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Lower antibody response to *Porphyromonas gingivalis* associated with immunoglobulin G Fcγ receptor II*B* polymorphism

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Background and Objective: Human FcγRIIB is one of the receptors for immunoglobulin G (IgG) and suppresses the activation of B lymphocytes through cross-linking with the B cell receptor via immune complexes. This function of FcγRIIB is essential for the negative regulation of antibody production. Our previous study has demonstrated the gene polymorphism FcγRIIB-I232T to be associated with periodontitis. The polymorphism FcγRIIB-232T has been reported to inhibit B-cell antigen receptor signaling more effectively compared to FcγRIIB-232I, while other groups concluded that FcγRIIB-232T had no ability to inhibit activatory receptors. In this study, we examined whether FcγRIIB-I232T polymorphism would change the IgG antibody response to the periodontopathic bacteria *Porphyromonas gingivalis*.

Material and Methods: Forty-seven patients with periodontitis were genotyped with the direct sequencing of genome DNA. Serum IgG and specific IgG subclass levels for the sonicate of *P. gingivalis* and the recombinant 40 kDa outer membrane protein (OMP) were determined.

Results: No significant difference in the total IgG level and IgG response to *P. gingivalis* sonicate were observed between sera from FcγRIIB-232T carriers and non-carriers. The FcγRIIB-232T carriers revealed a significantly lower IgG_2 response to *P. gingivalis* 40 kDa OMP compared to non-carriers ($p = 0.04$, Mann–Whitney *U*-test). Lower responses of FcγRIIB-232T carriers were also observed in specific IgG and IgG_1 levels. The FcγRIIB-232T carriers revealed a low level of IgG_2 response to *P. gingivalis* 40 kDa OMP, even with a high average probing pocket depth.

Conclusion: These results suggest that association of the FcγRIIB-232T allele with periodontitis might be related to the lower levels of antibody response to *P. gingivalis*.

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The Fcγ receptor IIB (FcγRIIB) is one of the immunoglobulin G (IgG) Fc receptors that has immunoreceptor tyrosine-based inhibitory motif (ITIM) on the cytoplasmic tail (1). The FcγRIIB has been shown to act as a negative feedback regulator of antibody production by inhibiting B-cell antigen receptor (BCR)-elicited activation signals via ITIM through IgG immune complexes (2,3). Deficiency of FcγRIIB in mice was shown

to be associated with severe inflammation triggered by immune complexes (4,5).

Our previous studies have reported novel polymorphisms in the human FcγRIIB gene (6). Enrichment of the 232T allele in the FcγRIIB-I232T polymorphism was associated with susceptibility to periodontitis in a Japanese population (7). The combination of stimulatory FcγRIIA and inhibitory FcγRIIB genotypes may also increase susceptibility to systemic lupus erythematosus and periodontitis (8).

In this study, we examined the possibility that FcγRIIB-I232T polymorphism could change the IgG antibody responses to periodontopathic bacteria *Porphyromonas gingivalis* in patients with periodontitis.

Material and methods

Subjects

Forty-seven Japanese patients with chronic periodontitis (21 males and 26 females; mean age 43.9 years old; age range 26–61 years) were referred to the Periodontics Clinic of the Niigata University Medical and Dental Hospital between April 2002 and March 2004 in this study. None of the participants had a history or current signs of systemic disease, nor had they used any medication for 3 months prior to this study. Informed consent was obtained from all participants with the signed format that had been reviewed and approved by the Ethical Committee for the Use of Human Subjects in Research, Niigata University Faculty of Dentistry.

Clinical assessments

Patients were clinically evaluated at the first visit by several periodontists for the following conditions: (1) number of teeth; (2) probing pocket depth, which was expressed as the mean distance from the free gingival margin to the bottom of the pocket; and (3) clinical attachment level (CAL), which was expressed as the mean distance from the cemento-enamel junction to the bottom of the pocket. Probing pocket depth and CAL were assessed using a Williams probe at six sites around each tooth: mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual locations. Measurements were recorded to the nearest millimetre, and every observation close to 0.5 mm was rounded to the lower whole number. Full-mouth radiographs were evaluated by two calibrated readers to examine alveolar bone levels on the mesial and distal aspects of each tooth. Based on the clinical and radiographic data, patients with chronic periodontitis were identified as having more than four diseased sites with probing pocked depths and CAL >5 mm, together with radiographic evidence of bone loss. Smokers were determined by a standard questionnaire. Clinical characteristics of patients are summarized in Table 1.

