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Polymorphisms in the 5′ flanking region of *IL12RB2* are associated with susceptibility to periodontal diseases in the Japanese population

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Abstract

Objectives: The expression of interleukin (IL)-12Rβ2 molecule is a crucial regulatory factor in the T-helper type (Th) 1 differentiation of T cells. To elucidate the role of the cell-mediated immune (CMI) response in the pathogenesis of periodontitis, Japanese periodontal patients were subjected to single nucleotide polymorphism (SNP) analyses of the 5′ flanking region of *IL12RB2*, whose variants are frequently detected in lepromatous leprosy patients, in which the very weak cellular immune response is caused by low expression of IL-12Rβ2.

Material and Methods: The gene polymorphisms of the 5′ flanking region of *IL12RB2* were examined in subjects with several types of periodontal disease and in healthy controls. Serum immunoglobulin (Ig) G antibody titres against periodontopathic bacteria were measured and compared in periodontal patients with and without variant alleles of *IL12RB2*.

Results: The frequencies of variant alleles of *IL12RB2* were significantly higher in aggressive periodontitis patients as compared with healthy controls or chronic periodontitis patients. Serum IgG titres against all periodontal bacteria examined in subjects carrying variant alleles were higher than those in subjects without variant alleles.

Conclusion: IL-12Rβ2 SNPs could be useful as genetic markers to access the susceptibility of the general population to periodontal disease. Low CMI responses or high humoral responses are associated with the pathogenesis of inflammatory periodontal diseases.

Key words: CMI responses; IgG; *IL12RB2*; periodontitis; SNPs

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Conflict of interest and source of funding statement

The authors declare that they have no conflict of interests

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Periodontitis is an infectious disease initially caused by the infection of the dental plaque (biofilm) with anaerobic, Gram-negative bacteria. In the lesions, host defence functions against these microorganisms are evoked by recruiting many kinds of immunoregulatory cells. In such immune systems, it has been postulated that cytokines produced by $CD4^+$ T-helper (Th) cells play an important role in host defense against

periodontal infection. In particular, the degree of differentiation of naïve $CD4^+$ T cells into Th1/Th2 cells greatly influences the effectiveness of the subsequent immune responses against pathogens, as well as the progression of the disease (Seymour et al. 1993, Dennison & Van Dyke 1997). Several previous studies have demonstrated that Th2 responses are likely to be induced in the gingival tissues of periodontitis patients (Yamazaki et al. 1994, Tokoro et al. 1997). Moreover, it has been demonstrated that peripheral blood mononuclear cells (PBMCs) from periodontitis patients have decreased Th1 responses and increased Th2 responses (Gemmell & Seymour 1994). In contrast, several conflicting findings have reported that Th0 and/or Th1 responses are dominantly evoked in the gingival tissues of periodontal patients (Ebersole & Taubman 1994, Roberts et al. 1997, Takeichi et al. 2000). Additionally, a recent report has shown that both Th1 and Th2 cytokines play an important role in maintaining alveolar bone homoeostasis (Alayan et al. 2007).

Periodontitis patients have also been subjected to genetic analysis for several kinds of single nucleotide polymorphisms (SNPs) located in the genes regulating the Th1/Th2 immune response, including interleukin (*IL*) *2* (Scarel-Caminaga et al. 2002), *IL4* (Michel et al. 2001, Kang et al. 2003), *IL6* (Park et al. 2003, Brett et al. 2005) and *IL10* (Yamazaki et al. 2001, Berglundh et al. 2003).

Based on these previous observations, it seems likely that people who are susceptible to periodontal diseases may have decreased Th1 responses in their systemic immune system, although this logic has to be firmly confirmed from various points of view by employing effective approaches. In our recent study, we found that the frequencies of SNPs, including $-1035A>G$, $-1023A>G$, $-650delG$ and $-464A>G$, in the 5′ flanking region of the gene coding IL-12 receptor $\beta2$ (IL-12R$\beta2$) molecules (*IL12RB2*) were higher in patients with lepromatous type of leprosy than in normal controls. The hereditary background of the lepromatous leprosy patients was characterized by a very low level of cellular immune response caused by the low expression of IL-12R$\beta2$ (Ohyama et al. 2005).

