differs in EOP risk compared to the (1,2) heterozygote.

The transmission disequilibrium for G-EOP cases was of approximately equal magnitude in Caucasian and African American subjects. At the IL-1ß+3953 polymorphism we observed transmissions of allele 1 and allele 2 of 7 and 1, respectively, for Caucasian subjects and 10 and 1 for African American subjects. The (1,1) genotype was more frequent (71%) in African American family members (including both affected and unaffected subjects) compared to Caucasians (54%). Genotype frequencies for G-EOP affected and unaffected subjects from our collection of families are presented separately for the 2 racial groups in Figure 4. The higher frequency of the G-EOP associated allele 1 in African American subjects is consistent with the higher prevalence of EOP in this population. However, statistical analyses of these data using the SIB-PAIR program to account for family relationships indicated that the marker allele frequencies were not significantly different between the races. This was true for comparisons of the racial groups done separately for both unaffected subjects and G-EOP subjects ($P = 0.34$ and

0.32, respectively). We did obtain evidence of marginal statistical significance (P = 0.057) of a higher frequency of allele 1 of this marker in the G-EOP cases versus in unaffected subjects (African American and Caucasian subjects pooled).

We noted that the (1,1) IL-1ß+3953 genotype frequency difference between G-EOP affected and unaffected subjects (combining subjects of both races) is about 16.6%. This is very nearly equal in magnitude to the difference of 17.6% we find between G-EOP versus unaffected family members in the frequencies of the 2-locus combination IL-1α and IL-1ß markers calculated according to the formula specified by Kornman et al.[44] The IL-1α polymorphism does not appreciably increase the frequency contrast between affected and unaffected subjects beyond that accounted for by the IL-1ß polymorphism. This is consistent with our TDT findings suggesting that variation at the IL-1α marker may be less closely linked with EOP risk than genetic variation at the IL-1ß+3953 site.

Our next tests were aimed at further establishing whether genetic variation at the IL-1α gene or the IL-ß gene is the actual source of the association with EOP disease risk. Since these genes are known to reside ca. 50 kbp apart on the chromosome, it is quite possible that the alleles at the 2 markers may be closely correlated with each other (i.e., in disequilibrium). If the correlation was close to 1.0, it would be impossible to distinguish which marker was more related to disease risk, since they would each give precisely the same evidence of association. As presented above, we obtained different TDT results for the 2 markers, so we know that they are not in complete disequilibrium, but it remains possible that they may be partially correlated. First, we performed LOD score linkage analysis between the 2 markers (assuming each is a fully penetrant codominant system). We obtained a very highly significant LOD score of 7.84 maximizing at a recombination fraction of 0.0. In addition to confirming the 2 markers very close linkage, this finding validates our marker typing and pedigree structures. Typing errors or pedigree structure mis-specifications will, on average, tend to create the appearance of genetic recombinations and thus to drive the maximum recombinant fraction away from the value of 0.0 that we observed in our data. This assurance that our data are of high quality allowed us to proceed with confidence to our next analysis, assessing whether the two marker polymorphisms are in linkage disequilibrium.

We used maximum likelihood methods to estimate the extent of linkage disequilibrium between the two polymorphisms. We found highly significant evidence of disequilibrium between the IL-1α and IL-1ß polymorphic sites in the Caucasian population (permutation based P = 0.0013). The haplotypes with 1 alleles at both markers or with 2 alleles at both markers occurred about 60% and 28% of the time, respectively, elevated above their expected frequencies under linkage equilibrium of 44% and 11%. Linkage disequilibrium is not complete in the Caucasian population since we estimated that the mixed haplotypes of 1 and 2 alleles at the 2 markers occur about 11% of the time. By contrast, no significant evidence of disequilibrium was observed in the African American population, although there was a small excess of 1/1 and 2/2 haplotypes.

