Oral Diseases (2007) 13, 308–313. doi:10.1111/j.1601-0825.2006.01285.x
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ORIGINAL ARTICLE

Interleukin-1 receptor antagonist gene polymorphism and gingivitis in children

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AIM: To investigate the role of the polymorphism of a variable numbers of tandem repeats of interleukin-1 receptor antagonist gene (IL-1RN) on gingivitis in children.

MATERIALS AND METHODS: A total of 146 Caucasian subjects (98 subjects with gingivitis and 48 controls) aged 8–12 years, were enrolled. Plaque and Calculus Indices were recorded to assess the oral hygiene. Gingival and Bleeding on Probing Indices were used to identify patients with gingivitis. DNA was extracted from epithelial cells of the cheek. Normal polymerase chain reaction was used for IL-1Ra genotyping.

RESULTS: A significant association was observed between IL-1Ra gene polymorphism and gingivitis in children ($P = 0.008$). The IL-1RN*2 allele (A2) was significantly more frequent in controls (37% *vs* 22% in children with gingivitis). In addition, the carriage of A2 seemed to be protective against gingivitis, and it was more frequent in controls (60% *vs* 40% in children with gingivitis, $P = 0.008$). Moreover, multiple logistic regression analysis showed that the association between IL-1Ra gene polymorphism and gingivitis in children remained significant ($P = 0.014$) regardless of the significant influence of plaque ($P = 0.013$).

CONCLUSION: IL-1Ra gene polymorphisms could have an active role in the pathogenesis of gingivitis in Caucasian children and IL-1RN*2 allele could be a protective marker against gingivitis.

Oral Diseases (2007) **13**, 308–313

Keywords: IL-1 receptor antagonist; cytokine; gene polymorphism; gingivitis; children

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Received 14 December 2005; revised 7 February 2006, 21 March 2006; accepted 24 April 2006

Introduction

Gingivitis, like some other forms of periodontal disease, is a result of the interaction between microbiological components of the plaque together with the genetic factors of the host. A large number of studies have been published on the role of pro-inflammatory cytokines such as interleukin (IL)-1 in periodontal disease. In the last few years, researchers have investigated ways to establish a safe way of blocking these pro-inflammatory cytokines.

Interleukin-1 receptor antagonist (IL-1Ra) is an endogenous receptor and an anti-inflammatory cytokine (Tseng *et al*, 2001), which is able to block the action of IL-1α and IL-1β by modulating their biological effects and preventing signal transduction (Abramson and Amin, 2002). The balance between IL-1 and IL-1Ra has been found to play an important role in the normal physiology of various organs and tissues. Under-production of local IL-1Ra and over-production of IL-1 have been implicated in a range of human diseases such as rheumatoid arthritis, inflammatory bowel disease, pulmonary disease, renal disease, acute myeloblastic leukemia, graft-*vs*-host disease, osteoporosis, diabetes, coronary artery disease, premature labor, ischemia, and periodontal disease (Arend, 2002; Delima *et al*, 2002). After infection, inflammation or even postsurgical conditions, IL-1Ra level increases in the circulation and diffuses into the tissue, increasing the local ratio of IL-1Ra to IL-1, and blocks the biological effects of the locally produced IL-1. A 10- to 100-fold excess of IL-1Ra has been found to be essential to block the biological effects of IL-1 (Arend, 2002). The protective role of IL-1Ra has been investigated in different human diseases including periodontitis. Researchers have shown an increased level of IL-1Ra in healthy sites when compared with periodontitis sites and an increased level of IL-1β in periodontitis sites, compared with healthy sites (Rawlinson *et al*, 2000).

The production of IL-1Ra is thought to be genetically determined (Hurme and Santtila, 1998). A polymorphism of a variable number of 86 bp tandem repeat

(VNTR) located in the second intron of IL-1Ra gene has been identified (Tarlow *et al* 1993; Vamvakopoulos *et al*, 2002). The presence of two repeats (IL-1RN*2 allele) has been found to be associated with increased production of IL-1Ra *in vitro*, increased plasma levels in diabetic patients and in healthy donors (Hurme and Santtila, 1998; Santtila *et al*, 1998).