In this study, to understand the role of cell-mediated immune (CMI) responses in susceptibility to periodontal diseases, we investigated the frequencies of SNPs in the 5′ flanking region of *IL12RB2* in patients suffering from different forms of periodontal diseases and in healthy controls. Additionally, to elucidate the effects of the SNPs on humoral immune responses against periodontal pathogens, we compared the serum immunoglobulin (Ig) G antibody titres against many species of periodontal bacteria in subjects with and without these SNPs.

Material and Methods

Study population

One hundred and ten Japanese patients with periodontitis, including 30 aggressive periodontitis (AP) patients (10 males and 20 females, average age 30.4 ± 6.7 years), 44 severe chronic periodontitis (sCP) patients (17 males and 27 females, average age 58.9 ± 7.6 years) and 36 mild CP (mCP) patients (14 males and 22 females, average age 59.5 ± 10.1 years), were enrolled in the study for gene analysis of the 5′ flanking region of *IL12RB2*. The patients were recruited from a pool of patients who visited Okayama University Medical and Dental Hospital. Patients were categorized into AP or CP groups. AP patients were clinically diagnosed according to the classification of the American Academy of Periodontology (Armitage 1999). In addition to that, we made a final diagnosis of AP among those who fulfilled the criteria described in our previous study (Takahashi et al. 2001). Briefly, patients, who were <35 years of age at examination, exhibited marked bone loss and had supragingival plaque levels not commensurate with the periodontal bone loss present. The criteria for severe and mCP were as follows: (1) the number of remaining teeth was at least 15 in both groups; (2) mCP patients had a pocket probing depth of more than 4 mm at up to three sites (six sites were probed per tooth), while sCP patients had a pocket probing depth of more than 4 mm at more than four sites; and (3) sCP patients had on average an alveolar bone loss of over 30%, while mCP patients had a bone score of under 20%. In this study, we excluded subjects with a bone score of between 20 and 30% in order to clearly delineate mCP and sCP subjects. Bone loss was measured from periapical radiographs as described previously (Schei et al. 1959). The patients had no significant medical history. As controls, 43 healthy Japanese volunteers (21 males and 22 females, average age 27.7 ± 4.1 years), who had no history of periodontitis and systemic diseases, were enrolled. The clinical features of these subjects are summarized in Table 1. Serum IgG titres against periodontal bacteria were evaluated in 55 subjects, including all of the 27 AP patients who participated in this study and 28 CP patients who were randomly selected from the sCP patients.

The study protocol was approved by the Human Ethics Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, and informed consent was obtained from all the donors.

Detection of SNPs of *IL12RB2*

DNA sequences for polymorphisms in the 5′ flanking region of *IL12RB2* were determined by using a direct sequencing method, which was described in our previous report (Ohyama et al. 2005). Briefly, genomic DNA was isolated from peripheral blood using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After amplification, the polymerase chain reaction products were sequenced using the ABI 3730 DNA Sequencer (PerkinElmer Life Science, Wellesley, MA, USA). The sequence data obtained were compared

Table 1. Clinical findings

	AP patients	sCP patients	mCP patients	Healthy controls
Distribution	(30) 20f/10m	(44) 27f/17m	(36) 22f/14m	(43) 22f/21m
Age (years)	30.4 ± 6.7	58.9 ± 7.6	59.5 ± 10.1	27.7 ± 4.1
Number of teeth	26.4 ± 4.1	23.3 ± 4.1	26.4 ± 2.9	28.1 ± 1.5
Bone loss (%)	33.0 ± 13.8	46.6 ± 9.1	11.6 ± 5.3	–
Bone loss $\geqslant 50\%$	25.0 ± 21.2	50.6 ± 23.0	0.0 ± 0.0	0.0 ± 0.0
Probing depth (mm)	4.1 ± 0.9	4.1 ± 1.1	2.3 ± 0.4	2.2 ± 0.3
Bleeding on probing (%)	50.9 ± 26.9	48.5 ± 27.1	18.6 ± 13.9	9.5 ± 3.0

Values represent the mean ± standard deviation.