Since disequilibrium between IL-1α-889 and IL-1ß+3953 is incomplete, we sought to distinguish which of the 2 polymorphisms is more closely correlated with EOP. To do this, we converted the genotypes at the 2 IL-1 loci into 2-locus haplotypes, and repeated the TDT

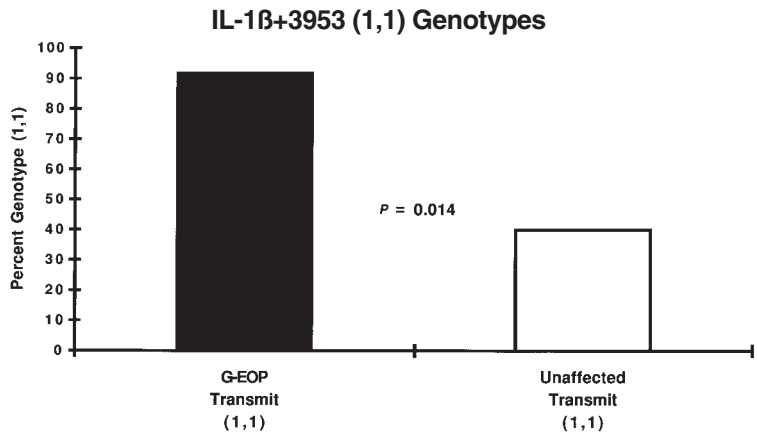

Figure 3.
Transmission disequilibrium test results showing that the (1,1) IL-1ß+3953 genotype is transmitted to G-EOP affected subjects 92% of the time which is significantly greater than the frequency of transmission of 40% to unaffected subjects.

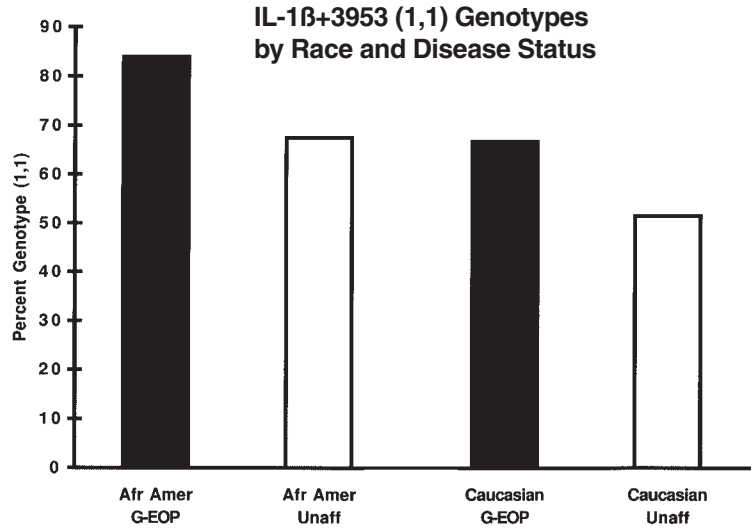

Figure 4.
Frequencies of the (1,1) IL-1ß+3953 genotype by race and G-EOP disease status.

Table 2.

Haplotype Analyses of G-EOP Subjects

Informative Marker*	T 1†	T 2
IL-1α -889 only	9	7
IL-1ß+3953 only	7	1
Both α-889 & (+3953	8	1

* One or both parents of affected offspring is (are) heterozygous for the marker polymorphism and transmission to the offspring can be unambiguously inferred.
† Numbers of transmissions from heterozygous parents of the 1 or the 2 allele to affected offspring. Where both markers were informative, only the 1-1/2-2 parental haplotype was present in our data, so either both markers' 1 alleles or both 2 alleles were transmitted.

Table 3.

