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The interleukin-1 genotype as a severity factor in adult periodontal disease

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Abstract. Although specific bacteria, dental plaque, and age are associated with periodontal disease, there are currently no reliable predictors of periodontitis severity. Studies in twins have suggested a genetic contribution to the pathogenesis of periodontitis, but previous attempts to identify genetic markers have been unsuccessful. The pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα) are key regulators of the host responses to microbial infection. IL-1 is also a major modulator of extracellular matrix catabolism and bone resorption. We report a specific genotype of the polymorphic IL-1 gene cluster that was associated with severity of periodontitis in non-smokers, and distinguished individuals with severe periodontitis from those with mild disease (odds ratio 18.9 for ages 40–60 years). Functionally, the specific periodontitis-associated IL-1 genotype comprises a variant in the IL-1B gene that is associated with high levels of IL-1 production. In smokers severe disease was not correlated with genotype. In this study, 86.0% of the severe periodontitis patients were accounted for by either smoking or the IL-1 genotype. This study demonstrates that specific genetic markers, that have been associated with increased IL-1 production, are a strong indicator of susceptibility to severe periodontitis in adults.

Key words: genetics; severe periodontitis; periodontal disease; IL-1; TNF; periodontitis

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Periodontitis is initiated by specific bacteria, predominately gram-negative anaerobes (Haffajee & Socransky 1994), that activate tissue mechanisms that produce a series of inflammatory and immunologic changes leading to destruction (Page 1991, Genco 1994) of connective tissue and bone. In adults, these bacteria routinely colonize the teeth when tooth cleaning is not performed on a regular basis. Although bacteria are essential for the initiation of periodontitis, there is currently no mechanism for determining the clinical trajectory of the disease for individual patients, i.e., differentiating those patients who will have a mild to moderate form of disease and respond well to simple professional care from those who are likely to develop a more severe periodontitis that demands more extensive therapy and results in tooth morbidity. Individual differences in disease progression are dramatic and are often not predictable by currently known mechanisms (Löe et al. 1986). Recently, studies in twins have shown that a significant part of the variance in clinical measures of periodontitis may be attributable to genetic factors (Michalowicz et al. 1991, Michalowicz 1994), but attempts to identify specific genetic markers in adults have not been successful (Hart 1994).

The cytokines tumor necrosis factor alpha (TNFα) and interleukin 1 (IL-1) are key mediators of the inflammatory process and modulate the extracellular matrix components and bone which comprise the periodontal tissues. The TNFα gene lies within the Class III region of the major histocompatibility complex on the short arm of human chromosome 6. There is a base-transition polymorphism in the promoter region (Wilson et al. 1992), and the rarer allele forms part of the extended A1, B8, DR3, DQ2 haplotype (Wilson et al. 1993). There are 3 known IL-1 genes in a cluster on human chromosome 2q 13 (Nicklin et al. 1994). Two of the genes encode pro-inflammatory proteins (IL-1A and IL-1B producing IL-1α and IL-1β, respectively) while the 3rd gene (IL-1RN) encodes a related protein that binds IL-1 receptors but acts as a receptor antagonist (IL-1ra) since it does not trigger intracellular signaling. Additional regulation of the IL-1 system is provided by the soluble type II receptor that binds IL-1β but loses affinity for IL-1ra after cleavage from the cell surface (Symons et al.

1995). Recently several genetic polymorphisms have been described in the genes of the *IL-1* cluster and, in case control studies, associations have been reported with increased severity of several chronic inflammatory diseases (Duff 1994). Some of the polymorphisms may contribute to the recognized stable inter-individual variation in cytokine production rates upon disease challenge (Duff 1993). In particular, one of the 3 genotypes of IL-1B at +3953 is associated with a 4-fold increase in IL-1β production (Pociot et al. 1992, Di Giovine et al. 1996). In view of the chronic inflammatory nature of periodontitis, the association of specific cytokine genotypes with the clinical severity of periodontitis was investigated.