Preparation of sonicated *P. gingivalis* and 40 kDa outer membrane protein (OMP)

Porphyromonas gingivalis 381 was cultured in tripticase soy broth (Becton Dickinson, Cockeysville, MD, USA) supplemented with 0.25% yeast extract (Difco, Detroit, MI, USA), hemin (5 μg/mL) and menadione (0.5 μg/mL) in anaerobic conditions (80% N_2–10% H_2–10% CO_2) at 37°C up to the mid-logarithmic phase (9).

Porphyromonas gingivalis sonicate was obtained using an ultrasonic homogenizer (Sonifier) for 5 min. Recombinant 40 kDa OMP of *P. gingivalis* 381 was purified by the method of Kawamoto *et al.* (10).

Measurement of serum IgG levels

Peripheral venous blood samples were obtained by venipuncture from all participants. Serum was collected by centrifugation at 1500*g* for 20 min and stored at −20°C until used. Serum total IgG and subclass antibodies specific for sonicated *P. gingivalis* and 40 kDa OMP were determined by enzyme-linked immunsorbent assay (ELISA; 11). Briefly, each of the 96-well flat-bottomed microtitre plates was coated with the capture monoclonal antibody for the antigens human IgG or 50 μL of sonicated *P. gingivalis* or 40 kDa OMP (1 μg/mL) in 50 mM sodium carbonate coating buffer (pH 9.6) for 1 h at 37°C. After removal of the antigen solution, 200 μL blocking reagent was added to each well for 20 min at room temperature. After washing thrice with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST; pH 7.4), 50 μL of the serum (1:1000 dilution) in PBST containing 1% bovine serum albumin was added to each well and incubated for 1 h at 37°C. Each well was washed thrice with PBST and incubated with 50 μL horseradish peroxidase-conjugated sheep antihuman IgG or each subclass of antibody (1:1000 dilution) in PBST for 30 min at room temperature. After washing thrice with PBST, color development was performed with 50 μL of 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) in 0.1 M sodium acetate–citric acid buffer (pH 5.5) containing 0.004% hydrogen peroxide for 10 min at 37°C and stopped by addition of 50 μL 2 M H_2SO_4. Optical density at 450 nm (OD

Table 1. Clinical characteristics of patients

	FcγRIIB-232T non-carriers (n = 34)	FcγRIIB-232T carriers (n = 13)
Gender (male/female)	13/21	8/5
Age (years)	43.5 ± 10.1	45.1 ± 10.0
Smoker (%)	12	8
Number of teeth	27.6 ± 2.8	26.4 ± 2.8
Probing depth (mm)	3.6 ± 1.1	3.7 ± 1.1
Clinical attachment level (mm)	4.2 ± 1.5	4.2 ± 1.5
Bleeding on probing (% sites)	21.8 ± 21.9	25.3 ± 22.2
Bone loss (%)	35.2 ± 14.1	36.0 ± 14.0

Data are expressed as means ± SD.
No statistically significant difference was detected between FcγRIIB-232T non-carriers and carriers (Mann–Whitney U-test, $p > 0.05$).

450 nm) was measured with a microplate reader. In a preliminary experiment, we obtained a standard titration curve by plotting the mean absorbance of the reference serum and developed a straight line equation of the regression. The IgG antibody levels obtained by ELISA were calculated by relating OD values from each serum sample from subjects to a reference serum and are expressed as ELISA units. The appropriate dilution of subject sera was determined from preliminary experiments involving serial dilutions of sera and measurements of antibody responses to absorbed *P. gingivalis* sonicate or 40 kDa OMP.

