Changing Trends in Periodontal Diagnosis, Disease Recognition & Management
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Chapter 1

High Risk Individuals for Periodontitis & Their Genetic Markers

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Introduction

Although microbial factors are essential for the initiation of periodontitis (Haffajee and Socransky 1994), they alone do not predict the presence or severity of the disease. Epidemiological studies have indicated that not everyone is equally susceptible to periodontitis (Beck *et al* 1990). Among the population, there is a group of high-risk individuals who are highly susceptible to periodontitis. Twin analyses by Michalowicz *et al* suggested that 38% to 82% of the population variance for the periodontal measures of the disease may be attributed to genetic factors (Mizhalowicz *et al* 1991, Michalowicz *et al* 1991). Now, the questions which remain to be answered are:

1. Are there any genetic marker(s) for high risk individuals for periodontitis?
2. What kind of gene(s) is associated with increased susceptibility to periodontitis?

Understanding these issues will help us to identify high-risk individuals for periodontitis among the population.

To elucidate the genetic background of chronic periodontitis (CP) in the Chinese, the gene polymorphisms of IL-1 cluster (including IL-1A-889, IL-1B-511, IL-1B+3954, IL-1RN intron 2/VNTR), FcγRIIA, FcγRIIIB, TNFA-308, IL-6, Vitamin D receptor gene (VDR), and their association with chronic periodontitis in a Chinese population were investigated.

Materials and Methods

Selection of subjects

All subjects were of Han nationality, in good general medical health, had at least 14 natural teeth and were non-smokers. Clinical attachment loss (CAL) was measured on 6 surfaces of all remaining teeth. The intra-examiner reliability for reproducibility was calibrated (kappa ≥0.75). Subjects were selected according to dental history, radiographic and clinical criteria (Armitage 1999, Armitage *et al* 2000) and were placed into 4 groups based on their average full-mouth CAL measurements:

Healthy control (periodontal healthy or gingivitis): Mean CAL ≤0.5 mm, no interproximal sites with CAL ≥3 mm. No more than 2 missing teeth with exception of extracted third molars, teeth extracted for orthodontic purpose, teeth lost as a result of trauma or extensive decay.

Initial CP: Mean CAL ≥0.6 mm to 1.6 mm and no interproximal sites with CAL ≥3 mm. No more than 3 missing teeth.

Moderate CP: Mean CAL ≥1.6 mm to 2.4 mm and ≤8 sites with interproximal CAL >3 mm distributed through at least 3 quadrants or at least 6 teeth. No more than 5 missing teeth.

Severe CP: Mean CAL ≥2.5 mm and 1 or more sites in 3 out of 4 quadrants with interproximal CAL ≥5 mm. No more than 14 missing teeth.

Sample collection

One sample of exfoliating epithelial cells was taken from every subject using a cotton swab. One cotton swab was rubbed lightly four times on the subject's inside cheek and left to dry at room temperature overnight, then stored at 4°C for DNA isolation.

Preparation of DNA templates

The surface of the cotton was cut from the buccal swab stem, transferred to an Eppendorf tube, and 200 µl Chelex-100 and 10 µl Proteinase K were added. Then, the standard phenol/chloroform extraction procedure was used. The genomic DNA yield was calculated by spectrophotometry at 260nm.

Analysis of genetic polymorphisms

PCR-RFLP or PCR-SSP procedures were used to analyze blindly for polymorphisms. All PCR reactions performed in 20 µl reaction mix which contained 20mM Tris-HCl, 50mM KCl, 1.6mM $MgCl_2$, 0.35mM each dNTP, 0.6 µl each primer, Taq polymerase 1.25U and DNA template 3 µl. The PCR conditions for different gene loci were justified respectively.

IL-1A –889 (Mc Dowell *et al* 1995)**:** Primers: 5‘-AAG CTT GTT CTA CCA CCT GAA CTA GGC-3‘ and 5‘-TTA CAT ATG AGC CTT CCA TG-3‘ were used. The PCR product (5 µl) was digested overnight at 37°C with 2U Nco I, and the restriction pattern visualized by electrophoresis through a 6% PAGE. The two sizes of the PCR products were 83bp+16bp (allele 1) or 99bp (allele 2).

