Interleukin-10 Haplotype Frequencies in Children With Gingivitis

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Background: Polymorphisms in the promoter region of the interleukin (IL)-10 gene have been identified at positions –1082 (G→A), –819 (C→T), and –592 (C→A). Linkage disequilibrium between alleles –819*C, and –592*C was observed. A previous study addressed the association between the –1082*A allele and gingivitis in white Caucasian children. The aim of this case-control study was to test whether differences could exist between children with and without gingivitis in the distribution of IL-10 alleles and haplotypes at positions –1082, –819, and –592.

Methods: A total of 248 subjects aged 8 to 12 years from the University Dental Hospital of Manchester were enrolled in this study. According to gingival and bleeding on probing indices, 84 children were classified as controls and 164 as children with gingivitis. Amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) was used for genotyping IL-10 polymorphisms. Allele and haplotype frequencies were calculated by direct counting and by a haplotype frequency estimation (EH) program. Differences between subjects with gingivitis and controls in the frequency of haplotypes were determined by a χ^2 test of homogeneity.

Results: The GCC/GCC genotype, which has been associated with increased production of IL-10, was significantly more frequent in controls than in children with gingivitis (36% versus 23%) ($P = 0.036$). In addition, there was a marginally significant difference between controls and cases in the frequency of haplotypes ($P = 0.06$). The GCC haplotype was more frequent in controls than in children with gingivitis (60% versus 50%). In contrast, the ACC and ATA haplotypes were more frequent in children with gingivitis (27% and 23% versus 19% and 21% in controls).

Conclusion: These findings suggest that IL-10 gene promoter polymorphisms could have an active role in the pathogenesis of gingivitis in Caucasian children. *J Periodontol 2006;77:1503-1509.*

KEY WORDS

Caucasoid race; children; cytokine; haplotype; gingivitis; interleukin-10; polymorphism.

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Interleukin (IL)-10 is an anti-inflammatory cytokine. Twin and family studies have shown that ~75% of IL-10 production is genetically determined.[1] The IL-10 promoter region is highly polymorphic. Three single nucleotide polymorphisms (SNPs) in the promoter region of IL-10 have been identified at positions –1082 (G to A), –819 (C to T), and –592 (C to A).[2] SNPs at position –1082 lie within a putative Ets transcription factor binding site,[3] SNPs at position –819 are thought to affect an estrogen responsive element,[4] and SNPs at –592 lie within a negative regulatory function.[3]

A previous study by Turner et al.[5] has shown that allele G at position –1082 is associated with an increased production of IL-10, whereas the –1082*A allele is associated with a decreased production of IL-10.[5] Moreover, previous work has also noted that IL-10 polymorphism at position –1082 is associated with the production of IL-10 by lymphocytes, whereas the IL-10 gene polymorphism at position –592 is associated with the production of IL-10 by monocytes and macrophages.[6] In addition, a more recent work has also shown that these three SNPs at positions –1082, –592, and –819 are also associated with the production of IL-10 and can form haplotypes.[7] It has been shown that positive subjects with the GCC/GCC genotype are high producers for IL-10; ATA/ATA, ACC/ATA, and ACC/ACC genotypes represent the low producers; and GCC/ACC and GCC/ATA genotypes represent the intermediate

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producers for IL-10.[7] Haplotype is a set of closely linked genetic markers present on one chromosome, which tend to be inherited together. The IL-10 –592 polymorphism has been found to be in a linkage disequilibrium with IL-10 –819 polymorphism: C at –592 is always present with C at position –819, and A at position –592 is always present with allele T at position –819.[8] Three haplotypes of IL-10 (GCC, ACC, and ATA) have been identified in Caucasians.[5]

The implication of the ATA haplotype has been noted in different human diseases including chronic progressive liver disease,[9] herpes zoster virus infection,[10] and sudden infant death syndrome.[11] The association between IL-10 haplotypes and different forms of periodontal disease has been investigated. However, there are no studies on the role of IL-10 haplotype at positions –1082, –819, and- 592 in gingivitis in children.

In previous work, we have found a significant association between the IL-10 gene polymorphism at position –1082 and gingivitis in Caucasian children.[12] The multivariate logistic regression analysis showed that the –1082*A allele remained a risk factor for gingivitis regardless of the influence of age or plaque.[12] In this study, we aimed to determine whether differences could exist between Caucasian children with and those without gingivitis in the distribution of IL-10 alleles and haplotypes at positions –1082, –819, and –592.

MATERIALS AND METHODS

Subjects

A case-control study was undertaken in the University Dental Hospital of Manchester. A total of 248 subjects aged 8 to 12 years were enrolled in this study.