Several studies in different ethnic groups have been undertaken to determine the role of IL-1Ra gene polymorphism in different human diseases including chronic and aggressive periodontitis (Parkhill *et al*, 2000; Laine *et al*, 2001; Duan *et al*, 2002; Meisel *et al*, 2002; Tai *et al*, 2002; Zhong *et al*, 2003). However, to date, there are no studies on the role of this polymorphism on gingivitis in children.

Materials and methods

Subjects

This was a case–control study. A total of 146 Caucasian subjects (98 subjects with gingivitis and 48 controls) aged 8–12 years, who were referred to the University Dental Hospital of Manchester (UDHM), UK, were enrolled.

Clinical evidence of gingivitis in the 98 subjects (56 males and 42 females), was based on the presence of bleeding on probing at any site, as determined by gingival and papillary bleeding on probing indices, when assessing gingival health.

The 48 healthy control children (22 males and 26 females) were those who had healthy gingiva and had neither evidence of bleeding on probing nor clinical signs of inflammation when obtaining the clinical assessment, and were matched by age, ethnicity and gender with the gingivitis cases. A minimum of a fully erupted first molar and central incisor in each quadrant was considered an essential inclusion criterion. Subjects were excluded if they had a history of systemic disease or medical, physical or developmental problems. Children, who had taken antibiotics in the previous 2 weeks, those who were under orthodontic treatment, or had endodontic lesions, were excluded from this study.

Clinical examination

This study was reviewed and approved by Bury and Rochdale Ethics Committee, and informed consent was obtained from parents of children enrolled in the study.

A general health questionnaire was designed to make sure that the inclusion criteria were met, and to record general information such as age, gender, and ethnicity. The clinical assessment was carried out by one investigator (MD). All fully erupted permanent teeth were assessed at midbuccal and midlingual/midpalatal aspects on each selected tooth. A diagnostic kit and WHO probe was used for the assessment (Diagnostic Kit: PA007; University Dental Hospital of Manchester (UDHM), Manchester, UK).

Plaque Index and Calculus Index were recorded dichotomously at two sites of the tooth to determine the presence or absence of plaque deposits and calculus without using any disclosing agents. Each index was divided into three scores corresponding to the level of plaque or calculus present; one, two or three. For each individual, the scores were totaled and divided by the number of the surfaces scored (Greene and Vermillion, 1964; Silness and Löe, 1964). Gingival Index (GI) was used to reveal the presence of gingivitis depending on the clinical appearance of the gingiva (Löe and Silness, 1963). Bleeding on Probing Index (BOPI) was used together with GI to identify patients with gingivitis, and to determine the severity of gingivitis (Greenstein *et al*, 1981). According to these two indices, cases were grouped into one of three disease categories: the mild category ($n = 48$), where mild inflammation included slight changes in color, slight edema, together with bleeding occurring as a single point after 20–30 s of probing; the moderate category ($n = 33$), where bleeding covered the gingival margin of the tooth after probing, in addition to the presence of some redness, edema, and glazing; the severe category ($n = 17$), marked redness and edema together with spontaneous bleeding covering the interdentally triangle and some portions of the corresponding tooth.

DNA extraction

Buccal cells were collected from the cheeks, using four sterile plain swabs per child. These samples were placed immediately in 50 ml sterilized tubes containing 5 ml of 2% sucrose. Automatic DNA extraction was performed with a KingFisher^{TM} Purification Kit, and a KingFisher^{TM} machine mL (Thermo Labsystems, Life and Laboratory Sciences, Basingstoke, UK), in accordance with the manufacturer's instructions, and selecting the Genomic_DNA_1 program.

Polymerase chain reaction

A total of 100 ng genomic DNA were amplified in a 25 μl final volume polymerase chain reaction (PCR) reaction mixture containing 10x NH_4SO_4 buffer (Bioline, London, UK), $MgCl_2$ (2.5 mM, Bioline), 0.2 mM of each dNTPs (Bioline), 0.3 pM of each primers and 1 U of Taq polymerase (Bioline). PCR amplification was performed using the primers (forward 5'-TCCTGGTCTGCAGGTAA-3') and (reverse 5'-CTCAGCAACACTCCTAT-3'). Primer design used sequence data for the IL1_RN alleles from GeneBank (accession number X64532).