IL-1B-511 (Pociot *et al* 1992)**:** Primers: 5'-TGG CAT TGA TCT GGT TCA TC-3' and 5'-GTT TAG GAA TC T TCC CAC TT-3' were used. The PCR product (5 µl) was digested with 3U Ava I at 37°C overnight, and the restriction pattern visualized through a 6% PAGE. This gave products of 190bp+114bp (allele 1) or 304bp (allele 2).

IL-1B +3954 (Kornman *et al* 1997)**:** Primers: 5‘-CTC AGG TGT CCT CGA AGA AAT CAA-3‘ and 5‘-GCT TTT TTG CTG TGA TCC CG-3‘ were used. The PCR product (5 µl) was digested with 3U Taq I at 65°C overnight, and the restriction pattern visualized by electrophoresis through a 6% PAGE. This gave products of 12bp+85bp+97bp (allele 1) or 12bp+182bp (allele 2) .

IL-1RN (intron 2) VNTR (Tarlow *et al* 1993)**:** Primers: 5‘-CTC AGC AAC ACT CCT AT-3‘ and 5‘-TCC TGG TCT GCA GGT AA-3‘ were used. Electrophoresis in 8%PAGE was performed following PCR. Allele 1 (4 repeats) was 412bp; allele 2 (2 repeats) was 240bp; allele 3 (3 repeats) was 326bp; allele 4 (5 repeats) was 500bp; and allele 5 (6 repeats) was 595bp.

FcγRIIA (Warmerdamn *et al* 1990)**:** PCR-SSP was used. Primer #1: 5‘-ATC CCA GAA ATT CTC,CCA-3‘ and Primer #2: 5‘-ATC CCA GAA ATT CTC CCG-3‘; Primer #3: 5‘-CAA TTT TGC TAT GGG C-3‘. The PCR product (253bp) was visualized by electrophoresis through a 6% PAGE.

FcγRIIIB (Ory *et al* 1989): Primer for NA1: 5'-CAG TGG TTT CAC AAT GTG AAA-3', 5'-CAT GGA CTT CTA GCT GCA CCG-3'; Primer for NA2: 5'-CTC AAT GGT ACA GCG TGC TT-3', 5'-CTG TAC TCT CCA CTG TCG TT-3'. The PCR products of NA1 was 141bp and NA2 was 169bp and were visualized by electrophoresis through a 6% PAGE.

TNFA -308 (Wilson *et al* 1992)**:** Primers: 5'-AGG CAA TAG GTT TTG AGG GCC-3'; 5'-ACA CTC CCC AT C CTC CCG GCT-3'. The PCR product (5 µl) was digested with Nco I at 37°C overnight, and the restriction pattern visualized by electrophoresis through a 6% PAGE. This gave the products of 87bp+20bp (allele 1) or 107bp (allele 2).

IL-6 –174 (Zheng *et al* 2000)**:** Primers: 5'-TTG TCA AGA CAT GCC AAA GTG-3'; 5'-TCA GAC ATC TCC AGT TCC TAT A-3'. The PCR product (5 µl) was digested with Nal III at 37°C overnight, and the restriction pattern visualized by electrophoresis through a 7% PAGE. This gave the products of 246bp+54bp (allele G) or 135bp +111+54bp(allele C).

VDR/BsmI (Morrison *et al* 1994)**:** Primers: 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3'; 5'-AAC CAG CGG GAA GAG GTC AAG GG-3'. The PCR product (5 µl) was digested with BsmI at 37°C overnight, and the restriction pattern visualized by electrophoresis through a 7% PAGE. This gave the products of 800bp (allele B) or 650bp +150bp(allele b).

VDR/Apal (Bell *et al* 2001)**:** Primers: 5'-CAG AGG ATG GAC AGG GAG CAA-3'; 5'-GCA ACT CCT CAT GGC TGA GGT CTC-3'. The PCR product (5 µl) was digested with ApaI at 37°C overnight, and the restriction pattern visualized by electrophoresis through a 7% PAGE. This gave the products of 740bp (allele A) or 535bp +205bp(allele a).

VDR/TaqI (Bell *et al* 2001)**:** Primers and PCR conditions are same as for VDR/ApaI. The PCR product (5 µl) was digested with TaqI at 37°C overnight, and the restriction pattern visualized by electrophoresis through a 7% PAGE. This gave the products of 495bp+245bp (allele T) or 495bp +290bp+245bp+205bp (allele t).

All PCR products were stained with silver nitrate. All PCR screening methods used in this study have been extensively validated. 10% randomly selected samples were subjected to an additional PCR procedure operated by another technician in a blinded test. These samples were used as laboratory quality controls to assess the reproducibility of the genotyping.