The study group (N = 164) consisted of children with clinical evidence of gingivitis assessed by gingival and bleeding on probing indices. The control group (N = 84) included children who had healthy gingiva and no evidence of bleeding on probing or clinical signs of inflammation, and the two groups were matched by age, ethnicity, and gender. 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, were under orthodontic treatment, or had endodontic lesions were also excluded from the study.

Clinical Examination

The study was approved by Bury and Rochdale Ethics Committee, and informed consent was obtained from parents of children enrolled in this study. The clinical assessment was carried out by one investigator (MD). All fully erupted permanent teeth were assessed at mid-buccal and mid-lingual/mid-palatal aspects on each selected tooth. A World Health Organization (WHO) probe was used for the assessment. The plaque index (PI) and calculus index (CI) were recorded dichotomously at two sites of the tooth to measure the presence of plaque deposits and calculus without using any disclosing agents.[13,14] The gingival index (GI) was used to reveal the presence of gingivitis depending on the clinical appearance of the gingiva.[15] The bleeding on probing index (BOPI) was used together with GI to identify patients with gingivitis. According to these two indices, gingival inflammation, including changes in color, edema, and bleeding on probing, was present in 164 children, whereas 84 had healthy gingiva.

IL-10 Genotyping

Genomic DNA was extracted from buccal cells of the cheeks as previously described.[11] Amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) was used to genotype the IL-10 gene at three positions: –1082, –819, and –592.[8] DNA was amplified in a 10-μl PCR. The PCR master mix‡ was prepared with 10 μM human growth hormone primer mix (HGH).

A volume of 5 μl DNA plus master mix was aliquoted into a 96-well PCR plate containing 5 μl of either one or the other specific primer mix (10 μM each). The specific primer sequences used for each polymorphism, together with those for HGH primer, are presented in Table 1.

The PCR was carried out using a DNA thermal cycler.§ Cycles of ARMS-PCR were as follows: 1 minute at 95°C followed by 10 cycles of 15 seconds at 95°C, 50 seconds at 65°C, and 40 seconds at 72°C followed by 20 cycles of 20 seconds at 95°C, 50 seconds at 59°C, and 50 seconds at 72°C.

The amplified products were visualized on a 2% agarose gel against a 200–base pair (bp) DNA ladder and stained with 0.5μg/ml of ethidium bromide. Two readings of the gel were taken on different occasions by one investigator (MD), who was blinded to the clinical classification.

Statistical Analysis

The frequency of gingivitis, alleles, and genotypes was calculated using statistical software,‖ a spreadsheet program,¶ and a haplotype frequency estimation (EH) program. Allele and haplotype frequencies were calculated on patients and control groups by direct counting and the EH program. The association between IL-10 genotype or haplotype and gingivitis

‡ ABgene, Epson, Surrey, U.K.
§ Programmable Thermal Cycler, PTC-100, MJ Research Inc. Technical Sales, Edmonton, AB, Canada.
‖ SPSS 11.5, SPSS, Chicago, IL.
¶ Microsoft Excel, Microsoft, Redmond, WA.

Table 1.

Primers Used for the Analysis of the IL-10 Gene at Positions –1082, –819, and –592

–1082 G→A (PCR product size = 258 bp)	Generic primer (antisense)	5′-cagtgccaactgagaatttgg-3′
	Primer G (sense)	5′-ctactaa ggcttctttgggag-3′
	Primer A (sense)	5′-actactaaggcttctttgggaa-3′
–592 (C→A), –819 (C→T): (PCR product size = 233 bp)	Generic primer (antisense)	5′-aggatgtgttccaggctcct-3′
	Primer C (sense)	5′-cccttgtacaggtgatgtaac-3′
	Primer T (sense)	5′-acccttgtacaggtgatgtaat-3′
HGH	Antisense	5′-tcaggatttctgttgtgtttc-3′
	Sense	5′-gccttcccaaccattccctta-3′

Table 2.

Clinical Characteristics of Controls and Children With Gingivitis

Characteristic Sample Size: N = 248 (% total)	Control 84 (34%)	Case 164 (66%)	*P* Value*
Males/females (n)	43/41	98/66	0.16
Age in yrs (mean ± SD)	9.4 ± 1.38	9.94 ± 1.4	0.02
Plaque (%)	62 (74%)	155 (95%)	0.00
Plaque (mean ± SD)	0.52 ± 0.47	1.04 ± 0.73	
Calculus (%)	6 (7%)	58 (35%)	0.00
Calculus (mean ± SD)	0.02 ± 0.77	0.32 ± 0.63	

* *P* values (Pearson χ^2) of differences between cases and controls.

was assessed by the odds ratio (OR) and confidence interval (CI). The χ^2 tests were also used to detect any association between gingivitis and IL-10 gene polymorphisms at positions –1082, –819, and –592. A corrected *P* value <0.05 was considered statistically significant.