AP, aggressive periodontitis; sCP, severe chronic periodontitis; mCP, mild CP; f, female; m, male.

with the GenBank database (GenBank accession number AL389925) to determine the SNPs, including −1035A>G, −1023A>G, −650delG and −464A>G, in the 5′ flanking region of *IL12RB2*.

Measurement of serum IgG titres against periodontopathic bacterial antigens

Serum IgG titres against periodontal bacteria were measured by enzyme-linked immunosorbent assay (ELISA) as described (Murayama et al. 1988). All sera were obtained from subjects at their first visit to the hospital. Sonicated extracts of periodontal bacteria, including *Actinobacillus actinomycetemcomitans* Y4 (Aa), *Capnocytophaga ochracea* S3 (Co), *Eikenella corrodens* FDC1073 (Ec), *Fusobacterium nucliatum* ATCC-25586 (Fn), *Porphyromonas gingivalis* SU63 (Pg) and *Prevotella intermedia* ATCC25611 (Pi), were employed as antigens for the ELISA assay. We defined ELISA units (EU) as the ratio of the antigen-specific serum IgG titre in each individual to that of the control serum, which was pooled from five periodontally healthy donors.

Statistical analysis

The allele frequencies of each SNP on *IL12RB2* between the groups were compared by the χ^2 test. The Mann–Whitney *U*-test and/or Student's *t*-test was adopted to evaluate the differences in the antigen-specific serum IgG titres and in the clinical data of the subjects.

Results

Genotype and allele frequencies of *IL12RB2* SNPs in periodontitis patients and in healthy controls

To test for an association between gene polymorphisms in the 5′ flanking region of *IL12RB2* and susceptibility to various forms of periodontal diseases, we investigated the frequencies of *IL12RB2* SNPs, including −1035A>G, −1023A>G, −650delG and −464A>G, in subjects with several types of periodontal diseases. In our previous study, we found that SNPs in the 5′ flanking region of *IL12RB2* affect the level of the expression of these receptors, suggesting that they may be the cause of individual differences in CMI responsiveness to mycobacterial antigens. The determination of the genotypes of the 5′ flanking region of *IL12RB2* in 110 Japanese periodontal patients, including 30 AP patients, 44 sCP patients and 36 mCP patients, revealed that the frequencies of all SNPs, including −1035A>G, −1023A>G, −650delG and −464A>G, were significantly higher in AP patients than in healthy controls (Table 2). In addition, when we compared the allele frequencies of SNPs between AP and sCP patients, between AP and mCP patients, and between AP patients and healthy controls, significant differences were also detected for the frequencies of the SNPs −1035A>G, −1023A>G, −650delG and −464A>G (p values were 0.0048, 0.0048, 0.0010 and 0.0028 between AP and sCP, 0.0008, 0.0008, 0.0002 and 0.0008 between AP and mCP, and 0.0048, 0.0048, 0.0040 and 0.0021 between AP and healthy controls). However, no differences were detected in the frequencies of the SNPs between sCP and mCP patients or between CP and healthy controls (Fig. 1).

Next, we examined the clinical data, including the numbers of remaining teeth, bone loss, probing pocket depth and bleeding on probing, among all the periodontitis patients enrolled in this study, and compared the clinical data of the patients subdivided into two groups, a ''carrier'' group and a ''non-carrier'' group. We defined ''carrier'' subjects as those who carried at least one variant allele of *IL12RB2* (−1035, −1023, −650 or −464) SNPs and ''non-carrier'' subjects as those who did not carry any of these alleles. The ''carrier'' subjects with periodontal diseases possessed significantly deeper mean periodontal pockets than ''non-carrier'' subjects (Table 3; $p = 0.0303$).