Statistical analysis

The distributions of the genotypes were calculated as percentage of the study population. The differences of gene frequency between each group were determined by x^2 test. All the analyses were performed with the SSCP statistical package.

Results

Comparison of DNA yields and concordance of genotyping

The average quantity of DNA isolated from one buccal swab was 63.8$\pm$ 18.7µg, which was sufficient to repeat PCR-based genetic analysis 10 times. When the code was broken for the duplicate samples used for the laboratory quality control, the genotyping results matched in all subjects.

IL-1 genotype distribution

The distribution of the sampled subjects according to age and disease is shown in Table 1. IL-1A-889/Nco and allele 2 was carried by 46% of the subjects, all of them were heterozygous. The frequency of allele 2 in both severe and initial/moderate CP groups was significantly higher than in healthy/gingivitis group ($p < 0.01$) (Table 2).

IL-1B+3954/Taq and allele 2 was carried by 12.9% of the subjects, all of them were heterozygous. The frequency of allele 2 in both severe and initial/moderate CP groups was significantly higher than in healthy/gingivitis group ($p < 0.05$) (Table 3).

IL-1B-511/Ava and allele 2 was carried by 83% of the subjects, 39% were heterozygous and 44% were homozygous. The distribution of allele 2 homozygote was significantly higher in both severe and initial/moderate CP groups than in healthy/gingivitis group ($p< 0.05$) (Table 4).

There were only 3.69% of the subjects carried of the composite genotype of IL-1A-889 allele 2 and IL-1B+3953 allele 2 (Table 5).

There were 42.44% of the subjects carrying the composite genotype of IL-1A-889 allele 2 and IL-1B-511 allele 2. The distribution of this composite genotype was significantly higher in both severe and initial/moderate CP groups than in healthy/gingivitis group ($p < 0.01$) (Table 6).

For the composite genotype of IL-1B+3953 allele 2 and IL-1B-511 allele 2, there was only 9.23% of the subjects carrying this composite genotype. The distribution of this composite genotype was significantly higher in both severe and initial/moderate CP groups than in healthy/gingivitis group ($p < 0.05$) (Table 7).

For gene frequency and distribution of IL-1RN (intron2)/VNTR allele, there was no significant difference for the distribution of the genotypes among the three groups (Table 8).

FcγRIIA, FcγRIIIB and TNFA-308 genotype distribution

The distribution of the sampled subjects according to age, gender and disease is shown in Table 9. The genotype of FcγRIIA R/R131 distributed significantly higher in severe CP group than in the healthy control (Table 10). There was no significant difference for the distribution of the FcγRIIIB genotype between the groups (Table 11). It was also found that the frequency of the composite genotype of FcγRIIA R131 and FcγRIIIB NA2 was

Disease category	Age group (years)				
	≤29	≥30-39	≥40-49	≥50-59	≥60
Severe CP n = 85	1	1	16	51	16
Initial to moderate CP n = 97	6	5	27	47	12
Healty/Gingivitis n = 89	7	46	36	0	0
Totals n = 271	14	52	79	98	28

Table 1. Distribution of sampled population according to age and disease category

Disease category	Size	Distribution of genotype I/I (%)	II/I (%)	II/II (%)	Frequency of allele I	II
Severe CP	85	32(37.65)	53(62.35)	0	0.688	0.312
Initial/Moderate CP	97	46(47.42)	51(52.58)	0	0.737	0.263
Healthy/Gingivitis	89	69(77.53)	20(22.47)	0	0.888	0.112

Table 2. Gene frequency and distribution of IL-1A-889/Nco I allele

Disease category	Size	Distribution of genotype I/I (%)	II/I (%)	II/II (%)	Frequency of allele I	II
Severe CP	85	68(80.00)	17(20.00)	0	0.900	0.100
Initial/Moderate CP	97	83(85.57)	12(14.43)	0	0.928	0.072
Healthy/Gingivitis	89	85(95.51)	4(4.49)	0	0.978	0.222

Table 3. Gene frequency and distribution of IL-1B+3954/Taq I allele

Disease category	Size	Distribution of genotype I/I (%)	II/I (%)	II/II (%)	Frequency of allele I	II
Severe CP	85	12(14.12)	28(32.94)	45(52.94)	0.306	0.694
Initial/Moderate CP	97	13(13.4)	30(30.93)	54(55.67)	0.289	0.711
Healthy/Gingivitis	89	21(23.6)	49(55.06)	19(21.35)	0.511	0.489