Linkage Analysis of G-EOP with IL-1 Haplotypes

Sibling Pair Type*	N Pairs	% IBD†	*P* Value
Affected/affected	44	50.4	0.457
Unaffected/unaffected	29	60.3	0.008
Affected/unaffected	43	47.2	0.221
Regression analysis‡	N/A	N/A	0.061

* Pairs of full siblings where either both are affected (concordant), both unaffected (concordant) or one is affected and the other unaffected (discordant).
† Percentage of parental alleles shared by the sibling pairs of each type that have been inherited identical by descent.
‡ Test of whether the mean % IBD for the concordant sibling pairs is higher than for the discordant pairs.

analyses using the haplotypes instead of alleles at a single locus. As shown in Table 2, these analyses indicate that transmission disequilibrium is primarily associated with the IL-1ß+3953 genetic polymorphism. When a parent varies only at the IL-1α-889 site, transmission of allele 1 versus allele 2 is approximately equal (9 versus 7 transmissions, respectively). In contrast, when a parent is heterozygous at the IL-1ß+3953 site only, allele 1 is transmitted 7 times and allele 2 only once. The contrast of the 9:7 transmission ratio for IL-1α-889 only versus the 15:2 ratio for IL-1ß with or without IL-1α heterozygous is of borderline statistical significance (Fisher's exact test, 2-tailed, $P = 0.057$). Again, it appears that the IL-1ß+3953 variation may be more important in influencing risk of this disease.

Results of our affected sibpair and regression nonparametric linkage analyses using the IL-1α and IL-1ß combined haplotypes are presented in Table 3. Similar results were found for analyses of each marker separately (data not shown). We found only marginally significant evidence of a single major gene for G-EOP risk in this chromosomal region, with elevation of allele sharing only present in pairs of unaffected siblings ($P = 0.008$) and a nearly statistically significant regression statistic ($P = 0.061$).

DISCUSSION

Our finding of an association between genetic variation at a polymorphic site in the IL-1ß gene and risk of G-EOP (and possibly also LJP) for the first time definitively demonstrates that an important genetic component underlies the etiology of this disease. For many years, recognition that EOP "runs in families" and formal segregation analyses have suggested the possible existence of a gene or genes of major effect. These observations, however, could not rule out the possibility that some component of the family environment, such as diet, oral hygiene, or transmission of virulent biotypes of bacteria might explain aggregation of the disease in family members. Our use of the transmission disequilibrium test strategy rules out these non-genetic alternatives. The TDT approach for family-based experimental designs also eliminates false positive associations which might occur in case-control designs due to imperfect matching or due to the presence of racial or ethnic subgroups within the population.

Our report of IL-1ß disequilibrium with EOP differs in several major ways from the finding reported by Kornman et al.[44] regarding severity of adult periodontitis. Most importantly, we found EOP strongly associated with allele 1 at this locus, while they reported severity of adult periodontitis was associated with allele 2. Thus, for diagnostic or prognostic purposes, our data indicate a completely opposite conclusion regarding the identification of individuals at increased risk of disease. Second, while the finding of Kornman and colleagues[44] was limited to non-smokers, our association with EOP appears to apply to both smoking and non-smoking subjects. Third, our data indicate an effect in both Caucasian and African American subjects, while the previous study's conclusions were limited to Caucasians of Northern European heritage. Fourth, our data indicate that the IL-1ß polymorphism is more important than the IL-1α variant, while the previous study suggested both markers were associated with adult disease severity. It remains possible that either our finding for EOP or the previous IL-1 association reported for adult periodontitis may be a false positive (i.e., not actually valid in the whole population but a chance deviation found in the samples studied). This could explain why the 2 results differ in which allele is associated with periodontal disease. Alternatively, both findings might be valid. A genetic predisposition to high IL-1 levels (influenced by the IL-1 genetic polymorphisms we have studied) may predispose to periodontitis at one age but be protective for a different age group. Much additional data will be necessary to determine which of these alternative explanations is actually true.

We applied 3 slightly different TDT tests to the analyses of our data using 3 different computer programs.