Determination of FcγRIIB genotypes

Genomic DNA was isolated from peripheral blood (Easy-DNA Kit; Invitrogen, San Diego, CA, USA). Isolated DNA was stored at 4°C until genotyping was performed. Polymerase chain reactions (PCR) for FcγRIIB and FcγRIIB-exon 5 were performed as described previously (7). Briefly, we first performed FcγRII*B*-specific PCR with primer set on introns 3 and 6, because the FcγRII*C* gene shows a high similarity to FcγRII*B*. After purification of the FcγRII*B*-specific fragment, PCR with primers specific for exon 5 was performed using the purified fragment as a template. The FcγRII*B*-exon 5 PCR was performed in a 25 μL reaction mixture containing 0.5 U Ex *Taq*™, and 30 ng of the purified PCR products was denatured for 5 min before 35 cycles of amplification (94°C for 30 s, 60°C for 30 s and 72°C for 30 s) and followed by a final extension at 72°C for 5 min. Nucleotide sequences of these amplified fragments were determined with the ABI PRISM Big Dye Terminator Cycle Sequencing Kits and ABI PRISM 377 DNA sequencer.

Statistical analysis

Differences in clinical parameter values and serum antibody levels between the FcγRIIB-232T carrier and non-carrier groups were assessed by Mann–Whitney *U*-tests.

The Spearman rank correlation coefficient was used to determine the relationship between serum antibody responses of 47 patients to *P. gingivalis* antigens and mean probing pocket depth. Statistical significance was accepted at 5% ($p < 0.05$).

Results

Thirteen subjects were determined to be FcγRIIB-232T carriers, whereas 34 were non-carriers. To examine the functional difference in serum IgG responses between the FcγRIIB-I232T polymorphism, clinical characteristics of the subjects were matched in this study (Table 1, $p > 0.05$, Mann–Whitney *U*-test).

There was no significant difference in total IgG concentration or IgG subclass responses to the *P. gingivalis* sonicate between FcγRIIB-232T carriers and non-carriers (Figs 1 and 2). Among the four IgG subclass responses to *P. gingivalis* 40 kDa OMP, FcγRIIB-232T carriers revealed significantly lower IgG_2 response compared to non-carriers ($p = 0.04$, Mann–Whitney *U*-test, Fig. 3). Distributions of subjects in each quadrant related to FcγRII*B* genotypes, and the IgG_2 response to *P. gingivalis* 40 kDa OMP showed statistically significant skewing ($p = 0.004$, 2×2 contingency table, Table 2). Lower responses of FcγRIIB-232T carriers were also observed in IgG, IgG_1 and IgG_4 to *P. gingivalis* 40 kDa OMP, though the difference from the responses of non-carriers did not reach statistical significance (Fig. 3). Relationships between serum IgG subclass response to *P. gingivalis* 40 kDa OMP and mean probing pocket depth are shown in Fig. 4. In the FcγRIIB-232T non-carrier group, some patients showed a high level of IgG_2 response in concert with the high average of probing pocket depth. In contrast, FcγRIIB-232T carriers revealed a low level of IgG_2 response in spite of high mean probing pocket depth. Positive or negative correlations were not statistically significant between IgG subclass responses to *P. gingivalis* 40 kDa OMP and mean probing pocket depth.

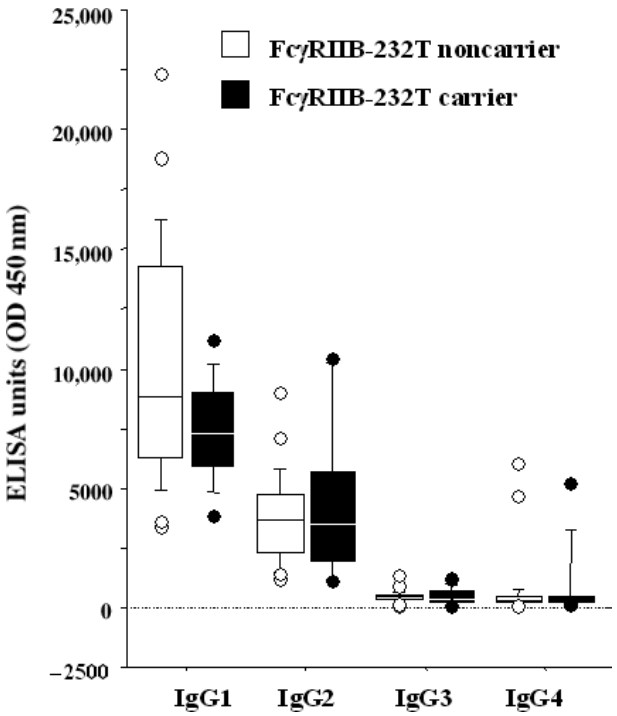

Fig. 1. Box plots for total IgG in sera from FcγRIIB-232T non-carriers and carriers. Sera from 47 patients with periodontitis (34 FcγRIIB-232T non-carriers and 13 carriers) were used in the ELISA. The optical density at 450 nm as an ELISA unit represents the IgG subclass concentration in the serum from each subject. No significant difference was observed between FcγRIIB-232T non-carriers and carriers ($p > 0.05$, Mann–Whitney *U*-test).