Table 4. Gene frequency and distribution of IL-1B-511/Ava I allele

Disease category	Size	Distribution of genotype Genotype + (%)	Genotype - (%)	Frequency of allele Genotype +	Genotype -
Severe CP	85	4(4.71)	81(95.29)	0.0235	0.9765
Initial/Moderate CP	97	5(5.15)	92(94.85)	0.0258	0.9742
Healthy/Gingivitis	89	1(1.12)	88(98.88)	0.0056	0.994

Table 5. Gene frequency and distribution of the composite genotype of IL-1A-889 allele 2 and IL-1B+3954 allele 2

Disease category	Size	Distribution of genotype Genotype + (%)	Genotype - (%)
Severe CP	85	50(58.82)	35(41.18)
Initial/Moderate CP	97	49(50.52)	48(49.48)
Healthy/Gingivitis	89	16(17.98)	73(82.02)

Table 6. Distribution of the composite genotype of IL-1A-889 allele 2 and IL-1B-511 allele 2

Disease category	Size	Distribution of genotype Genotype + (%)	Genotype - (%)
Severe CP	85	12(14.12)	73(85.88)
Initial/Moderate CP	97	10(10.31)	87(89.69)
Healthy/Gingivitis	89	3(3.37)	86(96.63)

Table 7. Distribution of the composite genotype of IL-1B+3954 allele 2 and IL-1B-511 allele 2

		Distribution of genotype					Frequency of allele	
Disease category	Size	I/I (%)	II/I (%)	III/I (%)	IV/I	V/I	I	II
Severe CP	85	42(49.41)	43(50.59)	0	0	0	0.747	0.253
Initial/Moderate CP	95	60(63.16)	35(36.84)	0	0	0	0.8158	0.1842
Healthy/Gingivitis	87	40(45.98)	47(54.02)	0	0	0	0.7299	0.2701

Table 8. Gene frequency and distribution of IL-1RN(intron2)/VNTR allele

Groups	N	Male(%)	Female(%)	Age range	Mean age
Severe CP	63	26(41.27)	37(58.73)	42-60	55
Initial/Moderate CP	103	27(26.21)	76(73.79)	27-60	52
Healthy/Gingivitis	80	32(40.00)	48(60.00)	35-69	53
Total	246	85(34.55)	161(65.45)	27-69	53

Table 9. Subject Data

Disease category	Size	Distribution of genotype H/H (%)	H/R (%)	R/R (%)	Frequency of allele H	R
Severe CP	63	4(6.35)	47(74.60)	12(19.05)	0.44	0.56
Initial/Moderate CP	103	13(12.62)	78(75.73)	12(11.65)	0.50	0.50
Healthy/Gingivitis	80	18(22.50)	60(75.00)	2(2.50)	0.60	0.40

Table 10. FcγRIIA genotype distribution and allele frequency

Disease category	Size	Distribution of genotype NA1/NA1 (%)	NA1/NA2 (%)	NA2/NA2 (%)	Frequency of allele NA1	NA2
Severe CP	63	6(9.52)	57(90.48)	0	0.55	0.45
Initial/Moderate CP	103	10(9.71)	93(90.29)	0	0.55	0.45
Healthy/Gingivitis	80	18(22.50)	62(77.50)	0	0.61	0.39

Table 11. FcγRIIIB genotype distribution and allele frequency

Disease category	Size	Distribution of genotype Genotype + (%)	Genotype - (%)
Severe CP	63	53(84.13)	10(15.87)
Initial/Moderate CP	103	83(80.58)	20(19.42)
Healthy/Gingivitis	80	52(65.00)	28(35.00)

Table 12. Distribution of the composite genotype of FcγRIIA-R131 and FcγRIIIB-NA2

Disease category	Size	Distribution of genotype I/I (%)	II/I (%)	II/II (%)	Frequency of allele I	II
Severe CP	63	53(84.13)	10(15.87)	0	0.92	0.08
Initial/Moderate CP	103	83(80.58)	19(18.45)	1(0.97)	0.90	0.10
Healthy/Gingivitis	80	70(87.50)	10(12.50)	0	0.94	0.06