The PCR reaction was carried out using a Thermal Cycler (PTC-100; MJ Research Inc. Technical sales, Alberta, Canada). Cycles of PCR were as follows: 5 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 57°C, and 1 min at 72°C followed by 5 min at 72°C.

Agarose gel (1.5%) stained with 0.5 μg ml^{-1} of ethidium bromide was used at 125 V for 1 h to separate the DNA for IL-1Ra intron 2 VNTR polymorphism. Ladder IV was also injected in each row to allow easy identification of the alleles in intron 2 of IL-1Ra gene. The four-repeat allele was detected as giving a 412-bp fragment and named A_1. The two repeats allele gave a 240-bp fragment and was named A_2. The five repeats allele was detected as a 498-bp fragment and named A_3. The three-repeat allele was detected as 326-bp fragment and named A_4. The A_5 with six repeats allele (595 bp) was not detected. Two readings of the gel were taken on

different occasions by two investigators (MD, MB), who were blinded to the clinical classification.

Statistical tests

A power analysis was performed to estimate the sample size required to detect a 2.5-fold increased risk for gingivitis that was associated with being negatives for IL-1RN*2. As the frequency of allele (A2) in controls was 37%, the power of the study was 82.95% and a minimum of 40 subjects were needed in each group. The frequency of gingivitis, alleles, and genotypes was calculated using SPSS 10.1 (SPSS Inc., Chicago, IL, USA) and Epi-Info 2002 (Centers for Disease Control and Prevention, Atlanta, GA, USA). For each subjects, the mean values of plaque, calculus, gingival, and bleeding on probing indices for all teeth were calculated. Using the Pearson chi-squared test, the Hardy–Weinberg Equilibrium (HWE) was tested for fitness of IL-1RN markers in all subjects. The chi-squared tests were also utilized to detect any association between gingivitis and IL-1Ra gene polymorphism. Multivariate logistic regression analysis was used to determine the role of IL-1Ra gene polymorphism in gingivitis in children, while adjusting for potential confounders. In this analysis, gingivitis, according to BOPI, was the dependent variable. It was coded as present or not present, whilst the independent variables were gender (male/female), plaque (present/not present), IL-1Ra allele 2 (positive/negative) and age (<10 years/>10 years). Odds ratios for allele frequency and allele 2 carriage rates were calculated with 95% confidence interval. A Mantel–Haenszel Corrected P-value <0.05 was considered statistically significant.

Results

A total of 146 children with mean age of 9.82 ± 1.43 were involved in this study. Plaque was present in 134 (92%) children examined and 36 (25%) had calculus. According to gingival and bleeding on probing indices, gingivitis was present in 98 (67%) children. A significant difference was observed between children with gingivitis and controls in the frequency of plaque and calculus ($P < 0.0001$). Table 1 summarizes the clinical characteristics of children with and without gingivitis.

Table 1 Summary of characteristics of children with gingivitis and controls

Characteristic	*Control*	*Case*	*P-value**
Sample size: $n = 146$ (% total)	48 (33)	98 (67)	
Males/females (*n*)	22/26	56/42	0.133
Age (mean ± s.d.)	9.73 ± 1.49	9.81 ± 1.47	0.88
Plaque (*n*)	39	95	<0.001
Plaque (mean ± s.d.)	0.63 ± 0.51	1.09 ± 0.72	
Calculus (*n*)	2	34	<0.001
Calculus (mean ± s.d.)	0.02 ± 0.06	0.31 ± 0.63	

**P*-values (Pearson chi-squared) of the differences between cases and controls.

The frequencies of IL-1Ra VNTR alleles and genotypes were calculated. The most frequent VNTR allele was A1 (four repeats) 60%, the next most common allele was A2 (two repeats) (27%). The A3 (five repeats) and A4 alleles (three repeats) were found less frequently (2% and 11% respectively). In addition, the IL-1Ra genotype frequencies were 40 (27%), 58 (40%), 4 (3%), 33 (23%), 2 (1%), and 9 (6%) for A1/A1, A1/A2, A1/A3, A1/A4, A2/A3, and A2/A2 respectively.