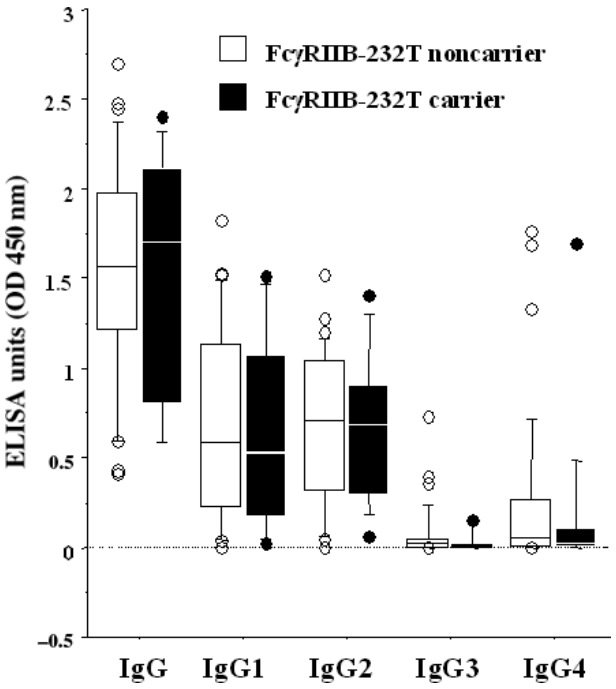

Fig. 2. Box plots for serum IgG responses to *P. gingivalis* sonicate. Sera from 47 patients with periodontitis (34 FcγRIIB-232T non-carriers and 13 carriers) were used in the ELISA. The optical density at 450 nm as an ELISA unit represents total IgG or each IgG subclass concentration reactive with *P. gingivalis* 381 sonicate in the serum from each subject. No significant difference was observed between FcγRIIB-232T non-carriers and carriers ($p > 0.05$, Mann–Whitney *U*-test).

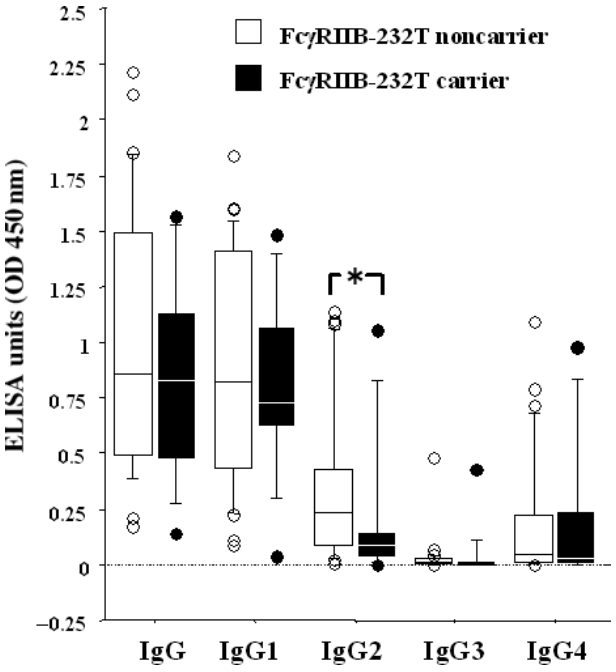

Fig. 3. Box plots for serum IgG responses to *P. gingivalis* 40 kDa OMP. Sera from 47 patients with periodontitis (34 FcγRIIB-232T non-carriers and 13 carriers) were used in the ELISA. The optical density at 450 nm as an ELISA unit represents total IgG or each IgG subclass concentration reactive with *P. gingivalis* 40 kDa OMP in the serum from each subject. The FcγRIIB-232T carriers revealed a significantly lower IgG_2 response to *P. gingivalis* 40 kDa OMP compared to non-carriers ($p = 0.04$, Mann–Whitney *U*-test).