Materials and Methods

Selection of subjects

Within a protocol approved by an institutional review board, subjects signed a consent form after being advised of the nature of the study. Subjects over age 35 years, in good general health were screened from three private dental practices. Subjects were selected based on radiographic and clinical criteria for one of 3 disease categories in a case-control study: mild to no periodontitis (mild n=49), moderate periodontitis (moderate n=42), and generalized severe periodontitis (severe n=43). Subjects completed personal and family medical and dental history questionnaires, and were excluded for a history of diabetes, requirement for antibiotic premedication, current pregnancy or lactation, chronic usage of anti-inflammatory drugs, a history of hepatitis or HIV infection. All subjects were required to have parents and grandparents of Caucasian Northern European heritage, to reduce genetic heterogeneity in the cohorts. Subjects were evaluated clinically by 1 of 2 calibrated examiners for probing depths (PD), clinical attachment level (CAL), plaque, and bleeding on probing. Full mouth radiographs of diagnostic quality, taken within 3 years of the baseline, were evaluated by a single calibrated reader for interproximal bone loss measurements from the cemento-enamel junction of the tooth to the bone crest, expressed as a % of the total root length. Patients were selected for clearly distinguishable clinical categories. The "mild" classification required no PDs >3 mm and no sites with bone loss >15%. The "moderate" classification required< 4 interproximal sites with ≥50% bone loss, and total mean bone loss of 17 to 28%. The "severe" classification required ≥7 interproximal sites with ≥50% bone loss, and total mean bone loss of >34%. Smoking status was classified as either current smoker (smokes) or not currently smoking and has not smoked in the last 5 years (non-smoker).

Analysis of genetic polymorphisms

The subject's finger was cleaned with antiseptic wipes and the skin was punctured with a sterile lancet. Finger-stick blood samples were collected on DNAase-free blotting paper (Tarlow et al. 1994) and analyzed blind for polymorphisms in the IL-1A gene at position -889 (McDowell et al. 1995), in the IL-1B gene at positions -511 (Di Giovine et al. 1992) and +3953 (Di Giovine et al. 1996), the IL-1RN gene intron-2 (Tarlow et al. 1993), and the TNFA gene at position -308 (Wilson et al. 1992). All screening methods are PCR-based and have been extensively validated. A reaction mix excluding *Taq* polymerase was prepared and 1 mm^2 dried blood spots were added prior to heating at 95°C for 15 min. *Taq* polymerase (1.25 u., GibcoBRL-UK) was then added and PCR started. All reactions were carried out in 20 mM Tris-HCl, 50 mM KCl, 0.2 mM each dNTP and 0.05% W-1 detergent. The $MgCl_2$ and primer concentrations varied in each type of reaction and are detailed below.

IL-1A (-889;) Primers: 5'-AAGCTTGTTCTACCACCTGAACTAGGC-3';5'-TTACATATGAGCCTTCCATG-3'; both at 0.8 μM; $MgCl_2$ (1 mM); cycling: 96°C for 2 min, 45 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 50°C for 1 min. PCR product is digested overnight at 37°C with 6 units per 30 μl reaction of *NcoI*, and restriction pattern visualized by electrophoresis through a 6% PAGE (150V for 2.5 hours). This gave products of 83bp+16bp (allele 1) and 99bp (allele 2).

IL-1B -511; Primers: 5'-TGGCATTGATCTGGTTCATC-3' and 5'-GTTTAGGAATCTTCCCACTT-3' (1 μM); $MgCl_2$ at 2.5 mM; Cycling at 95°C for 2 min, 53°C for 1 min, 74°C for 1 min 2× then 35 cycles of 95°C for 1 min, 53°C for 1 min, 74°C for 1 min and 3 cycles of 95°C for 1 min, 53°C for 1 min, 74°C for 1 min. Digestion with 3 units *AvaI* per 30 μl reaction at 37°C overnight, yields products of 190bp+114bp (allele 1) or 304bp (allele 2).