Table 2 shows the distribution of IL-1RN alleles in children with and without gingivitis. No deviation from Hardy–Weinberg expectations was observed for IL-1RN markers in controls and children with gingivitis except one mismatch for A_2/A_4 genotype; there was a false significant difference between observed and expected value for HWE (cases only). This is also well explained in a recent report, which has shown that chi-squared test used in HWE can have inflated type one-error rates (Wigginton *et al*, 2005).

In addition, a marginal significant difference was observed between controls and cases in the frequency of IL-1RN genotypes. The carriage of A2 seemed to be protective rather than a risk factor for gingivitis. A1/A2 and A2/A2 genotypes were higher in controls than cases (50% and 10% *vs* 36% and 4% respectively, $P = 0.087$). A significant difference was observed between controls and cases in the frequency of IL-1RN alleles ($P = 0.024$). Allele 2 was significantly more frequent in controls (37% *vs* 22% in children with gingivitis, $P = 0.008$, OR = 0.49; 95% CI: 0.28–0.87).

When comparing A2 positives with A2 negative children for the presence of gingivitis, a significant difference was observed between controls and children with gingivitis in the carriage rate of IL-1RN*2 allele. The findings are presented in Table 3. An increased risk of having gingivitis was observed in IL-1RN*2 allele negatives (60% *vs* 40% in IL-1RN*2 allele positive children, $P = 0.008$. The odds ratio for IL-1RN*2 allele negatives = 2.52; 95% CI: 1.17–5.47).

Table 3 also shows the carriage rate of IL-1RN*2 allele in controls and children with different levels of gingivitis. Severe gingivitis was more frequent in IL-1RN*2 negative children (59% *vs* 37% in controls $P = 0.036$). The odds ratio (severe compared with controls) was 2.38 (95% CI: 0.67–8.57, $P = 0.12$).

In order to determine the most important confounders associated with the risk of gingivitis in children, we performed a multiple logistic regression considering age, gender, and plaque as independent variables. Findings are presented in Table 4. The association between IL-1RN VNTR polymorphism and gingivitis in children remained significant ($P = 0.014$) regardless of the significant effect of plaque ($P = 0.013$). Children at highest risk of having gingivitis were allele A2 negatives who had abundant plaque.

Discussion

Interleukin-1 receptor antagonist is an anti-inflammatory cytokine, which is able to inhibit the functions of IL-1 such as bone resorption and attachment loss by

Table 2 Allele and genotype frequencies of IL-1RN VNTR polymorphism in Caucasian controls and children with gingivitis comply with Hardy–Weinberg Expectations (HWE) with 95% confidence interval

	Controls			*Cases*			
IL-1RN VNTR 86	*Observed, n (%)*	*HWE, n (%)*	*P-value*	*Observed, n (%)*	*HWE, n (%)*	*P-value*	*Cases vs Controls (P-value)*
Genotype							
A_1/A_1	12 (25)	15 (31.64)	0.4959	28 (28)	37 (38.12)	0.1721	0.6494
A_1/A_2	24 (50)	20 (41.02)	0.4126	34 (36)	27 (27.1)	0.2802	0.0758
A_1/A_3	0 (0)	1 (1.17)	0.3148	4 (4)	3 (3.16)	0.7003	0.1558
A_1/A_4	6 (13)	3 (7.03)	0.2935	27 (27)	17 (17)	0.0869	0.0411
A_2/A_3	1 (2)	1 (0.76)	1.0	1 (1)	1 (1.1)	1.0	0.6037
A_2/A_2	5 (10)	6 (13.29)	0.7486	4 (4)	5 (4.81)	0.7329	0.1349
A_2/A_4	0 (0)	2 (4.56)	0.1530	0 (0)	6 (6.04)	0.0129	–
A_3/A_3	0 (0)	0 (0.01)	–	0 (0)	0 (0.06)	–	–
A_3/A_4	0 (0)	0 (0.13)	–	0 (0)	0 (0.7)	–	–
A_4/A_4	0 (0)	0 (0.39)	–	0 (0)	2 (1.9)	0.1552	–
Total = 146		48 (100)			98 (100)		
Allele							
A_1	54 (56)	54 (56)	–	121 (62)	121 (62)	–	0.3689
A_2	35 (37)	35 (37)	–	43 (22)	44 (22)	0.9033	0.0084[a]
A_3	1 (1)	2 (2.08)	0.5606	5 (2)	4 (2)	0.7359	0.3931
A_4	6 (6)	5 (5.21)	0.7562	27 (14)	27 (14)	–	0.0564[b]
Total = 292		96 (100)			196 (100)		