Using the Pearson χ^2 test, the Hardy-Weinberg equilibrium (HWE) was tested for fitness for IL-10 gene polymorphisms at positions –1082, –819, and –592. Allele and haplotype frequencies of IL-10 were calculated by the direct-counting method and using the EH program. To calculate the frequency of IL-10 haplotype by the direct-counting method, all data were transferred from the statistical software to the spreadsheet program. Direct counting for IL-10 gene polymorphisms was performed three times for each control group and patient group, and all samples were pooled together. The difference between cases and controls in the frequency of haplotypes was also determined by the EH program. This program was used three times: on cases alone to determine the frequency of alleles and haplotypes and the log likelihood in cases (in L_{case}); on controls to calculate the frequency of alleles and haplotypes and the log likelihood in controls (in $L_{controls}$); and on cases and controls pooled together to obtain the frequency of alleles, haplotypes, and the log likelihood in cases and controls (in $L_{case\text{-}controls}$). Considering N a degree of freedom (df), which is the number of haplotypes in the target population, a χ^2 with N – 1 was obtained using the following formula: 2([log likelihood (in L_{cases}) + log likelihood (in $L_{controls}$) – (–log likelihood [in $L_{combined}$])]).[16] The *P* value was determined using an online calculator.

RESULTS

A total of 248 children (141 males and 107 females) with a mean age of 9.79 years (SD: 1.44) were enrolled in this study. The clinical characteristics of all participants are shown in Table 2. Plaque was present in 217 of 248 (87.5%) children examined, and 64 of 248 (26%) subjects had calculus. Based on the GI and BOP, gingivitis was present in 164 (66%) children, and 84 (34%) children were considered controls. A significant difference was observed between controls and children with gingivitis in the distribution of plaque and calculus ($P<0.0000$).

The Pearson χ^2 test showed no significant difference between observed and expected frequencies of genotypes at position –1082, –819, and –592.

The frequency of IL-10 haplotypes was calculated by direct-counting and EH-program methods. Similar frequencies were reported after pooling the data from cases and controls. Six genotypes: GCC/GCC (28%), GCC/ACC (24%), GCC/ATA (28%), ACC/ACC (7%), ACC/ATA (11%), and ATA/ATA (2%) were observed. Consequently, three possible haplotypes: GCC (54%), ACC (24%), and ATA (22%), were found. No GTA haplotype in all samples was observed. Table 3 summarizes the distribution of IL-10 genotypes and haplotypes at position –1082, –819, and –592 using the direct-counting method.

The difference between cases and controls in the frequency of IL-10 haplotypes was also determined by direct-counting and EH-program methods. Table 4 summarizes the estimated frequencies of IL-10 alleles and haplotypes in controls, cases, and all samples combined calculated by the EH program.

Considering N a df, which is the number of haplotypes in the target population, a χ^2 with N – 1 was obtained using the following formula:

$$\chi^2 \text{ with } N-1 = 2([\log \text{likelihood}\,(\text{in } L_{cases}) + \log \text{likelihood}\,(\text{in } L_{controls}) - (-\log \text{likelihood}\,[\text{in } L_{combined}])]).$$

$$\chi^2 = 2(127.47 - 266.34 - [-396.62]) = 2 \times 2.81 = 5.62.$$

Table 3.

Observed Frequencies and Percentages of IL-10 Haplotypes at Positions –1082, –592, and –819

	Number	Percentages
Genotype		
GCC/GCC	68	28
GCC/ACC	59	24
GCC/ATA	69	28
ACC/ACC	18	7
ACC/ATA	26	11
ATA/ATA	8	2
Total	248	100
Haplotype		
GCC	264	54
ACC	121	24
ATA	111	22
Total	496	100

Table 4.