These programs differ in how they handle families with only one parent available for genotyping. This can be important for some (rare) circumstance where a very common allele might exhibit false positive evidence of transmission disequilibrium.[61] We first ran SIB-PAIR using an option which ignores this possibility and fully utilized all available data for the families, but consequently increased the chance of false positives. TDTEX deletes all families with a missing parent, and, therefore, is conservative but suffers a considerable loss of power for data sets such as ours where frequently only one parent is available for genotyping. TRANSMIT uses maximum likelihood methods to estimate the probability distribution of the missing parental genotypes, thus potentially eliminating false positives while retaining power. This latter method has the disadvantage of requiring that we assume reliability of marker allele frequency estimates derived from the collection of families under study. Since our finding has been confirmed by all 3 methods, however, we believe it is highly reliable. In the course of these analyses, we have performed multiple statistical tests. We tested for disequilibrium for both LJP and G-EOP, in African American and Caucasian subjects and in the combined data set for both races, for IL-1α and IL-1ß markers and their haplotypes, and (for some of the tests) using the 3 alternative methods of accounting for missing parents just noted above. Clearly, some adjustment for these multiple comparisons is warranted. However, many of the tests are very highly correlated. For example, the 3 TDT methods that differ in the way they account for missing parents produce very similar results, the polymorphisms at IL-1α and IL-1ß are themselves partially correlated, and the data set pooling results for both racial groups is highly correlated with the outcomes found in each race analyzed separately. Consequently, a simple Bonferroni adjustment would be inappropriate since it would produce *P* values that were biased in a very overly conservative direction. In lieu of making such adjustments, we simply note that our most significant finding of disequilibrium between the IL-1ß marker and G-EOP (with nominal $P = 0.0005$) is robust to a Bonferroni adjustment equivalent to 100 independent statistical tests, far more than actually performed in this study.

We emphasize the value of additional data from family studies and/or case control designs to clarify the nature of the association we reported here between EOP and variation at IL-1 genes and to conclusively identify the specific DNA variation associated with EOP disease risk. For example, an association of these IL-1 variants with LJP is strongly suggested but not conclusively demonstrated by our data. It will also be of major interest to revisit the wealth of information about oral bacteria, responses to different preventative or restorative therapies, and the previously reported differences in IgG_2 levels in LJP versus G-EOP smokers versus non-smokers in the context of these IL-1 genotypes. Another key goal will be to determine whether the IL-1ß+3953 polymorphism or another polymorphic site nearby in strong disequilibrium with this site underlies the observed EOP disease risk association. The IL-1ß+3953 polymorphism lies within an exon in the coding region of the gene, but it does not result in an amino acid substitution (i.e., a missense substitution). However, the variation might influence mRNA splicing, nuclear RNA stability, or (conceivably) levels of mRNA expression. The IL-1α-889 polymorphism is within the regulatory region of the gene. It may also result in differences in expression. Alternatively, either or both of these polymorphic sites may be in strong linkage disequilibrium with another polymorphic site either within the coding or regulatory regions of these genes. Studies to address these questions may potentially include large scale DNA sequence analyses of EOP affected and unaffected subjects, as well as a wide range of in vitro and in vivo experimentation.

If the TDT is applied using only simplex families (one affected offspring per family) a significant transmission bias can only be caused by linkage disequilibrium. However, a positive TDT finding using multiplex families such as ours could potentially be caused by linkage only, without any linkage disequilibrium.[46,62] This means that there could be a disease gene in the vicinity of the marker, but that no one marker allele is associated with the disease in the population. Thus, in one family the disease may "travel" with allele 1, while in another family with allele 2. The artifactual appearance of allelic association could conceivably be attributable to the analysis of multiple affected relatives in the same (very large) families where all relatives happened to be linked to the same disease allele. Such a finding of linkage would still be of great value in that it maps a disease gene to a chromosomal region. However, the size of the candidate region where the gene might be located under a finding of linkage could potentially be much larger (ca. 20 to 30 Mbp) than if the finding is attributable to linkage disequilibrium (<< 1 Mbp for outbred populations). Whether our TDT finding is attributable to linkage disequilibrium or to linkage without disequilibrium, therefore, has very important implications for the next steps in gene mapping, the identification of the specific gene and mutation(s) actually biologically involved in mediating effects on disease risk.