Table 2. Genotypes of *IL12RB2* in periodontal patients

Clinical diagnosis	AP patients ($n = 30$)	sCP patients ($n = 44$)	mCP patients ($n = 36$)	Healthy controls ($n = 43$)
−1035 SNP genotype				
A/A	10 (33.3%)	28 (63.6%)	25 (69.4%)	27 (62.8%)
A/G	14 (46.7%)	13 (29.5%)	10 (27.8%)	14 (32.6%)
G/G	6 (20.0%)	3 (6.8%)	1 (2.8%)	2 (4.7%)
p-value	0.0133*,†	0.9348†	0.5346†	
−1023 SNP genotype				
A/A	10 (33.3%)	28 (63.6%)	25 (69.4%)	27 (62.8%)
A/G	14 (46.7%)	13 (29.5%)	10 (27.8%)	14 (32.6%)
G/G	6 (20.0%)	3 (6.8%)	1 (2.8%)	2 (4.7%)
p-value	0.0133*,†	0.9348†	0.5346†	
−650 SNP genotype				
G/G	8 (26.7%)	26 (59.1%)	24 (66.7%)	24 (55.8%)
G/del	14 (46.7%)	15 (34.1%)	10 (27.8%)	15 (34.9%)
del/del	8 (26.7%)	3 (6.8%)	2 (5.6%)	4 (9.3%)
p-value	0.0262*,‡	0.7572†	0.3252†	
−464 SNP genotype				
A/A	10 (33.3%)	29 (65.9%)	25 (69.7%)	28 (65.1%)
A/G	14 (46.7%)	12 (27.3%)	10 (27.8%)	13 (30.2%)
G/G	6 (20.0%)	3 (6.8%)	1 (2.8%)	2 (4.7%)
p-value	0.0075*,†	0.9380†	0.8042†	

The frequencies of the four SNPs were compared between patients with periodontitis and healthy controls.
*Significant differences ($p < 0.05$, χ^2 test) were found between AP patients and healthy controls.
†Because the homozygotes of the rare allele were too few to perform the 2×3 χ^2 test, the homozygotes of the dominant allele and variant allele carriers were compared by the 2×2 χ^2 test.
‡2×3 χ^2 test.
AP, aggressive periodontitis; sCP, severe chronic periodontitis; mCP, mild CP; SNP, single nucleotide polymorphism.

Comparison of serum IgG titres against periodontopathic bacteria between ''carrier'' and ''non-carrier'' subjects of *IL12RB2* SNPs

Because it has been postulated that CMI responses function as a negative regulator of humoral immune responses, we expected that *IL12RB2* SNPs would also affect humoral immune responses against periodontopathic bacteria. In order to determine this, we evaluated the serum IgG titres against Aa, Co, Ec, Fn, Pi and Pg in 55 periodontal patients, including 27 AP patients and 28 CP patients, whose genotypes of the 5′ flanking region of *IL12RB2* had been identified. When we compared

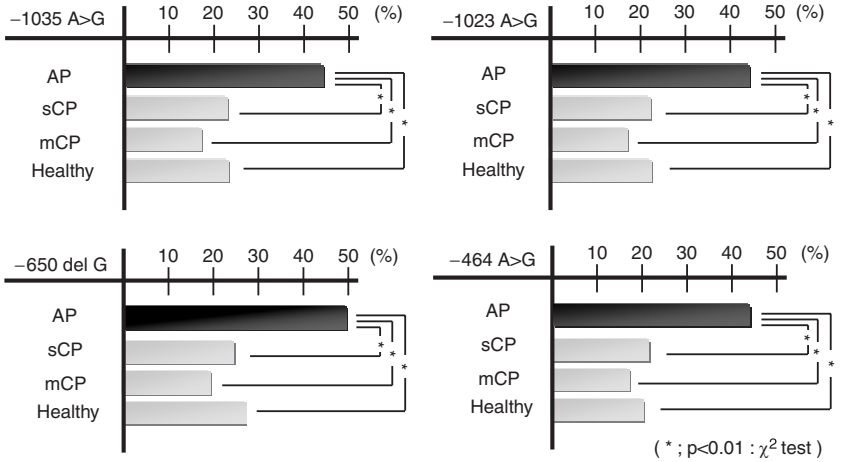

Fig. 1. Allele frequencies of *IL12RB2* in periodontal patients. The allele frequencies of four single nucleotide polymorphisms ($-1035A>G$, $-1023A>G$, -650delG and $-464A>G$) on *IL12RB2* in the groups were compared. *Significant differences ($p<0.01$, χ^2 test) were detected between aggressive periodontitis (AP) patients and both healthy controls and patients with other types of periodontal diseases including severe chronic periodontitis (sCP) and mild CP (mCP).