IL-1B +3953; Primers: 5'-CTCAGGTGTCCTCGAAGAAATCAAA-3' and 5'-GCTTTTTTGCTGTGAGTCCCG-3' (2 μM) with 2.5 mM $MgCl_2$. Thermocycling was performed thus: 95°C for 2 min, 67.5°C for 1 min, 74°C for 1 min cycled 2× followed by 35 cycles of 95°C for 1 min, 67.5°C for 1 min, 74°C for 1 min 35×, and 3× at 95°C for 1 min, 67.5°C for 1 min, 74°C for 5 min. The products were digested with 10 units per 30 μl reaction of *TaqI* at 65°C overnight. The resulting products of 12bp+85bp+97bp (allele 1) and 12bp+182bp (allele 2) are diagnostic.

IL-1RN (intron 2) VNTR; Primers: 5'-CTCAGCAACACTCCTAT-3' and 5'-TCCTGGTCTGCAGGTAA-3' (1 μM); with 1.75 mM (final concentration) $MgCl_2$ and cycling at 96°C for 1 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 70°C for 2 min and finally 70°C for 5 min and 55°C for 5 min. Electrophoresis in agarose was performed at 90V for 45 min. Allele 1 (4 repeats) was 412bp; allele 2 (2 repeats), 240bp; allele 3 (3 repeats), 326bp; allele 4 (5 repeats), 498bp; and allele 5 (6 repeats), 584bp.

TNFA-308; Cycling: 94°C for 3 min, 60°C for 1 min and 72°C for 1 min; followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min; with a final cycle of 94°C for 1 min, 60°C for 1 min and 72°C for 5 min; Primers: 5'-AGGCAATAGGTTTTGAGGGCCAT-3' and 5'-TCCTCCCTGCTCCGATTCCG-3' (2 μM) with 1.5 mM $MgCl_2$. Digestion was performed at 37°C overnight with 6 units per 30 μl reaction of *NcoI*. Products of 87bp+20bp (allele 1) and 107bp (allele 2) were diagnostic.

All PCR products were stained with ethidium bromide 0.2 μg/ml and visualized under ultra-violet light following electrophoresis. All PCR screening methods used in this study have been extensively validated.

Statistical methods

Data analyses were performed in a sequential process as follows. All the variables were analyzed following multiple logistic regression stepwise selection to determine their prediction power for severe periodontal disease. This information was then used to determine which variables would be

further analyzed. Smoking was shown to be the strongest risk factor, which had been well established from other studies (Haber 1994, Schenkein et al. 1995). When the smoking factor was present, the predictive value of other variables became statistically insignificant. Because the number of smokers in the Mild and Moderate groups was so small (only 10% and 12% in each group, respectively) statistical analyses were only performed on the non-smoking population.

In the 2nd stage of analyses, the frequencies of individuals who carried at least one copy of the less frequent allele for each DNA polymorphism were compared between the disease groups and the mild group. In the third stage of analyses, composite genotypes combining pairs of DNA polymorphisms in the IL-1 gene cluster were then constructed for comparison. The above sequence produced 26 statistical comparisons.

The associations were determined by the χ^2 test and, in certain analyses, by the Fischer exact test. The analyses were adjusted for multiple comparisons using a Bonferroni correction (Miller 1981) which gave an adjusted P-value of 0.00192. We therefore considered any P-value <0.002 as statistically significant. Odds ratios with 99.81% confidence intervals, estimating the relative risk, were calculated (Woolf 1955).

The difference of the clinical parameters between the groups were determined using the Wilcoxon test. All the analyses were performed with the SAS statistical package.