With our moderately large collection of multiplex families, we expect to have high statistical power to detect a gene of very major effect on EOP risk if present in most of our families when applying linkage analysis approaches. However, the only significant evidence we found of linkage was based on pairs of unaffected siblings (Table 3). When working with complex diseases with reduced penetrance, one usually places

greater emphasis on the results for the affected pairs of siblings versus the unaffected sibling pairs. In this study, due to the disease association, the 29 unaffected/unaffected pairs more often share the 2 allele, since the disease is associated with the 1 allele. Because the 2 allele is the rarer allele it may be easier to discern whether the sharing is identical by descent or not. By contrast, the affected/affected pairs share the 1 allele, which is so common that it is very difficult to discern identity by descent sharing status, and so power is quite low to detect linkage in these 44 sibling pairs. Nevertheless, since we found only weak or no evidence of linkage in the sib pair linkage test, we conclude that our finding is most likely attributable to linkage disequilibrium. It is not likely to be an artifact of linkage without disequilibrium and our use of multiplex families.[62] If linkage disequilibrium is present, this means that the actual DNA variation causing increased risk of EOP is either the IL-1ß+3953 site or another site located very close nearby.

Our data are just what we expect to find for a disease susceptibility gene of moderate effect, a gene that increases risk in the range of 4-fold up to about 20-fold. Risch and Merikangas[48,49] have used mathematical modeling to demonstrate that such genes of moderate effect are unlikely to be detected by linkage analysis of samples of families of realistic size, but can often be found by transmission disequilibrium tests. Our evidence indicates that the IL-1ß genetic variant does not act as a single major gene that accounts for all or nearly all of the difference among individuals in EOP risk. Instead, our finding of linkage disequilibrium without evidence of linkage is most consistent with an interpretation of EOP as a complex, oligogenic disorder, with IL-1 genetic variation contributing an important but not exclusive influence on disease risk. This is precisely the picture that has been emerging for other common diseases such as diabetes, many forms of cancer, cardiovascular diseases and mental illnesses.[14,15] Unlike simple genetic diseases such as cystic fibrosis or muscular dystrophy, where inheriting the gene means getting the disease for certain, some EOP affected individuals may have the low risk IL-1ß genotype and some unaffected individuals may have the high risk genotype (Fig. 4). The reason these IL-1 genetic variants may occur at such a high frequency in the population even though the disease is quite rare (ca. 1%) is because inheritance of the 1 allele increases susceptibility to EOP but does not inevitably lead to development of the disease.

It is important to recognize, however, that the absence of a simple one-to-one mapping between disease gene and disease phenotype does not diminish the value of identifying these susceptibility genes. They may still have great potential to explain a substantial portion of disease risk in the population and to predict risk for individuals. For example, such knowledge can be applied to optimize health care resources by allocating greater monitoring and/or prophylactic treatment to individuals identified to be at high risk of developing the disease. Knowledge of disease etiology at the level of specific genes may also lead to improved therapies designed to correct the specific biochemical aberration caused by the gene defect. It may also be beneficial to classify subtypes of disease using a system based on gene defects rather than solely on differences in clinical presentation. Different therapies, in turn, might be most optimally targeted to the different gene defect subtypes of the disease. Our search for additional genes located elsewhere in the genome that have a major effect on risk of EOP may be substantially enhanced by adjusting for the effect of these IL-1 polymorphisms in our statistical analyses. This strategy has been applied successfully in gene mapping studies of other complex disorders such as insulin-dependent diabetes, with stratification based on HLA haplotypes.[63] Finally, one of the biggest benefits that might accrue from identifying gene mutations in a complex disease such as EOP could be greatly enhanced power to understand the environmental components of disease etiology. Once we can adjust for the genetic sources of variation in disease risk by direct measurement of appropriate gene mutations, removal of this "noise" from our studies should greatly enhance our ability to identify environmental influences on the disease.

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