Table 3. Comparison of the clinical findings of periodontitis patients with and without *IL12RB2* SNPs

	Carriers ($n = 55$)	Non-carriers ($n = 55$)	p-value
Number of teeth	24.9 ± 4.2	25.5 ± 3.6	0.5640
Bone loss (%)	33.6 ± 17.3	29.2 ± 18.2	0.1350
Bone loss ⩾50%	29.4 ± 28.2	24.7 ± 28.2	0.3651
Probing depth (mm)	3.7 ± 1.2	3.3 ± 1.2	0.0303
Bleeding on probing (%)	41.6 ± 25.4	36.4 ± 29.2	0.1601

Carriers indicate the subjects who carried at least one variant allele in *IL12RB2* (-1035, -1023, -650 or -464) SNPs. Non-carriers indicate subjects without SNPs. Significant differences were detected between carriers and non-carriers with respect to probing depth (Mann–Whitney *U*-test). Each value represents the mean ± standard deviation.
SNP, single nucleotide polymorphism.

IgG titres of ''carrier'' and ''non-carrier'' subjects, the IgG titres against Aa, Co, Ec and Fn were significantly higher in the ''carrier'' subjects than in the ''non-carrier'' subjects (Co: $p<0.01$; Aa, Ec, Fn: $p<0.05$; Fig. 2). Although no statistically significant differences were found between carriers and non-carriers with respect to the antibody titre against Pg and Pi, the mean titres against these bacteria in the carrier group were much higher.

Discussion

IL-12 is secreted from macrophages and dendritic cells and is a potent inducer of interferon (IFN)-γ from Th1 cells, which is partially dependent upon the expression level of IL-12R on macrophage cell surface (Sieling et al. 1994, Trinchieri 1994, Gately et al. 1998). IL-12R is composed of two protein subunits, referred to as the $\beta1$ and $\beta2$ chains. The expression of the latter is a crucial determinant of Th1/Th2 development as STAT4 is activated through an interaction with the tyrosine residue at the cytoplasmic domain of the IL-12R$\beta2$ subunit (Szabo et al. 1997, Naeger et al. 1999, Rogge et al. 1999). We previously hypothesized that susceptibility to several disease-related mycobacterial pathogens would be determined by the expression level of IL-12R$\beta2$. For this reason, we examined for SNPs on *IL12RB2* and detected several SNPs, including $-1035A>G$, $-1023A>G$, -650delG and $-464A>G$, in the 5′ flanking region of *IL12RB2*, which affected the expression level of IL-12R$\beta2$ molecules (Ohyama et al. 2005). Thus, it is possible that these SNPs are not only associated with the establishment of certain clinical types of leprosy, but are also associated with susceptibility to other infectious diseases, whose pathogenesis is greatly aggravated by an impaired Th1/Th2 balance against pathogens. Indeed, these SNPs could be one of the possible hereditary factors that determine differences in the intensities of CMI responses among individuals. In this study, we surveyed the frequencies of these SNPs in subjects with several clinical types of periodontal diseases and found a significantly higher frequency of the SNPs in AP patients than in CP patients and healthy controls. Based on these observations, it is likely that subjects prone to periodontal diseases and those with LL leprosy share a hereditary background that confers similar immunological response properties, which are characterized by a tendency to have low Th1 responses against pathogens. Although it is still too early to conclude that the immunological response that results in a skew towards Th1 could lead to resistance against periodontal diseases, we believe that a more refined study of the immunogenetic background of lepromatous leprosy patients could provide a good model for unravelling the features of immunological aspects associated with the susceptibility to AP, and that the SNPs studied here could be one of the possible genetic markers for determining susceptibility to periodontal diseases.