[a]OR = 2.04, 95% CI = 1.15–3.61, $P = 0.0084$.
[b]OR = 2.40, 95% CI = 0.92–7.35, $P = 0.056$.

Table 3 The carriage rate of IL-1RN*2 allele in controls and children with different levels of gingivitis

IL-1Ra gene polymorphism	*Controls*	*Mild*	*Moderate*	*Severe*	*P-value*
A2 positives	30 (63%)	22 (46%)	10 (30%)	7 (41%)	0.036
A2 negatives	18 (37%)	26 (54%)	23 (70%)	10 (59%)	
Total ($n = 146$)	48	48	33	17	

OR for A2 negatives (severe *vs* controls) = 2.38 (0.67–8.57), $P = 0.127$.
OR for A2 negatives (total cases *vs* controls) = 2.52 (1.17–5.47), $P = 0.008$.

Table 4 Multiple logistic regression analysis for gingivitis in Caucasians

Logistic regression analysis	*Odds ratio (95% CI)*	*P-value*
Plaque	5.08 (1.42–18.23)	0.013
IL-1Ra gene polymorphism	2.36 (1.19–4.67)	0.014
Age	0.95 (0.71–1.26)	0.712
Gender	0.57 (0.28–1.14)	0.113

preventing signal transduction. There is increasing evidence suggesting that part of IL-1Ra production is genetically determined and IL-1R VNTR polymorphism is of particular interest.

Similar frequencies of IL-1Ra alleles to those previously reported on Caucasians by Tarlow *et al* (1993) and Tseng *et al* (2001) were reported. However, the frequencies of allele 1 and allele 2 were different from those published on Korean (92%, 6.4%), Chinese (93%, 6%), and Japanese populations (95.1%, 2.7%) by Pyo *et al* (2003) and Tseng *et al* (2001).

Several studies have implicated the IL-1RN*2 allele in different immune diseases such as ankylosing spondylitis (Van der Paardt *et al*, 2002), SLE (Blakemore *et al*, 1995), Sjögren syndrome (Perrier *et al*, 1998) and alopecia areata (Clay *et al*, 1994).

In our study, however, we have observed that IL-1RN*2 is a protective rather than a risk factor against gingivitis in Caucasian children.

This allele was significantly more frequent in controls than in children with gingivitis ($P = 0.008$) whilst, an increased risk of having gingivitis was observed in IL-1RN*2 allele negatives than in allele 2 positive children.

The subjects were described as free from systemic diseases, medical, physical or developmental problems, not taking antibiotics, not under orthodontic treatment nor had endodontic treatment. Given these criteria, the gingivitis presented by the subjects could be strongly attributed to dental plaque, which is the most common cause, feature and exacerbating factor of the disease (Mariotti, 1999). Therefore, we performed a multiple logistic regression after adjusting for the most important confounders including plaque, age, and gender. The analysis showed that being negative for allele 2 increases the risk of having gingivitis ($P = 0.014$) regardless of the significant influence of plaque ($P = 0.013$).

Based on a study which has shown that IL-1RN*2 allele is associated with increased production of IL-1Ra

(Hurme and Santtila, 1998; Santtila *et al*, 1998), it can be suggested that allele 2 of IL-1RN gene has a protective role against gingivitis in Caucasian children rather than being a risk factor for gingivitis.

Our results are in agreement with other studies, which have reported that the IL-1RN*2 allele is a protective genetic marker against *Ureaplasma urealyticum* and *Mycoplasma hominis* (Witkin *et al*, 2002). The present findings are also consistent with those which reported that IL-1RN* allele 2 is a protective marker which can increase the immune defense against HIV replication, as well as increases the resistance against human cytomegalovirus and Epstein–Barr virus (Witkin *et al*, 2002), and reduce the bone loss in the spine of females (Langdahl *et al*, 2000).