Estimated Frequencies of IL-10 Alleles and Haplotypes in Controls, Cases, and All Samples Calculated by the EH Program

Allele	Controls (n = 84)	Cases (n = 164)	Cases and Controls (N = 248)
Position –1082			
–1082*G	0.6012	0.4970	0.5323
–1082*A	0.3988	0.5030	0.4677
Positions –819/–592†			
–819*C/–592*C	0.7917	0.7683	0.7762
–819*T/–592*A	0.2083	0.2317	0.2238
Haplotype (frequency)			
GCC	0.601188	0.496948	0.532251
GTA	0.000002	0.000004	0.000007
ACC	0.190479	0.271345	0.243959
ATA	0.208331	0.231704	0.223783
Log likelihood‡ (in $L_{subjects}$)	$L_{controls}$: –127.47	L_{cases}: –266.34	$L_{cases\text{-}controls}$: –396.62

† A linkage disequilibrium between position –819 and –592.
‡ χ^2 = 2([log likelihood (in L_{cases}) + log likelihood (in $L_{controls}$) – (–log likelihood [in $L_{combined}$])]) = 2(–127.47 – 266.34 – [–396.62]) = 2 × 2.81 = 5.62. df = number of haplotypes – 1 = 3; P = 0.0602.

Assuming there were three haplotypes (GCC, ACC and ATA), there would be three degrees of freedom, and the P value would be 0.06.

There was a marginally significant difference between cases and controls in the frequency of haplotypes. The GCC haplotype was more frequent in controls at 60% versus 50% in children with gingivitis, whereas ACC and ATA haplotypes were more frequent in children with gingivitis (27% and 23% versus 19% and 21% in controls, respectively).

Similar values were obtained by the direct-counting method. The χ^2 test also showed that there was a marginal significant difference (P = 0.06) between controls and those with gingivitis in the frequency of haplotypes. The GCC haplotype was found to be a protective marker against gingivitis. It was more frequent in controls (60% versus 50% in children with gingivitis). The GCC/GCC genotype was also more frequent in controls (36% versus 23% in children with gingivitis (P = 0.036) (Table 5). In contrast, the ACC and ATA haplotypes were more frequent in children with gingivitis (27% and 23% versus 19% and 21% in controls, respectively), and the risk of having gingivitis was noted to increase two-fold in children positive for ACC and ATA haplotypes (Table 5). The ACC/ATA genotype was more frequent in cases (13% versus 6% in controls) (OR for the genotype carriage = 2.32; 95% CI: 0.81 to 8.16; P = 0.09) (Table 5).

DISCUSSION

Haplotype analysis has recently become a powerful tool in gene-disease studies as it is easier to determine the association between a particular region of the genome with the disease rather than using a single marker whereby some single-nucleotide polymorphisms, from which haplotypes are constructed, may be closely linked.

In this study, three SNPs at positions –1082, –592, and –819, which form IL-10 haplotypes, have been analyzed. A previous study found a closed linkage between alleles –819*C and –592*C and between alleles –819*T and –592*A.[8] Similar to a previous study on Caucasians, three observed haplotypes: GCC, ACC, and ATA, have been observed. No GTA haplotype has been identified.[5] Previous work by Mok et al.[17] has addressed the presence of GTA among a southern Chinese population. In addition, Scarel-Caminaga et al.[4] described new haplotypes including the ATC, GTC, and ACA haplotypes together with the GTA haplotype among Brazilians. They explained these new haplotypes by the heterogeneity among the Brazilian population.

Studies have shown that people who have the GCC/GCC genotype are high producers for IL-10; the ATA/ATA, ACC/ATA, and ACC/ACC genotypes represent the low producers; and the GCC/ACC and GCC/ATA genotypes represent the intermediate producers for IL-10.[7] In this study, the GCC haplotype, which

Table 5.

Observed Frequencies of IL-10 Alleles and Haplotypes Calculated by the Direct-Counting Method

	Controls (n = 84)	Cases (n = 164)	Significance for Carriage (*P* value; OR [CI])	*P* Value*
Genotype				
GCC/GCC	30 (36%)	38 (23%)	*P* = 0.036; OR: 0.54 (0.29 to 1.00)	0.13
GCC/ACC	15 (18%)	44 (27%)	*P* = 0.12; OR: 1.69 (0.84 to 3.43)	
GCC/ATA	26 (31%)	43 (26%)	*P* = 0.43; OR: 0.79 (0.43 to 1.47)	
ACC/ACC	6 (7%)	12 (7%)	*P* = 0.96; OR: 1.03 (0.34 to 3.46)	
ACC/ATA	5 (6%)	21 (13%)	*P* = 0.09; OR: 2.32 (0.81 to 8.16)	
ATA/ATA	2 (2%)	6 (4%)	*P* = 0.72; OR: 1.56 (0.28 to 16.08)	
Total = 248	84 (34%)	164 (66%)		
Haplotype				
GCC	101 (60%)	163 (50%)		0.06
ACC	32 (19%)	89 (27%)		
ATA	35 (21%)	76 (23%)		
Total = 594	168 (34%)	328 (66%)		

* Pearson χ^2 test; corrected $P > 0.05$.