Results

The distribution of polymorphisms in the genes for IL-1α, IL-1β, IL-1 receptor antagonist, and TNFα was evaluated in 3 groups of subjects with clearly distinguishable severities of periodontitis. Of subjects with severe periodontitis (severe), 58.1% were smokers, compared with 10.2% and 11.9% in the mild and moderate groups, respectively (odds ratio: severe versus mild=12.22 (2.14–69.87), $P<0.002$).

In non-smokers, a strong association was observed between severity of periodontitis and the composite genotype comprising allele 2 of the IL-1A -889 polymorphism plus allele 2 of the +3953 polymorphism of the IL-1B gene (odds ratio severe versus mild=6.8 (1.01–45.62), $P<0.002$) (Fig. 1). The clinical characteristics of non-smokers who were positive for the composite genotype and those who were negative are shown in Table 1.

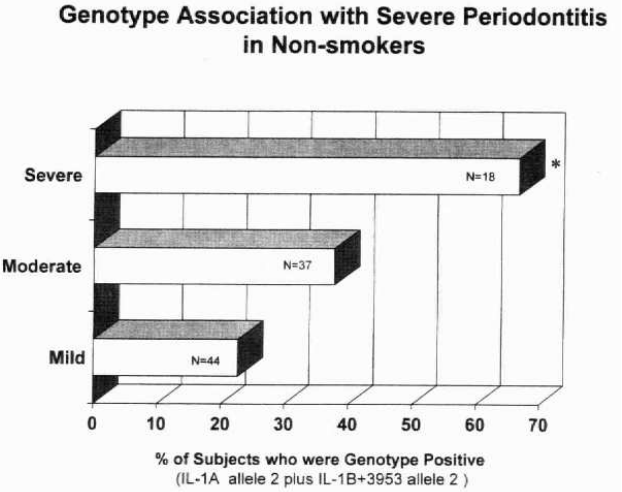

Fig. 1. The occurrence of the composite genotype (IL-1A allele 2 plus IL-1B+3953 allele 2) for non-smokers in the different disease groups. Severe $n=18$, moderate $n=37$, mild $n=44$. * Odds ratio: severe versus mild=6.80 (1.01–45.95), $P<0.002$.

To control the effect of age on disease severity, data were analyzed separately for non-smokers aged 40–60 years. In this age range, the composite genotype was present in 78% of severes ($n=9$), 26% of moderates ($n=30$), and 16% of milds ($n=32$) (odds ratio: severe versus mild=18.90 (1.04–343.05), $P<0.002$). The influence of genotype on disease severity is evident in the cumulative frequency distribution (Fig. 2) that shows an increased prevalence of severe periodontitis in the genotype positive non-smokers at all ages. Severe disease in genotype-negative individuals was most pronounced only after the age of 60 years, but was present 20 years earlier in those who were genotype positive. No composite genotype association with disease was evident in smokers.

For single alleles (Table 2), there were no significant associations between the prevalence of the less frequent allele (allele 2) and disease severity. IL-1A carriage rate of allele 2 by the severe group tended to be higher than for the Mild group, but this relationship was not statistically significant when the P-value was corrected for multiple comparisons using the Bonferroni method.

Table 1. Clinical data for non-smokers based on genotype

	Non-smoker genotype negative[1] mean±SD, $n=63$	Non-smoker genotype positive[2] mean±SD, $n=36$	P-value[8]
BOP%[3]	16.24±12.02	19.10±12.02	NS
PD[4]	3.32±0.59	3.62±0.63	0.015
CAL[5]	3.70±1.41	4.52±1.80	<0.01
%bone loss[6]	18.13±8.94	25.61±12.62	<0.001
#≥50%[7]	1.43±4.60	4.47±7.30	<0.001

[1] Subjects who were non-smokers and not carrying allele 2 for both IL-1A and IL-1B+3953. [2] Subjects who were non-smokers but were carrying allele 2 for both IL-1A and IL-1B+3953. [3] Mean % of sites that bleed on probing. [4] Mean probing depth. [5] Mean clinical attachment level. [6] Mean % radiographic bone loss. [7] Mean number of teeth with ≥50% bone loss. [8] Wilcoxon test.