Discussion

We used recombinant proteins of 40 kDa OMP as specific antigens of *P. gingivalis* (10,12) which is a gram-negative anaerobe frequently isolated from the lesions of periodontitis and considered to be an etiological agent of the disease (13). The 40 kDa OMP is a key virulence factor for coaggregation and is found on many strains of *P. gingivalis* (14,15). Administration of monoclonal antibody directed to the *P. gingivalis* 40 kDa OMP has been shown to inhibit aggregation of *P. gingivalis* with *Actinomyces viscosus* cells (14,16,17) and to have bactericidal and opsonic activities (18–21). Serum IgG subclass distribution for patients with periodontitis and healthy control subjects was $IgG_1 > IgG_4 > IgG_2 > IgG_3$ in the responses against *P. gingivalis* 40-kDa OMP; the patients showed significantly higher serum IgG responses to the 40 kDa OMP than the control group (22).

In this study, FcγRIIB-232T carriers revealed a significantly lower IgG_2 response to *P. gingivalis* 40 kDa OMP compared to non-carriers. Lower responses of FcγRIIB-232T carriers were also observed in anti-*P. gingivalis* 40 kDa OMP IgG, IgG_1 and IgG_4 levels (not statistically significant).

The frequency of *P. gingivalis* in subgingival plaque from chronic periodontitis has been reported to be 72% in Japanese (23), but 50–60% in Japanese subjects with 4.6 mm of mean probing pocket depth (24). A significant association was found between the detection of *P. gingivalis* and probing pocket depth (24), suggesting that a high average probing pocket depth would correlate with the presence of *P. gingivalis* in the patient. Among FcγRIIB-232T non-carriers, there was a subgroup of patients showing higher levels of IgG_2 with a higher average of probing pocket depth, whereas most FcγRIIB-232T carriers revealed lower levels of IgG_2 even combined with a higher average of probing pocket depth (Fig. 4).

The positive correlation between the clinical severity of periodontitis and serum IgG level against *P. gingivalis* has been reported in previous studies (25,26). The patients with periodontitis

Table 2. Distributions of subjects in each quadrant related to FcγRIIB genotypes and the IgG_2 response to *P. gingivalis* 40 kDa OMP

		FcγRIIB-232T non-carriers (n = 34)	FcγRIIB-232T carriers (n = 13)
Serum anti-*P. gingivalis* 40 kDa OMP IgG_2 antibody levels (OD 450 nm) median: 0.178	High	44.7% (21)	4.3% (2)
	Low	27.7% (13)	23.4% (11)

Medians were used as cut-off levels for serum anti-*P. gingivalis* 40 kDa OMP IgG antibody. Values represent the percentage (number) of subjects.
Statistical difference (2 × 2 contingency table): p = 0.004.

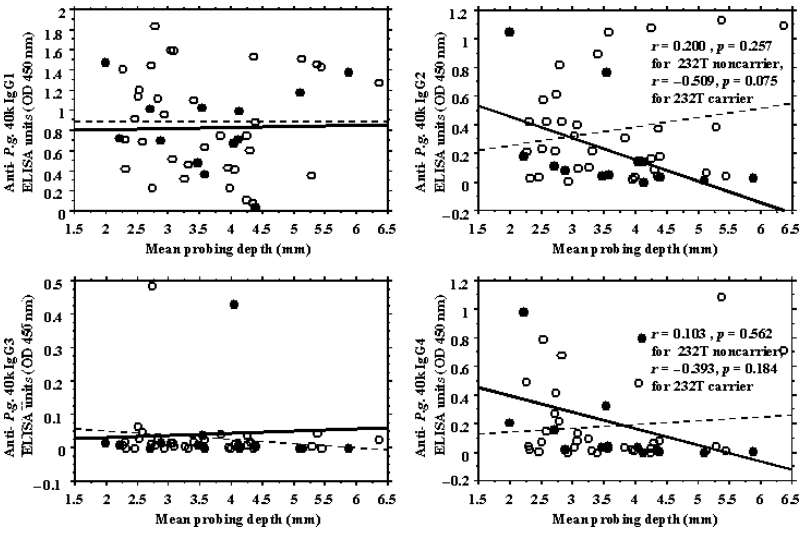

Fig. 4. Relationship between serum IgG subclass responses to *P. gingivalis* 40 kDa OMP and mean probing pocket depth. Sera from 47 patients with periodontitis (34 FcγRIIB-232T non-carriers represented by open circles and thin regression line, and 13 FcγRIIB-232T carriers represented by filled circles and thick regression line) were used in the ELISA. Each circle represents the IgG subclass response to *P. gingivalis* 40 kDa OMP and mean probing pocket depth (in mm) for one patient. No significant association was observed between serum IgG subclass responses to *P. gingivalis* 40 kDa OMP and mean probing depth, assessed by the Spearman's rank correlation coefficient.