Table 6.

Percentage Frequencies of the IL-10 Haplotype in Different Populations

Population	GCC	ACC	ATA	References
Present study	54	24	22	Dashash et al.
England (Manchester)	51	28	21	Turner et al.[5]
Australia	51	28	21	Edwards-Smith et al.[20]
England (North West)	51	25	24	Summers et al.[6]
Finland	42.9	35.1	22	Haanpaa et al.[10]
Ireland	57.5	24.2	18.3	Meenagh et al.[23]
Kwa-Zulu	38.1	28	33.9	Meenagh et al.[23]
Oman	34.8	40.5	24.7	Meenagh et al.[23]
China	2.5	30.2	67.3	Meenagh et al.[23]
Mexico	35.9	23.1	41	Meenagh et al.[23]
England	49	24	26	Constantini et al.[21]
Poland	44	34	23	Constantini et al.[21]
Japan	4	27	69	Miyazoe et al.[9]
Spain	38.2	36.1	25.7	Suarez et al.[30]
Norway	51.4	28.7	19.9	Opdal[11]
Korea	7.4	24.8	67.8	Pyo et al.[22]

represents the increased production of IL-10, was at the highest frequency (54%) compared to the frequency of the ACC (24%) and ATA (22%) haplotypes (Table 3).

This result was in agreement with a study that reported that the GCC haplotype was more frequent in Caucasians when compared to African Americans.[18,19] When haplotype frequencies were compared to other studies, there were similarities with those reported on English Caucasians from Manchester,[5] Australia,[20] England,[6,21] and Norway.[11] However, these frequencies were different from those reported on Koreans,[22] Japanese,[9] or Chinese[23] (Table 6).

The GCC/GCC genotype has a protective role against gingivitis in Caucasian children. In addition, a marginally significant difference was observed between controls and gingivitis cases in the frequency of IL-10 haplotypes. The GCC haplotype was more frequent in controls than children with gingivitis. In contrast, the carriage of ACC and ATA, which is associated with decreased production of IL-10, increased the risk of having gingivitis.

Our study population also included 35 Asians and 15 Afro-Caribbean children, and a similar significant association ($P = 0.005$) between IL-10 haplotypes and gingivitis in Afro-Caribbean children was found, but these data were not included as the sample size of this racial grouping was not large enough to present meaningful conclusions. A further study with a larger size sample would be helpful to determine the role of IL-10 haplotypes in these ethnic groups.

Previous work on patients with chronic periodontitis reported similar findings to ours; it was found that the ATA haplotype could increase the risk of chronic periodontitis by 2.5×.[4] However, the same study reported that the GCC/ACC genotype was protective against chronic periodontitis rather than the GCC/GCC genotype.

In fact, the implication of the ATA haplotype has also been noted in several human diseases including chronic progressive liver disease,[9] herpes zoster virus infection,[24] sudden infant death syndrome (SIDS),[6,11] renal involvement in patients with systemic lupus erythematosus (SLE),[17] and severe juvenile rheumatoid arthritis.[25]

Our study findings were consistent with reports that ACC and ATA haplotypes are responsible for the severity of Epstein-Barr virus (EBV) infections and the reactivation of the varicella zoster virus.[24,26]

There is also an agreement with the published work on the increase of the ACC haplotype in patients positive for immunoglobulin A (IgA) of rheumatoid factor (RF). This factor has been associated with extraarticular manifestations and bone erosion in patients with rheumatoid arthritis (RA).[27] In contrast, our study was not in agreement with Lazarus et al.,[28] who reported that GCC has been a risk factor for SLE, or with the research of Hulkkonen et al.[29] on Sjögren's syndrome. This may be explained by the fact that IL-10 is a protective cytokine, but too much of a good thing can be detrimental. Overproduction of IL-10 has been implicated in various autoimmune diseases including SLE and Sjögren's syndrome.

CONCLUSIONS

Our study confirmed the protective role of IL-10 in gingivitis in Caucasian children, as we have shown that the GCC/GCC genotype, which is associated with an increased production of this cytokine, was more frequent in controls than in children with gingivitis. However, a functional study, which investigates the association between the level of IL-10 and GCC/GCC genotype, would certainly be helpful to further confirm the protective role of IL-10 in gingivitis in Caucasian children.

In addition, a further study with a larger sample size would be helpful to ascertain the genetic association between IL-10 gene polymorphisms and gingivitis in Caucasians and children from different ethnic groups.

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