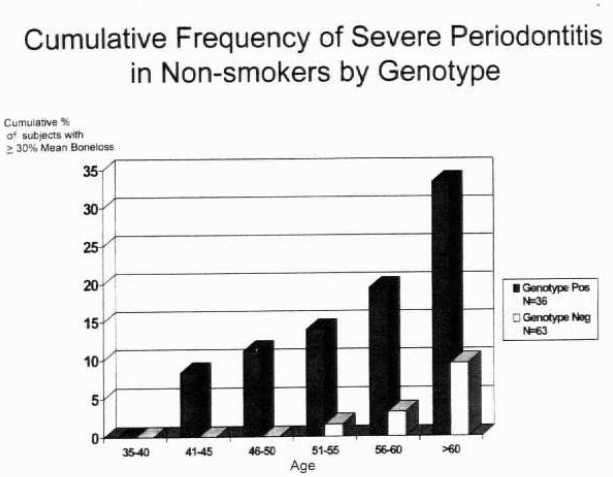

Fig. 2. The cumulative frequency distribution of non-smokers with ≥30% mean bone loss (severe) at different ages. The bars represent the cumulative % of subjects who had severe disease by the indicated age. Genotype positive (n=36). Genotype negative (n=63).

Discussion

Periodontitis involves multiple clinical patterns including various severities of adult periodontitis, uncommon early onset forms that affect children and young adults, periodontitis associated with systemic diseases, and patients who do not respond predictably to conventional therapy (refractory periodontitis). The most common form, adult periodontitis, has been reported to affect over 30% of the adult population, with severe disease reported in 7–13% of adults (Brown & Löe 1993, Clark & Löe 1993, Papapanou 1994). In this study, we examined only adults with no known history of early onset disease, and most likely included subjects with both adult periodontitis and refractory periodontitis.

The association of the IL-1 genotype with periodontitis severity appears to connect multiple lines of research. Although periodontitis initiation requires specific bacterial accumulations (Haffajee & Socransky 1994), the quantity and types of bacteria have not fully explained the differences in disease severity in adults (Kornman et al. 1994). Classic studies in beagle dogs showed a dramatic difference in disease response to plaque accumulation, with some animals developing minimal disease progression and others developing severe generalized periodontitis (Lindhe et al. 1975). Similarly, some markers of the host immuno-inflammatory response correlate with periodontitis once the disease has initiated (Offenbacher et al. 1993), but none predict an individual's disease susceptibility, nor is there a reliable mechanism for determining the trajectory of the disease to identify patients who require more aggressive therapy. Although several studies have suggested there is a genetic influence in periodontal disease (Löe et al. 1986, Michalowicz et al. 1991, Offenbacher et al. 1993), and some specific genetic markers have been identified in the juvenile forms of periodontitis (Shapira et al. 1994) previous studies of specific genetic markers in adults with periodontitis have not been encouraging (Hart 1994). The finding that a specific genotype in the IL-1 gene cluster correlates with severe periodontitis suggests a genetic mechanism by which some individuals, if challenged by bacterial accumulations, may have a more vigorous immuno-inflammatory response leading to more severe periodontitis.

Gene polymorphisms are a mechanism by which individuals may exhibit variations within the range of what is considered biologically normal. At IL-1 and TNF loci, some allelic variants have been found to be significantly over-represented in inflammatory diseases or in inflammatory complications of infectious diseases. For example, allele 2 of the IL-1 receptor antagonist gene is significantly elevated in ulcerative colitis (Mansfield et al. 1994), systemic lupus erythematosus (Blakemore et al. 1994), alopecia areata (Tarlow et al. 1994), and lichen sclerosus (Clay et al. 1994). A polymorphism in the IL-1A gene is associated with juvenile arthritis (McDowell et al. 1995), and a polymorphism in the TNFα promoter region has been associated with cerebral malaria (McGuire et al. 1994).