In this study, we found that the IgG levels against several periodontal bacteria were significantly higher in ''carrier'' patients than in ''non-carrier'' patients, and that the SD of EU against the periodontopathic bacterial antigens investigated in this study showed a wider range in ''carrier'' patients than in ''non-carrier'' patients. From these observations, we judged that the humoral immune responses against periodontal bacteria in ''carrier'' patients would be hyper-activated as a result of the low CMI response against periodontal bacteria. Thus, it is likely that the immune system in the ''carrier'' group is skewed towards Th2 responses and produces higher amounts of immunoglobulins after infection with periodontal bacteria. Based on the current study, we suggest that low CMI responses or high humoral responses against periodontal bacterial infection might constitute a genetic factor that influences susceptibility to this disease. Interestingly, it has also been reported by others that PBMCs in AP

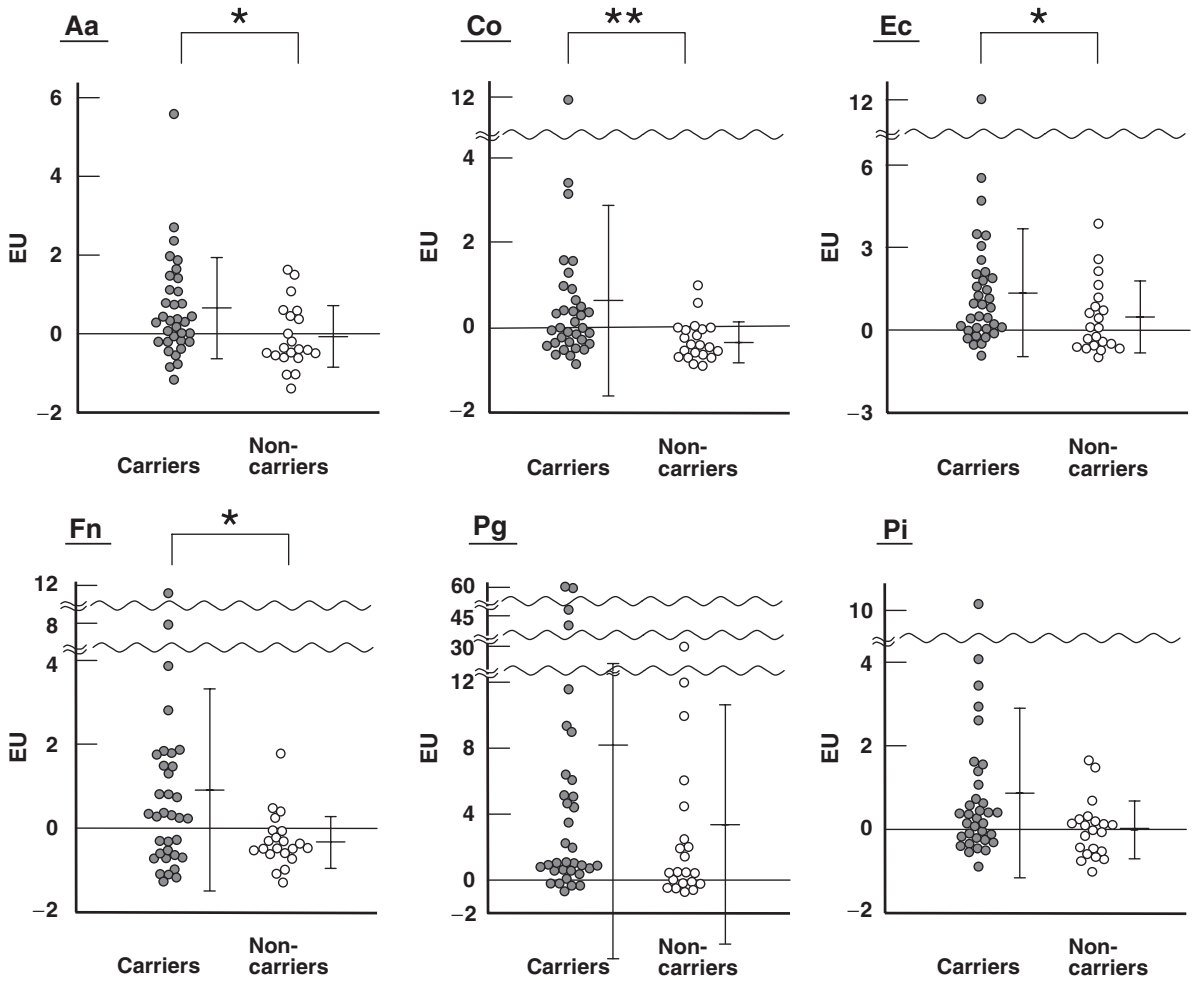

Fig. 2. Comparison of the serum immunoglobulin (Ig)G titres against periodontopathic bacteria in periodontal patients with and without single nucleotide polymorphisms in the 5′ flanking region of *IL12RB2*. Serum IgG titres against periodontal bacteria, including *Actinobacillus actinomycetemcomitans* (Aa), *Capnocytophaga ochracea* (Co), *Eikenella corrodens* (Ec), *Fusobacterium nucliatum* (Fn), *Porphyromonas gingivalis* (Pg) and *Prevotella intermedia* (Pi), were measured in the ''carrier'' and ''non-carrier'' groups of periodontal patients (''carriers'': $n = 34$; ''non-carriers'': $n = 21$). Enzyme-linked immunosorbent assay units (EU) were defined as the ratio of serum IgG titre specific to each antigen in each individual to that of the control serum that was pooled from five periodontally healthy donors. The Mann–Whitney *U*-test was adopted to evaluate the difference in EU at each bacterial species among the subjects.

patients produce low levels of Th1 cytokines, including IFN-γ, in response to mitogenic stimulation (Sigusch et al. 1998, Bartova et al. 2000).