The results presented are similar to those which demonstrated that the IL-1RN*2 allele is a protective role against rheumatoid arthritis (Lee *et al*, 2004) and graft-*vs*-host disease (Cullup *et al*, 2003).

Our study is the first to investigate the role of IL-1Ra gene polymorphism in gingivitis in children. Previous studies have investigated the role of IL-1Ra gene polymorphism in patients with either aggressive or chronic periodontitis, but these have yielded contradictory results.

Laine *et al* (2001) found a higher frequency of allele 2 carriage in IL-1α (-889), IL-1β (+3954) and IL-1RN in Caucasians with adult periodontitis who did not have *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, in their samples (OR = 5.7, $P = 0.0068$). Similarly, Meisel *et al* (2002) have shown that Caucasian smokers who were positives for allele 2 of IL-1RN and IL-1α gene exhibited the highest frequency of attachment loss.

Our findings were also different from those reported on Chinese and Uighur patients with adult periodontitis (Duan *et al*, 2002; Zhong *et al*, 2003).

Studies linking IL-1RN genotypes with aggressive periodontitis have also shown that allele 2 of IL-1RN (VNTR) polymorphisms are a risk factor in Japanese patients with generalized aggressive periodontitis ($P = 0.005$; OR = 3.81, 95% CI = 1.31–11.31) (Tai *et al*, 2002). However, findings of Parkhill *et al* (2000) were similar to ours. The IL-1RN* allele 1 was a risk factor in Caucasian patients with aggressive periodontitis. The OR for allele 1 *vs* allele 2 in EOP was 1.9 (95% CI: 1.4–2.5).

It should be emphasized that studies which have investigated other ethnic groups rather than Caucasians may explain the disagreement with our results, plus the other studies examined other forms of periodontal diseases rather than gingivitis which may well explain the differences between our study and those on aggressive or chronic periodontitis.

The present findings have provided evidence for the protective role of the IL-1RN*2 allele in gingivitis in Caucasian children, and that IL-1RN*2 allele negative children were at highest risk of having gingivitis. However, future research will need a larger sample, and investigating different ethnic groups will be essential to confirm these findings.

Although there are some publications, which have addressed the association between the IL-1RN*2 allele and increased production of IL-1Ra (Hurme and Santtila, 1998), a functional study, which confirms this association would certainly be helpful to further demonstrate the protective role of IL-1Ra in gingivitis in children.

Future work, involving family-based controls, is still required to confirm that the genetic association found is because of the chromosomal proximity of the marker and a trait causing mutation rather than an artifact of population admixture (Rice *et al*, 2000; Little *et al*, 2002).

To date, the ability of IL-1Ra to block the detrimental effects of IL-1 has enabled it to be used for the management of different inflammatory conditions. It has provided clinical and radiographic improvement in rheumatoid arthritis, septic shock, experimental glomerulonephritis and cerebral ischemia (Bresnihan *et al*, 1998; Abramson and Amin, 2002). Moreover, studies of IL-1 antagonists on periodontitis in animal models have shown that IL-1Ra is able to inhibit the osteoclast – like cell formation mediated by *A. actinomycetemcomitans* Y4 capsular polysaccharide in mouse marrow cultures – and also to inhibit the differentiation of osteoclasts progenitor into multinucleated osteoclasts induced by IL-1 (Nishihara *et al*, 1995).

In view of these encouraging studies, future efforts to address IL-1RN*2 allele as a functional genetic marker may hold promise in delivering effective therapy with IL-1 receptor antagonists to the IL-1RN* allele 2 negative patients with periodontal disease.

Acknowledgements

We are grateful to the staff in Pediatric Clinic, University Dental Hospital of Manchester, University of Manchester, for their invaluable assistance. This study was supported in part by Damascus University, Ministry of Higher Education, Syria. The work is also supported in part by British Society of Dental Research as a winning Senior Unilever poster prize given to M. Dashash at the BSDR meeting, Birmingham, UK, 2004.

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