had significantly higher serum IgG response to *P. gingivalis* 40 kDa OMP compared to healthy control subjects (22). We have analyzed the clinical parameters from the patients studied the previously by Yasuda *et al.* (7), and found that periodontitis was more severe in FcγRIIB-232T carriers compared to non-carriers (p = 0.0006 for mean probing pocket depth, p = 0.001 for mean CAL, Mann–Whitney U-test, n = 21 for carriers and 79 for non-carriers).

In the present study, clinical characteristics of the subjects were matched so that the different levels of IgG antibody to *P. gingivalis* between FcγRIIB-232T carriers and non-carriers could not be attributed to the different severity of periodontitis (Table 1). An *in vitro* study using the FcγRIIB-negative mouse B cell line IIA1.6 demonstrated that the FcγRIIB-232T allele mediated a high level of CD19 dephosphorylation and a greater degree of inhibition of the calcium response when co-engaged with BCR than did FcγRIIB-232I, independent of the presence of the ITIM (27). This might explain the relatively lower responses of IgG, IgG_1, IgG_2 and IgG_4 to *P. gingivalis* 40 kDa OMP in FcγRIIB-232T carriers. However, Kono *et al.* (28) and, later, Floto *et al.* (29) reported, using human cell lines lacking endogenous FcγRIIB, that FcγRIIB-232T was significantly less potent than FcγRIIB-232I in inhibitory functions because of the exclusion of this receptor from lipid rafts. In their discussion, these authors described how human FcγRIIB-232I may have exerted a greater constitutive inhibition than FcγRIIB-232T in mouse B cells, and in the study by Li *et al.* (27), the entire inhibitory effect of 232I might have been underestimated, because constitutive inhibition of FcγRIIB was not taken into consideration (28). Further investigation would be needed to elucidate the functional difference between FcγRIIB-I232T genotypes *in vivo*.

The ligand specificity of FcγRIIB for human IgG isotypes is $IgG_3 \geq IgG_1 > IgG_4 > IgG_2$ at 4°C with dimeric complexes; however, at 37°C, the binding of IgG_2 dimers increases significantly (30). More prominent differences of IgG_2 responses to *P. gingivalis* 40 kDa OMP between FcγRIIB-I232T genotypes compared to IgG_1 and IgG_3 might be related to this specificity of ligand binding.

Total IgG and IgG subclass concentrations and antibody responses to *P. gingivalis* sonicates did not differ between FcγRIIB-I232T genotypes. The function of FcγRIIB to inhibit B cell activation is mediated by the ligation with BCR, physically, via immune complexes. Thus, one of the possible explanations for the functional difference between FcγRIIB-232I and 232T is the presence of a large non-specific antibody responses *in vivo*.

In previous studies, we first identified many synonymous and non-synonymous substitutions in the human FcγRII*B* gene and determined the frequency of the genotypes in Japanese subjects (6). A significant difference was observed in the distribution of the FcγRIIB-I232T allele between the aggressive periodontitis and healthy groups, with enrichment of 232T in the aggressive periodontitis group (p = 0.006, odds ratio = 4.06; 7). A single nucleotide polymorphism in the intron of the FcγRII*B* gene was associated with susceptibility to chronic periodontitis (p = 0.011, odds

ratio = 2.51; 7). Additionally, the combination of FcγRIIA-R131 and FcγRIIB-232T alleles yielded a strong association with systemic lupus erythematosus and periodontitis (p = 0.0009, odds ratio = 11.2; 8).

In conclusion, this study suggests the possibility of functional differences among FcγRIIB-I232T genotypes, and may support a relationship between FcγRIIB-I232T polymorphism and periodontitis. Further studies should be undertaken to elucidate the functional roles of FcγRIIB and its genotypes in periodontitis and other diseases.

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