In the present study, severe disease in non-smokers was identified when allele 2 of the IL-1A -889 polymorphism was present with allele 2 of the +3953 polymorphism of the IL-1B gene. This composite genotype occurred in 29.1% of Northern European subjects of unknown periodontal status and with no overt medical conditions (G. W. Duff and FS di Giovine, data not shown). IL-1 activates the degradation of the extracellular matrix and bone of the periodontal tissues (Birkedal-Hansen 1993, Tewari et al. 1994), and elevated tissue or gingival fluid levels of IL-1β have been associated with periodontitis (Stashenko et al. 1991, Preiss & Meyle 1994, Yavuzyilmaz et al. 1995, Lee et al. 1995, Liu et al. 1996,). It is notable in our study that the IL-1B polymorphism associated with severe periodontitis is also known to correlate with IL-1β production rates. Specifically, one component of the periodontitis-related genotype (IL-1B +3953 allele 2) defines high

Table 2. Allele 2 carriage rate in all subjects

	IL-1A	IL-1B +3953	IL-1B −511	IL-1RN	TNFA
mild[1]	38.8[2]	40.8	55.1	59.2	26.5
moderate[3]	61.9	47.6	61.9	33.3	38.1
severe[4]	55.8	46.5	62.7	48.8	20.9

[1] Subjects classified as having minimal to no periodontitis. [2] % of individuals in the mild group who carry at least one copy of allele 2 on the IL-1A gene. [3] Subjects classified as having moderate periodontitis. [4] Subjects classified as having generalized severe periodontitis.

IL-1β production states (Pociot et al. 1992). Monocytes from individuals homozygous for the IL-1B +3953 allele 2 produce four-fold more IL-1β and heterozygous cells produce approximately two-fold more IL-1β than cells from individuals homozygous for allele 1 (Pociot et al. 1992, di Giovine et al. 1996).

It is of interest that the genetic association with periodontitis was evident only when smokers were excluded, confirming the importance of this risk factor (Haber 1994, Schenkein et al. 1995), and suggesting that its effect is strong enough to be seen even in subjects who are not genetically predisposed to severe disease. However, the IL-1 genotype identified here appears to be a marker of some biologic change that is sufficiently strong to lead to severe periodontitis in the absence of smoking and without regard for the level of bacterial challenge. In this study, the combination of either smoking or the specific composite genotype accounted for 86.0% of the severe periodontitis subjects. The IL-1 genotype was an especially strong predictor of severe disease in non-smokers between ages 40–60, with an odds ratio of 18.90. In addition, of subjects in that age group with mild periodontitis, 84% were genotype negative. Further studies to confirm this observation are in progress, including transmission disequilibrium testing with parental DNA to detect excess transmission of the disease associated genotypes to affected off-spring.

The diagnosis of moderate to severe periodontitis is a simple clinical process. However, there is currently no mechanism for determining which patients with mild or no disease will respond to bacterial plaque with progression to a more severe periodontitis that demands more extensive therapy. The lack of reliable markers for patient susceptibility to severe periodontitis has prevented the early identification of those at most risk and has prevented delivery of therapy appropriate for the degree of the risk. In addition, the development of new therapeutics has been hampered by clinical trials in which only a small segment of enrolled subjects exhibit disease progression, and by a lack of clarity as to which segments of the population would benefit from more complex therapies.

We are able, for the first time, to describe a genetic marker that identifies adults who, with a bacterial challenge, are highly susceptible to severe periodontitis. The association of severe periodontitis with smoking and the IL-1 genotype reported above suggest a rôle for these factors in the pathogenesis and clinical course of adult periodontitis.