Table 13. TNFA-308 genotype distribution and allele frequency

Groups	N	Male	Female	Age range	Mean age
Severe CP	63	26	37	42-60	55
Moderate CP	69	20	49	35-60	52
Initial CP	34	7	27	27-60	52
Healthy/Gingivitis	80	32	48	35-60	53

Table 14. Subject Data

		Distribution of genotype			Frequency of allele	
Disease category	Size	A/A(%)	A/ab(%)	a/a(%)	A	a
Severe CP	63	39(61.8)	12(19.1)	12(19.1)	0.71	0.29
Moderate CP	69	14(20.3)	41(59.4)	14(20.3)	0.50	0.50
Initial CP	34	8(23.5)	21(61.8)	5(14.7)	0.54	0.46
Healthy/gingivitis	80	6(7.5)	31(38.8)	43(53.7)	0.27	0.73

Table 15. VDR/ApaI genotype distribution and allele frequency

		Distribution of genotype			Frequency of allele	
Disease category	Size	B/B(%)	B/b(%)	b/b(%)	B	b
Severe CP	63	0	11(17.5)	52(82.5)	0.09	0.91
Moderate CP	69	1(1.58)	7(11.1)	61(88.4)	0.07	0.93
Initial CP	34	0	3(8.8)	31(91.2)	0.04	0.96
Healthy/gingivitis	80	1(1.2)	7(8.8)	72(90.0)	0.06	0.94

Table 16. VDR/BsmI genotype distribution and allele frequency

		Distribution of genotype			Frequency of allele	
Disease category	Size	T/T(%)	T/t(%)	t/t(%)	T	t
Severe CP	63	57(90.5)	6(9.5)	0	0.95	0.05
Moderate CP	69	60(86.9)	9(13.1)	0	0.93	0.07
Initial CP	34	28(82.4)	6(17.6)	0	0.91	0.09
Healthy/gingivitis	80	71(88.8)	9(11.2)	0	0.94	0.06

Table 17. VDR/TaqI genotype distribution and allele frequency

Disease category	Size	Distribution of genotype G/G(%)	G/C(%)	C/C(%)	Frequency of allele G	C
Severe CP	63	62(98.4)	1(1.6)	0	0.99	0.01
Moderate CP	69	69(100.0)	0	0	1.00	0
Initial CP	34	34(100.0)	0	0	1.00	0
Healthy/gingivitis	80	79(98.8)	1(1.2)	0	0.99	0.01

Table 18. IL-6-174 genotype distribution and allele frequency

significantly higher in severe CP group than in the healthy control (Table 12). There was no significant difference for the distribution of TNFA-308 genotype between the groups (Table 13).

VDR and IL-6-174 genotype distribution

The distribution of the sampled subjects according to age, gender and disease is shown in Table 14. It showed that there was a significant over-representation of VDR allele A in all CP groups than in healthy control, the carriage rate of genotype AA was significantly higher in the severe CP group than in the healthy control (61.8% VS 7.5%, $p<0.01$)(Table 15). No significant difference in the distribution of genotype of VDR BsmI, TaqI polymorphism between different groups was observed (Table 16,17). It was showed that no significant differences in distribution of genotype of IL-6-174 were observed among patients and controls. The IL-6 -174 allele C is rare in the Chinese Han nationality (Table 18).

Discussion

In this preliminary study, it was found that genotypes of IL-1A-889 II/I, IL-1B-511II/II, IL-1A-889 allele 2/IL-1B-511 allele 2, VDR/ ApaI A/A were significantly associated with severity of chronic periodontitis, and genotypes of IL-1B+3954 II/I, IL-1B+3954 allele 2/IL-1B-511 allele 2, FcgRIIA R/R were also associated with severity of chronic periodontitis, although their distribution in the population were relatively low. According to our data and others, it is believed that an individual may have increased susceptibility to periodontitis due to different factors, genotypes associated with increased susceptibility may be quiet heterogeneous and an individual with increased susceptibility may be influenced by multiple genes. Further studies are being undertaken on more potential candidate genes in large size of samples and with linkage analysis and associate studies in families.

Multifactorial diseases, such as periodontitis, are believed to be due to the combined effect of multiple genes, often interacting over long time periods with environmental factors. For systematic analysis of association of genetic polymorphisms with periodontitis and defining the role of the gentic polymorphisms in the disease pathogenesis, meaningful models to accommodate complexity and diversity of periodontitis due to interactions between genetic and environmental elements are needed.

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