IL-12 is known to be a powerful inducer of IFN-γ production in T cells. Recently, we found that activated T cells from subjects carrying *IL12RB2* SNPs showed significantly lower production of IFN-γ even in the presence of IL-12 than T cells from subjects with dominant alleles of *IL-12RB2* (unpublished data). IFN-γ may also act to suppress osteoclastogenesis by interfering with the RANKL–RANK signalling pathway (Takayanagi et al. 2000). Furthermore, it has been reported that IFN-γ strongly inhibits T-cell differentiation to Th17, which facilitates osteoclastogenesis caused by IL-17-mediated induction of RANKL on osteoblastic cells (Harrington et al. 2005, Sato et al. 2006). Thus, it is possible that subjects with genetic factors associated with low IFN-γ productivity, including those with low IL-12-producing ability, would be susceptible to periodontitis because of their inability to regulate osteoclastogenesis by IFN-γ.

A comparison of the clinical data of periodontal patients (numbers of remaining teeth, bone loss, probing pocket depth and bleeding) showed that pocket depths in the ''carrier group'' were significantly deeper than in the ''non-carrier'' group. We also found that ''carrier'' subjects showed more severe symptoms in other clinical parameters; however, the differences in these clinical parameters, excluding probing pocket depth, did not reach a significant level. At this time, the reason as to why a statistical difference was only found in pocket depth is not clear. We hope that the analysis of a larger number of subjects in a future study will throw light on this problem.

In summary, our study shows that novel *IL12RB2* SNPs, which influence differences in the intensities of CMI responses and is associated with the establishment of clinical types of leprosy, could be useful genetic markers to screen the Japanese population for susceptibility to AP. Additionally, low CMI responses against parasitic bacterial infection could be one of the factors contributing to periodontal disease susceptibility.

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Clinical Relevance

Scientific rationale for the study: The role of cellular immunity in the pathogenesis of periodontitis is still unclear. To try to understand this, we performed SNP analyses on the 5′ flanking region of *IL12RB2* in patients with periodontal diseases because SNP variants of *IL12RB2* were previously found to occur frequently in lepromatous leprosy patients, whose weak immune responses were caused by the low expression of IL-12 receptor $\beta 2$.

Principal findings: Variant SNPs were higher in patients with AP than in patients with CP or in healthy controls.

Clinical implications: *IL12RB2* SNPs could be useful as markers to assess the susceptibility to periodontal disease in the Japanese population.