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Zusammenfassung

Interleukin 1 Genotyp als Indikationsfaktor für den Schweregrad der Parodontalkrankheit des Erwachsenen

Obwohl spezifische Bakterien, dentaler Plaque und das Lebensalter mit der Parodontalkrankheit vergesellschaftet sind, liegen keine verläßlichen Werte für die Schätzung der Vorhersage (Prediktoren) des Schweregrades der Parodontitis vor. Ergebnisse der Zwillingsforschung lassen einen genetischen Beitrag zur Pathogenese der Parodontitis vermuten. Frühere Versuche genetische Marker zu identifizieren waren erfolglos. Die entzündungsförderuden Zytokine, Interleukin-1 und Tumornekrosefaktor Alpha (TNFα), werden als Schlüsselregulatoren für Wirtsantworten auf die mikrobielle Infektion angesehen. IL-1 ist außerdem hauptsächlicher Modulator des extrazellulären Matrixkatabolismus und der Knochenresorption. Wir berichten hier über einen spezifischen Genotyp des polymorphen IL-1 Genclusters, der bei Nichtrauchern mit dem Schweregrad der Parodontitis vergesellschaftet war und Patienten mit profunder Parodontitis von solchen mit milderen Formen dieser Krankheit unterschied (bei den Altersgruppen 40–60 Jahre lag ein Chancenverhältnis von 8.9 vor). Funktionell besteht dieser spezifische, mit der Parodontitis vergesellschaftete, IL-1 Genotyp aus einer, an hohe Niveaus der IL-1 Produktion gebundenen Variante des IL-1B Gens. Bei Rauchern war der Schweregrad der Krankheit nicht mit dem Genotyp korreliert. In dieser Studie gab bei 87% aller Patienten mit profunder Parodontitis entweder das Rauchen oder der IL Genotyp Anlaß zu ihre, Identifikation. Diese Studie zeigt, daß mit erhöhter IL-1 Produktion einhergehende, spezifische genetische Marker, entscheidende Indikatoren der Anfälligkeit für die profunde Parodontitis des Erwachsenen sind.

Résumé

Le génotype de l'interleukine-1, facteur de sévérité dans la maladie parodontale de l'adulte

Bien que des bactéries spécifiques, la plaque dentaire et l'âge soient associés avec la maladie parodontale, il n'existe actuellement pas de prédicteurs fiables de la sévérité de la parodontite. Des études faites chez des jumeaux ont semblé indiquer l'existence d'une contribution génétique à la pathogénie de la parodontite, mais les tentatives faites jusqu'ici pour identifier des marqueurs génétiques sont restées sans succès. L'interleukine-1 (IL-1) et el facteur nécrosant des tumeurs alpha (TNFα), cytokines pro-inflammatoires, sont les éléments clés de la régulation des réponses de l'hôte à l'infection microbienne. L'IL-1 est aussi un modulateur majeur du catabolisme de la matrice extracellulaire et de la résorption osseuse. Nous rendons compte ici d'un génotype spécifique du cluster des gènes polymorphes de l'IL-1, qui était associé avec la sévérité de la parodontite chez les non fumeurs, et distinguait les sujets ayant une parodontite sévère de ceux dont la maladie était bénigne (Mild) (risque relatif 18.9 pour les âges allant de 40 à 60 ans). Fonctionellement, le génotype spécifique de l'IL-1 associé à la parodontite contient dans le gène de l'IL-1B un variant, qui est associé avec des niveaux élevés de production de l'IL-1. Chez les fumeurs, il n'y avait pas de corrélation entre le génotype et la parodontite sévère. Dans cette étude, 86.0% des cas avec parodontite sévère étaient imputables au fait de fumer ou au génotype de l'IL-1. Cette étude montre que des marqueurs génétiques spécifiques, qui ont été associés avec une augmentation de la production de l'IL-1, sont un fort indicateur de susceptibilité à la parodontite sévère chez les adultes.

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